Aspartate 74 as a Primary Determinant in Acetylcholinesterase Governing Specificity to Cationic Organophosphonates†

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ABSTRACT: Through site-specific mutagenesis, we examined the determinants on acetylcholinesterase which govern the specificity and reactivity of three classes of substrates: enantiomeric alkyl phosphonates, trifluoromethyl acetophenones, and carboxyl esters. By employing cationic and uncharged pairs of enantiomeric alkyl methylphosphonyl thioates of known absolute stereochemistry, we find that an aspartate residue near the gorge entrance (D74) is responsible for the enhanced reactivity of the cationic organophosphonates. Removal of the charge with the mutation D74N causes a near equal reduction in the reaction rate constants for the R_p and S_p enantiomers and exerts a greater influence on the cationic organophosphonates than on the charged trimethylammonio trifluoromethyl acetophenone and acetylthiocholine. This pattern of reactivity suggests that the orientation of the leaving group for both enantiomers is directed toward the gorge exit and in apposition to Asp 74. Replacement of tryptophan 86 with alanine in the choline subsite also diminishes the reaction rates for cationic organophosphonates, although to a lesser extent than with the D74N mutation, while not affecting the reactions with the uncharged compounds. Hence, reaction with cationic OPs depends to a lesser degree on Trp 86 than on Asp 74. Docking of S_p and R_p cycloheptyl methylphosphonyl thiocholines and thioethylates in AChE as models of the reversible complex and transition state using molecular dynamics affords structural insight into the spatial arrangement of the substituents surrounding phosphorus prior to and during reaction. The leaving group of the R_p and S_p enantiomers, regardless of charge, is directed to the gorge exit and toward Asp 74, an orientation unique to tetrahedral ligands.

Acetylcholinesterase (AChE)[1] catalyzes the hydrolysis of the neurotransmitter acetylcholine (ACh) at a rate approaching a diffusion-controlled process (Rosenberry, 1975; Fersht, 1985; Quinn, 1987), thereby terminating neurotransmission. The X-ray crystal structure of AChE from *Torpedo californica* (TAChE; Sussman et al., 1991) and mouse (MAChE; Bourne et al., 1995) reveals that ACh must travel down a gorge of 20 Å in depth to reach the catalytic serine, the proximal nucleophile in the ester hydrolysis mechanism. The great speed at which the enzyme catalyzes the hydrolysis is thought to arise, in part, from a dipole aligned with the gorge and directed to the active site that electrostatically drives the positively charged substrate to the catalytic center (Tan et al., 1993; Ripoll et al., 1993). Trifluoromethyl acetophenone (TFK) transition state analogs of ACh, which conjugate with the active center serine of AChE at diffusion-controlled rates without requiring the departure of a leaving group (Nair et al., 1993, 1994), also demonstrate that the electric field of AChE accelerates quaternary ammonium binding to the active site (Quinn et al., 1995); in fact, the association rate constants for cationic and uncharged substituted TFK compounds differ by 20−70-fold (Nair et al., 1994). This electrostatic enhancement of catalysis agrees with kinetics of aromatic cation binding (Nolte et al., 1980) and calculations detailing the electrostatic attractions between AChE and the ligands (Tan et al., 1993).

Organophosphonates (OPs) also react rapidly with cholinesterase by phosphonylating the active site serine (Oosterbann & Cohen, 1964; Aldridge & Reiner, 1972). However, the resulting phosphonyl enzymes react slowly with water; thus, these compounds become irreversible inhibitors, rendering AChE inactive upon reaction (Froede & Wilson, 1971). Their reaction rates with AChE depend on the size, nature, and stereochemical arrangement of the substituents about the phosphate where the rates differ between cationic and uncharged alkyl phosphonates by 2−3 orders of magnitude with a preference for the charged OP. Moreover, an enantiomeric preference of greater than 200-fold has been found (Berman & Leonard, 1989). The acyl pocket region, comprised of two phenylalanines at 295 and 297, dictates the preference for the S_p isomer by sterically hindering the approach of the R_p isomer and its positioning for optimal phosphonyl transfer. However, the acyl pocket region has essentially no role in dictating AChE's selectivity for the cationic OPs (Hosea et al., 1995).

The tetrahedral geometry of OPs adds an additional dimension to analysis of specificity compared to the trigonal, planar carboxyl ester substrates and the TFK transition state analogs. Thus, the tetrahedral phosphonates possess a

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[1] Abbreviations: AChE, acetylcholinesterase; TAChE, *Torpedo californica* AChE; MAChE, mouse AChE; TFK, trifluoromethyl acetophenone; TFK$^+$, *m*-(*N*,*N*,*N*-trimethylammonio) TFK; TFK^o, *m*-(*tert*-butyl) TFK; OP, organophosphonate; ACh, acetylcholine; ATC, acetylthiocholine; CHMP, cycloheptyl methylphosphonyl; iPrMP, isopropyl methylphosphonyl; DMBMP, 3,3-dimethylbutyl methylphosphonyl; k_{on}, bimolecular rate constant of association for TFK; k_i, bimolecular rate constant for OP acylation of AChE.

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different spatial arrangement for acylation, and the contribution of residues to their stabilization will differ from those stabilizing ACh and TFKs. Nevertheless, parallels between the three classes of ligands should exist since they all react with a common serine in an equivalent, asymmetric environment (Sussman et al., 1991, 1995).

Three regions of AChE—the acyl pocket, the choline subsite, and the peripheral site—are highly aromatic in amino acid content. Whereas aromatic side chains in the acyl pocket sterically exclude ligands with a particular size and dimension, the choline subsite contributes to stabilization of a positively charged quaternary ammonium moiety as found in ACh, TFK, and edrophonium conferring selectivity to cationic ligands (Sussman et al., 1991, 1995; Harel et al., 1996). Additionally, interaction of an aromatic residue with a cationic substituent, coined a "cation$-\pi$" interaction, is suggested to arise through the quadrupole moment of the aromatic ring and to be electrostatic in nature (Dougherty & Stauffer, 1990; Mecozzi et al., 1996). This structural model has been supported kinetically by analysis of AChE mutations of Trp 86, where replacement by an Ala significantly diminishes catalytic efficiency with cationic ligands but only moderately alters reaction rates with uncharged ligands (Ordentlich et al., 1993a, 1995; Radić et al., 1995).

AChE also contains three nonvariant anionic residues (Cygler et al., 1993; Gentry & Doctor, 1995) in and near the active center: Asp 74 at the rim of the gorge and Glu 202 and 450 at the base of the gorge. The X-ray crystal structure of TAChE in complexes with edrophonium and tacrine reveals that Glu 202 and 450 hydrogen bond with water molecules in the active site (Harel et al., 1993). This network of hydrogen bonding is thought to contribute to efficient acylation of AChE (Radić et al., 1992) and dealkylation during "aging" of OP—AChE conjugates (Michel et al., 1967; Saxena et al., 1993; Ordentlich et al., 1993b; Qian & Kovach, 1993).

Herein, we report on a mutational analysis of AChE to elucidate the determinants responsible for its large selectivity for cationic ligands and assess the contribution by aromatic residues of the choline subsite and peripheral site regions. Information gained from the mutational analysis of the acyl pocket (Hosea et al., 1995) provided evidence for steric determinants dictating phosphonate reactivity and suggested an orientation of the leaving group of the isomers directed out of the gorge. Our findings reported here substantiate that orientation for both the R_p and S_p enantiomers and reveal Asp 74 as a primary determinant in dictating specificity to cationic OPs. In conjunction with kinetic data, computational docking of charged and uncharged enantiomeric alkyl methylphosphonates in a reversible complex and in a trigonal bipyramidal model of the transition state enables one to refine the spatial arrangements of the substituents about the phosphonate prior to and during the phosphonylation reaction.

MATERIALS AND METHODS

Materials. Acetylthiocholine (ATC) iodide and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were products of Sigma Chemical Co. (St. Louis, MO). (S_p)- and (R_p)-alkyl methylphosphonyl thioates were synthesized and isolated as resolved S_p and R_p enantiomers as described previously (Berman & Leonard, 1989). 7-[[(Methylethoxy)phosphinyl]-oxyl]-1-methylquinolinium iodide (MEPQ) was a gift of Drs. Y. Ashani and B. P. Doctor (Walter Reed Army Research Center, Washington, DC; Levy & Ashani, 1986). *m*-(*N*,*N*,*N*-Trimethylammonio) trifluoromethyl acetophenone and *m*-*tert*-butyl trifluoromethyl acetophenone (TFK^+ and TFK^o, respectively) were synthesized as described (Nair et al., 1993,1994).

Production of Enzymes. Wild-type and mutant mouse AChE cDNA constructs were generated as described in Radić et al. (1993). The pRCCMV (Invitrogen) expression plasmids were purified by standard procedures involving polyethylene glycol precipitation and centrifugation in CsCl gradients.

Human embryonic kidney (HEK-293) cells obtained from American Type Culture Collection (Atlanta, GA) were plated at 2×10^6 cells per 10 cm plate in 10% fetal bovine serum-supplemented Dulbecco's modified Eagle's (DME) medium 24 h prior to transfection. Cells were transfected with 15 μg of plasmid/plate of mutant or wild-type cholinesterase-pRCCMV using a standard HEPES-based calcium phosphate precipitation protocol (Ausubel et al., 1994). After 16—24 h, the transfected plates were washed with phosphate-buffered saline and maintained in serum free DME medium for 48—72 h. The medium, containing the expressed cholinesterase, was collected, and the transfected cells were replenished with serum free DME medium. This process was continued for three or four harvests of enzyme. Batches of media were then concentrated to approximately 1—2% of original volume and stored at 4 °C using Centriprep 30 Centricons (Amicon, Beverly, MA).

To produce larger quantities of enzyme for detailed kinetic studies, stable transfectants were generated by selecting transfected HEK cells with G418 for 2—3 weeks or until cell death subsided. Pools of selected cells were frozen in 10% serum and 5% DMSO-containing DME medium for future use.

Enzyme Activity Measurements and Active Site Quantitation. Wild-type and mutant AChE activities were measured in 0.1 M sodium phosphate buffer (pH 7.0) and 0.3 mM DTNB at 22 °C according to Ellman et al. (1961) using ATC as the substrate. Maximum concentrations of ATC did not exceed 100 mM due to appreciable spontaneous substrate hydrolysis. Active sites were quantitated according to Levy and Ashani (1986) and Radić et al. (1992) by titrating the enzyme samples with known concentrations of MEPQ.

Inhibition. Enzyme samples (tens of picomoles) were incubated for designated times with the (S_p)- and (R_p)-alkyl phosphonylthioates in the above assay mixture in the absence of substrate; typically, four inhibitor concentrations were used. The inhibition reaction was stopped by addition of 5—10 mM ATC (a concentration 30—50-fold above the K_m), and residual activity was measured. From the slopes of semilogarithmic plots of activity versus time, pseudo-first-order rate constants (k_{obs}) were plotted against inhibitor concentration to obtain the bimolecular rate constants (k_i) (Aldridge & Reiner, 1972; Radić et al., 1992). When semilogarithmic plots of inhibition versus time were nonlinear, the initial rates were taken as the k_{obs}.

Inhibition by TFK^+ and TFK^o was measured as above which parallels the stopped-time assay reported in Nair et al. (1994). The resulting activities analyzed as a function of time were then fit using nonlinear regression according to the following equation:

$$\%V = (\%V_0 - \%V_{ss})e^{-k_{obs}(t)} + \%V_{ss}$$

where $\%V$, $\%V_0$, and $\%V_{ss}$ are the AChE activities expressed as a percentage of control activity at times t, 0, and infinity, respectively, and k_{obs} is the observed first-order rate constant. The association rate constant, k_{on}, was determined by the following equation:

$$k_{obs} = k_{on}[\text{inhibitor}] + k_{off}$$

The dissociation rate constant, k_{off}, under the experimental conditions was negligible except for the E202Q mutation.

Computer Modeling. Two sequential stages in reaction of (S_p)- and (R_p)-cycloheptyl methylphosphonyl thiocholines and (S_p)- and (R_p)-cycloheptyl methylphosphonyl thioethylates with the enzyme were analyzed: the tetrahedral noncovalent reversible complex preceding phosphonylation and the pentavalent transition state during phosphonylation. Models of the inhibitor structures were built and energy minimized using the Insight II package (Biosym, San Diego, CA) with their partial charges calculated using the MOPAC module of Insight II. The best conformation for each inhibitor in the model of the reversible complex with *T. californica* AChE was obtained after simulated annealing calculations starting from a series of inhibitor orientations in the AChE active center. A mild distance restraint resulted in a harmonic force with a maximum of about 40 kcal/Å applied between the γ-oxygen of Ser 203 of AChE (200 of TAChE)[2] and the phosphorus atom of the inhibitors in order to reduce the number of resulting conformations to the productive ones that lead to phosphonylation. Transition state models were then built by covalent bonding of the inhibitor phosphorus atom to γ-oxygen of Ser 203. The geometry of the substituents attached to the phosphorus atom was adjusted to an ideal trigonal bipyramidal structure (Westheimer, 1968): 180° from apical to apical, 120° from equatorial to equatorial, and 90° from apical to equatorial positions. Simulated annealing calculations were then started (Ashani et al., 1995) to obtain a family of energetically favorable conformations reflecting the pentavalent transition state. In all calculations, residues of the *T. californica* AChE crystal structure were kept fixed except for Ser 203; only this side chain and the conjugated inhibitor were allowed free rotation.

RESULTS

Electrostatic Influences on Reaction Rates with Cationic and Uncharged Organophosphonates. The acyl pocket region of the active site of cholinesterases is the primary determinant in the enantiomeric specificity of chiral phosphonates; however, this region is not involved in distinguishing between the cationic and uncharged thioate leaving groups (Hosea et al., 1995). To explore the electrostatic influences of AChE on reaction rates with charged and uncharged phosphonates, rates of inhibition by enantiomeric alkyl methylphosphonates containing either a thiocholine or thioalkyl leaving group (Figure 1) were examined with MAChE which was mutated at three anionic residues near or within the active center gorge—Asp 74, Glu 202, and Glu

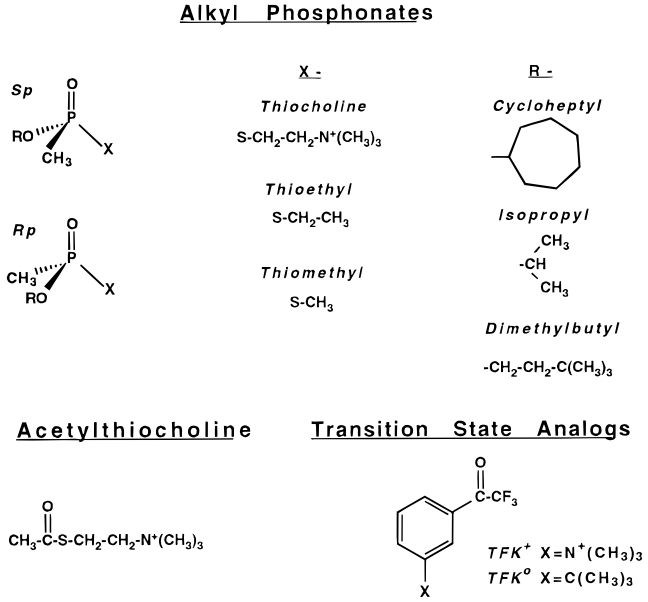


FIGURE 1: Structures of ligands used in the analysis. The leaving group is denoted by X, and the alkyl substituent is denoted by R for the alkyl phosphonates.

450—to the corresponding neutral isosteric residues, Asn and Gln, respectively. The resulting bimolecular rate constants (k_i values) for the inhibition by enantiomers of cycloheptyl methylphosphonyl, isopropyl methylphosphonyl, and 3,3-dimethylbutyl methylphosphonyl (CHMP, iPrMP, and DMBMP, respectively) thiocholines and of CHMP *S*-methyl- or *S*-ethylthioates (thiomethylate and thioethylate, respectively) are shown in Table 1a,b.

The differences in reaction rate constants between the cationic and uncharged CHMP compounds span nearly 3 orders of magnitude for AChE. These congeners differ only in the leaving group substituent which imparts the difference in charge (Figure 1). Mutations in which the anionic residues at the base of the gorge are replaced by isosteric neutral residues, E202Q and E450Q, reduce the reaction rate constants for the charged and uncharged CHMP congeners to approximately the same degree. Thus, Glu 202 and Glu 450 do not account for the large preference of AChE wild-type for the thiocholine-substituted phosphonates. On the other hand, the Asp 74 mutation to Asn reduces the rate constants for cationic CHMP-thiocholines at least 100-fold, whereas the mutation appears to slightly increase rate constants for uncharged S_p and R_p CHMP-thioates. The resulting bimolecular rate constants for the charged and uncharged compounds are of similar magnitude in the Asn 74 enzyme. Thus, the presence of Asp 74 at the rim of the gorge solely accounts for the 2−3 orders of magnitude enhancement of reaction with the thiocholine-substituted CHMP isomers. The effect of charge neutralization with the D74N mutation on the rates of reaction with the thiocholine compounds maintains the enantiomeric preference, suggesting that the quaternary ammonium moieties of the S_p and R_p isomers reside at similar distances from D74.

Contribution by Aromatic Residues to Alkyl Phosphonate Specificity. AChE mutants encompassing the choline binding site (Trp 86 mutated to Ala; Tyr 337 mutated to either Ala or Phe) and the peripheral site (Trp 286 mutated to either Ala or Arg) were analyzed with the cationic and uncharged alkyl phosphonates (Figure 1) to determine the contribution of the aromatic residues on the specificity of these com-

[2] The numbers in parentheses denote residue positions in the *T. californica* AChE sequence from which the molecular models were built.

Table 1: Bimolecular Rate Constants (10^3 M^{-1} min^{-1})[a] Determined for the Inhibition of Recombinant DNA-Derived Mouse Cholinesterases with Mutations of Anionic Residues by (a) Alkyl Methylphosphonyl Thiocholine Enantiomers and (b) Cycloheptyl Methylphosphonyl Thioate Enantiomers

(a) alkyl substitution	cycloheptyl			isopropyl			3,3-dimethylbutyl		
enzyme	S_p	R_p	S_p/R_p	S_p	R_p	S_p/R_p	S_p	R_p	S_p/R_p
AChE	190000 ± 20000	820 ± 50	230	16000 ± 1000	150 ± 10	110	400000 ± 40000	11000 ± 1000	36
D74N	1400 ± 200	8.0 ± 0.2	180	110 ± 10	1.7 ± 0.3	65	11000 ± 2000	230 ± 20	48
E202Q	21000 ± 2000	130 ± 10	160	490 ± 20	38 ± 2	13	120000 ± 10000	2700 ± 100	44
E450Q	1400 ± 300	23 ± 3	61	180 ± 20	5.9 ± 0.3	31	12000 ± 3000	490 ± 60	24

(b) thioate substitution	SCH_3			SCH_2CH_3		
enzyme	S_p	R_p	S_p/R_p	S_p	R_p	S_p/R_p
AChE	310 ± 20	1.7 ± 0.3	180	74 ± 5	0.16 ± 0.02	460
D74N	530 ± 50	2.3 ± 0.2	230	190 ± 30	0.41 ± 0.04	460
E202Q	14 ± 1	0.060 ± 0.004	230	2.3 ± 0.3	0.014 ± 0.001	160
E450Q	9.0 ± 0.4	0.050 ± 0.002	180	14 ± 2	0.018 ± 0.004	780

[a] Data shown as means ± standard error of the mean typically from three measurements.

Table 2: Bimolecular Rate Constants (10^3 M^{-1} min^{-1})[a] Determined for the Inhibition of Recombinant DNA-Derived Mouse Cholinesterases with Mutations of Aromatic Residues by (a) Alkyl Methylphosphonyl Thiocholine Enantiomers and (b) Cycloheptyl Methylphosphonyl Thioate Enantiomers

(a) alkyl substitution	cycloheptyl			isopropyl			3,3-dimethylbutyl		
enzyme	S_p	R_p	S_p/R_p	S_p	R_p	S_p/R_p	S_p	R_p	S_p/R_p
AChE	190000 ± 20000	820 ± 50	230	16000 ± 1000	150 ± 10	110	400000 ± 40000	11000 ± 1000	36
W86A	4800 ± 1100	25 ± 5	190	43 ± 6	23 ± 7	1.9	37000 ± 14000	1700 ± 200	22
Y337A	120000 ± 10000	840 ± 40	140	24000 ± 3000	340 ± 40	71	750000 ± 20000	19000 ± 1000	39
Y337F	720000 ± 30000	3700 ± 100	190	140000 ± 10000	1000 ± 100	140	1100000 ± 100000	30000 ± 1000	37
W286A	230000 ± 20000	2000 ± 100	120	20000 ± 1000	230 ± 10	87	470000 ± 30000	13000 ± 1000	36
W286R	45000 ± 2000	350 ± 10	130	8700 ± 600	98 ± 2	89	60000 ± 1000	1800 ± 100	33

(b) thioate substitution	SCH_3			SCH_2CH_3		
enzyme	S_p	R_p	S_p/R_p	S_p	R_p	S_p/R_p
AChE	310 ± 20	1.7 ± 0.3	180	74 ± 5	0.16 ± 0.02	460
W86A	170 ± 20	0.75 ± 0.01	230	63 ± 9	0.27 ± 0.002	230
Y337A	81 ± 4	0.46 ± 0.02	180	27 ± 2	0.047 ± 0.002	570
Y337F	320 ± 10	0.94 ± 0.04	340	92 ± 8	0.13 ± 0.02	710
W286A	530 ± 40	1.6 ± 0.2	330	160 ± 10	0.43 ± 0.09	370
W286R	710 ± 30	2.1 ± 0.2	340	200 ± 30	0.35 ± 0.09	570

[a] Data shown as means ± standard error of the mean typically from three measurements.

pounds; the bimolecular rate constants are summarized in Table 2a,b. Replacement of Trp 86 with Ala gives rise to a large reduction in reaction rate constants for both enantiomers of CHMP-thiocholine and, to a lesser extent, with DMBMP-thiocholines while conserving the enantiomeric preference seen for wild-type AChE. Although the rate constant of inhibition for iPrMP-thiocholine was also decreased, this mutation gave a significant shift in enantiomeric preference. A large decrease in the rate constant of reaction with the S_p isomer relative to the R_p isomer was evident, resulting in a 100-fold loss in selectivity for the S_p enantiomer.

Unlike that seen with the cationic alkyl phosphonates, the W86A mutation had a minimal influence on the reaction rate constant for the uncharged phosphonothioates where the k_i values for the thiomethylate and thioethylate compounds were affected at most 2-fold. This observation is consistent with W86 playing a role in stabilization of cationic ligands such as ATC while having little influence on the catalysis of uncharged ligands (Ordentlich et al., 1993a).

The second residue mutated in the choline subsite, Tyr 337 to either Ala or Phe, had a minimal effect on the rate constant of reaction for the thiocholine- and thioalkyl-substituted OPs as shown in Table 2a. Although small differences exist between rate constants for the wild-type and 337 mutant AChEs, it is noteworthy that the Y337A mutation reduced the reaction rate constants of the uncharged phosphonothioates 3−4-fold, indicating an aromatic residue at 337 slightly enhances the reaction.

The final aromatic residue analyzed, W286, resides in the peripheral site at the lip of the gorge and was mutated to either Ala or Arg. As shown in Table 2a, replacing Trp 286 with Ala at the rim of the gorge had little influence on the rate constants for the cationic OPs, showing at most a 2-fold enhancement for R_p CHMP-thiocholine. Introducing an Arg at the 286 position did, however, decrease the rate of inhibition by the cationic OPs. Most notably, the rate constants for CHMP- and DMBMP-thiocholines were affected more than those for iPrMP-thiocholine, indicating that Arg at the rim of the gorge impedes the rates of reaction for the bulkier alkyl-substituted OPs more than with the smaller isopropyl methylphosphonate. Furthermore, the changes in rate constants observed for the W286R mutation are conserved between enantiomers which is consistent with the cationic substitution restricting diffusional entry.

The rate constants for uncharged phosphonates were either maintained or enhanced with the W286A and W286R mutations (Table 2b), also suggesting that the 286 residue does not influence reaction rates through stabilization of the complex but rather may contribute through steric hindrance by the large indole ring of bulky ligands entering the gorge.

Influence on Diffusion of Ligands Entering the Gorge. The *meta*-substituted trifluoromethyl acetophenones (TFK) (Fig-

Table 3: Bimolecular Association Rate Constants (10^9 M^{-1} min^{-1})[a] Determined for the Inhibition of Mouse Wild-Type and Mutant AChEs by *m*-Trimethylammonio Trifluoromethyl Acetophenones (TFK^+) and *m*-*tert*-Butyl Trifluoromethyl Acetophenones (TFK^o)

enzyme	TFK^+	TFK^o
MAChE	180 ± 20	4.0 ± 0.5
D74N	5.1 ± 0.4	5.1 ± 0.1
E202Q	94 ± 4	4.5 ± 0.3
W286A	120 ± 10	2.6 ± 0.2
W286R	34 ± 2	2.3 ± 0.5
MAChE[b]	210 ± 20	3.0 ± 0.4
W86A[b]	19 ± 9	2.6 ± 0.8

[a] Values corrected for the ratios of hydrated to nonhydrated compound according to Nair et al. (1994). [b] Data of Radić et al. (1995).

ure 1), whose conjugates behave as transition state analogs to ATC, exhibit a diffusion-controlled association with bimolecular constants in the range of 10^9-10^{11} M^{-1} min^{-1} (Nair et al., 1993, 1994; Radić et al., 1995). In order to dissect the influence of residue substitutions on diffusion from the specific interactions that give rise to OP specificity, inhibition of AChE mutants at Asp 74, Trp 86, Glu 202, and Trp 286 by *meta*-substituted trimethylammonio and *tert*-butyl TFKs (TFK^+ and TFK^o, respectively) was analyzed (Table 3).

Mutations of anionic residues to neutral isosteres at the rim of the gorge and at the base of the gorge both resulted in decreases in the k_{on} of TFK^+; however, D74N exhibited a 35-fold reduction, whereas with E202Q, only a 2-fold reduction in the association rate constant was observed. The magnitude of these reductions is considerably less than that observed with the S_p and R_p CHMP-thiocholines. As for the uncharged *tert*-butyl-substituted TFK, the D74N and E202Q mutations essentially were without influence, a result which parallels that seen for uncharged CHMP-thioates with the D74N mutation but not for the E202Q mutation which reduces the OP reaction rate constant. Through comparison of on-rate constants for the cationic and uncharged analogs, it becomes apparent that among these residues Asp at position 74 solely accounts for the 50-fold faster association rate constant for the TFK^+ with AChE compared to that for TFK^o.

The most influential mutation from the choline subsite on inhibition rate constants for the cationic OPs, W86A, was reported previously to reduce the association rate constant of the TFK^+ analog with AChE while not altering that with the TFK^o analog (Radić et al., 1995). As with the mutations D74N and E202Q, the 11-fold reduction of k_{on} for trimethylammonio-substituted TFK with the W86A mutation is 3−4-fold less than that of the cationic CHMP-thiocholine compounds, suggesting that the rates of reaction of the cationic phosphonates have a greater dependence on Asp 74, Trp 86, and Glu 202 than does the cationic TFK^+ analog.

Substitution of an Arg for Trp at position 286 revealed that introducing a positive charge at the rim of the gorge reduced the bimolecular association rate constant of TFK^+ to a similar extent as that for S_p and R_p CHMP-thiocholines. However, the uncharged TFK analog was less affected with this mutation, indicating that TFK^+ association is hindered by electrostatic repulsive forces at the mouth of the gorge. The W286A mutation further supports this contention since Ala substitution slightly affects the association rate constant of the charged and uncharged TFKs to the same degree.

Effect of the Mutations on the Catalysis of Carboxyl Ester Hydrolysis. The changes in catalytic parameters for the hydrolysis of acetylthiocholine (ATC) were compared with data for the above substrates (Radić et al., 1993; Hosea et al., 1995). Velocities of hydrolysis for various ATC concentrations were fit according to Radić et al. (1993), and the resulting kinetic constants are given in Table 4. Related mutant ChEs reported previously have been included to allow for direct comparisons.

Table 4: Kinetic Constants[a] Calculated for the Catalysis of Acetylthiocholine by Mutant and Wild-Type AChEs

enzyme	K_m (μM)	k_{cat}[b] (10^3 min^{-1})	k_{cat}/K_m (10^6 M^{-1} min^{-1})
MAChE[c]	46 ± 3	140 ± 10	3000
D74N[d]	1300 ± 140	84 ± 11	65
E202Q[e]	130 ± 10	85 ± 13	650
E450Q[f]	140 ± 10	3.4 ± 0.4	24
W86A[g]	66 ± 19	2.8 ± 0.2	42
Y337A[h]	110 ± 20	52 ± 16	470
Y337F[i]	53 ± 5	68 ± 26	1300
W286A[j]	64 ± 3	140 ± 20	2200
W286R[k]	420 ± 60	160 ± 30	380

[a] Values are shown as the mean from three measurements ± the standard error of the mean. [b] k_{cat} was determined from titrations of the inhibition assay with MEPQ. Values for K_m, K_{ss}, and b were calculated using nonlinear regression to fit according to $v = [V_{max}(1 + b[S]/K_{ss})]/[(1 + K_m/[S])(1 + [S]/K_{ss})]$ (Webb, 1963) using Sigma Plot. [c] K_{ss} = 15 ± 2 mM, b = 0.23 ± 0.01; data of Radić et al. (1993). [d] K_{ss} = 530 ± 170 mM, b = 0; data of Radić et al. (1993). [e] K_{ss} = 103 ± 3 mM, b = <0.2. [f] K_{ss} = 59 ± 22 mM, b = 1.8 ± 0.1. [g] K_{ss} = 20 ± 9 mM, b = 8.2 ± 1.0; data of Radić et al. (1995). [h] K_{ss} = 29 ± 21 mM, b = 0.59 ± 0.08; data of Radić et al. (1993). [i] K_{ss} = 10 ± 2 mM, b = 0.42 ± 0.03; data of Radić et al. (1993). [j] K_{ss} = 46 ± 12 mM, b = 0.26 ± 0.13. [k] K_{ss} = 23 ± 8 mM, b = 0.24 ± 0.06; data of Radić et al. (1993).

The mutation, D74N, significantly affected the K_m value, unlike removing the charges located at the base of the gorge as with the E202Q and E450Q mutations (Table 4). E450Q however substantially reduced the maximum rate of turnover, k_{cat}, of ATC, whereas removing the negative charges at positions 202 and 74 had very little effect on k_{cat}. The combination of the reduced k_{cat} with E450Q and the increased K_m with D74N results in enzymes less efficient in catalyzing the hydrolysis of ATC as seen by comparing k_{cat}/K_m of the mutants and wild-type AChEs. Comparatively, the anionic character of Glu 202 seems to contribute less to binding and turnover of ATC since both the k_{cat} and the K_m are only slightly modified with the E202Q mutation. Replacement of Glu 202 with Gln in TAChE (Glu 199) resulted in a near 50-fold reduction in k_{cat}/K_m (Radić et al., 1992), while in human AChE, the mutation showed an 18-fold reduction in k_{cat}/K_m (Shafferman et al., 1992), revealing that species differences exist in the magnitude of influence of this conserved anionic residue.

Mutations in the choline binding domain (Sussman et al., 1991) that we examined are W86A, Y337A, and Y337F. As shown in Table 4, mutations at Tyr 337 and the W86A mutation have essentially no effect on the K_m, while substitutions at these two positions have very different effects on the maximum turnover of ATC since mutating the Tyr 337 slightly reduced the k_{cat} and W86A shows a 50-fold reduction in the maximum turnover and, consequently, in the efficiency of the catalytic hydrolysis of ATC. From these data, it was suggested that the aromaticity of Trp 86 stabilizes the positively charged choline group for the most efficient hydrolysis (Ordentlich et al., 1993a; Radić et al., 1993, 1995), but the effective removal of an indole side chain with this substitution will also create a substantial volume difference and may rather significantly perturb local structure and polarity in this region.

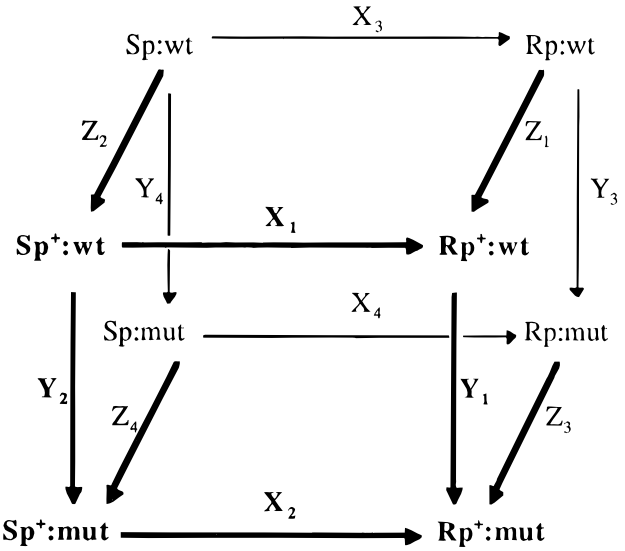
The third region of analysis at position 286 reveals that introducing a positively charged residue at the rim of the gorge as in W286R significantly increases K_m, whereas replacing the Trp with Ala has no effect. Additionally, the reduced k_{cat}/K_m value for W286R, but not for W286A, indicates that Trp 286 in AChE does not influence the catalysis of ATC hydrolysis by stabilization of the quaternary group but, as seen with the TFKs, may gate entry of cationic ligand passing into the gorge.

DISCUSSION

The chemical diversity of substrates catalyzed by AChE can be used advantageously to delineate distinct steps in the catalytic pathway and, in turn, residues governing the specificity of the component steps. The trifluoromethyl acetophenones conjugate with the active center serine to form a hemiketal without departure of a leaving group. The organophosphonates rapidly acylate the enzyme with loss of a leaving group, but subsequent deacylation is typically slow. The carboxyl esters show rapid acylation and deacylation rates. Of these three chemical classes, only the organophosphonates possess an asymmetric center at the point of nucleophilic attack by the enzyme, thus adding another dimension to the analysis of specificity. In this study, we employ charged and uncharged congeners of enantiomeric phosphonates and trifluoromethyl acetophenones with specific mutations in AChE to examine residues that influence substrate diffusion to and association with the AChE active center.

Recognition versus Phosphonylation. The absolute stereochemistry of the enantiomeric organophosphonates is known (Berman & Leonard, 1989), and substantial evidence has emerged from experimental data (Cygler et al., 1994; Hosea et al., 1995) and theoretical considerations (Sussman et al., 1991; Harel et al., 1991, 1996; Barak et al., 1992) to show that their rapid acylation of the enzyme requires insertion of the phosphonyl oxygen in the oxyanion hole. Our findings that the D74N mutated enzyme loses reactivity with the cationic phosphonates, but not with the uncharged agents (Table 1), implicate the importance of an electrostatic interaction in orientation of the phosphonate. Moreover, that reactions with the enantiomers are equally altered with the D74N mutation indicates that the cationic groups of the R_p and S_p enantiomers and D74 are separated by the same distances in their initial docking positions. We conclude, therefore, that the docking orientation of the tetrahedral phosphonate is such that the leaving group (thiocholine) is directed toward the mouth of the gorge. Of significance is that this orientation is achieved for both (R_p)- and (S_p)-methylphosphonyl thiocholines. With the positions of the P═O and the thiocholine groups identified, the differences in reactivity of the enantiomeric phosphonylthiocholines can now be seen to depend on the relative orientation of the remaining two substituents: the methyl and alkoxyl groups. Accordingly, the reduced reactivity of the R_p enantiomer relative to that of the S_p enantiomer must therefore reflect respective differences primarily in steric restrictions encountered by each enantiomer with proximal side chains in the active center gorge. In fact, the 230-fold preference for the S_p enantiomers arises from the steric constraints precluding the optimal fit of the more bulky alkoxyl group in the acyl pocket, an apparent requirement for efficient phosphonyl transfer (Hosea et al., 1995).

Scheme 1



Mutant Cycle Analysis and Orientation of the Phosphonates. Our data of AChE mutagenesis in relation to the charge on the leaving group of the organophosphonate may be combined with previous data on acyl pocket mutations and enantiomer selectivity (Hosea et al., 1995) to analyze the linkages between ligand structure and the mutations by a mutant cycle analysis (Scheme 1) (Wolfenden, 1978; Carter et al., 1984; Hidalgo & MacKinnon, 1995; Schreiber & Fersht, 1995). In Scheme 1, enantiomeric selectivities of the bimolecular rate constants for organophosphonate reaction with AChE [i.e. $k(S_p$:wt)] in relation to the mutations (*XY* planes) are defined by

$$\psi_+ = \frac{S_p^+}{R_p^+} = \frac{X_1}{X_2} = \frac{Y_2}{Y_1} = \frac{k(S_p^+\text{:wt})k(R_p^+\text{:mut})}{k(R_p^+\text{:wt})k(S_p^+\text{:mut})} \quad (1)$$

$$\psi_o = \frac{S_p}{R_p} = \frac{X_3}{X_4} = \frac{Y_4}{Y_3} = \frac{k(S_p\text{:wt})k(R_p\text{:mut})}{k(R_p\text{:wt})k(S_p\text{:mut})} \quad (2)$$

The electrostatic influence of charge on the leaving group with respect to the mutations (*ZY* planes) is defined by

$$\phi_S = \frac{S_p}{S_p^+} = \frac{Z_2}{Z_4} = \frac{Y_4}{Y_2} = \frac{k(S_p\text{:wt})k(S_p^+\text{:mut})}{k(S_p^+\text{:wt})k(S_p\text{:mut})} \quad (3)$$

$$\phi_R = \frac{R_p}{R_p^+} = \frac{Z_1}{Z_3} = \frac{Y_3}{Y_1} = \frac{k(R_p\text{:wt})k(R_p^+\text{:mut})}{k(R_p^+\text{:wt})k(R_p\text{:mut})} \quad (4)$$

These values are represented as absolute values without reference to sign. They may be converted to the change in free energy of activation for the corresponding reactions, $\Delta\Delta G^{\ddagger}$, where

$$\psi_+^{\ddagger} = RT \ln \psi_+ \quad (5)$$

$$\psi_o^{\ddagger} = RT \ln \psi_o \quad (6)$$

$$\phi_S^{\ddagger} = RT \ln \phi_S \quad (7)$$

$$\phi_R^{\ddagger} = RT \ln \phi_R \quad (8)$$

and $R = 1.99 \times 10^{-3}$ kcal mol^{-1} K^{-1} and $T = 298$ K.

Analysis of the coupling between enantiomer selectivity of cycloheptyl methylphosphonyl thiocholine and mutations

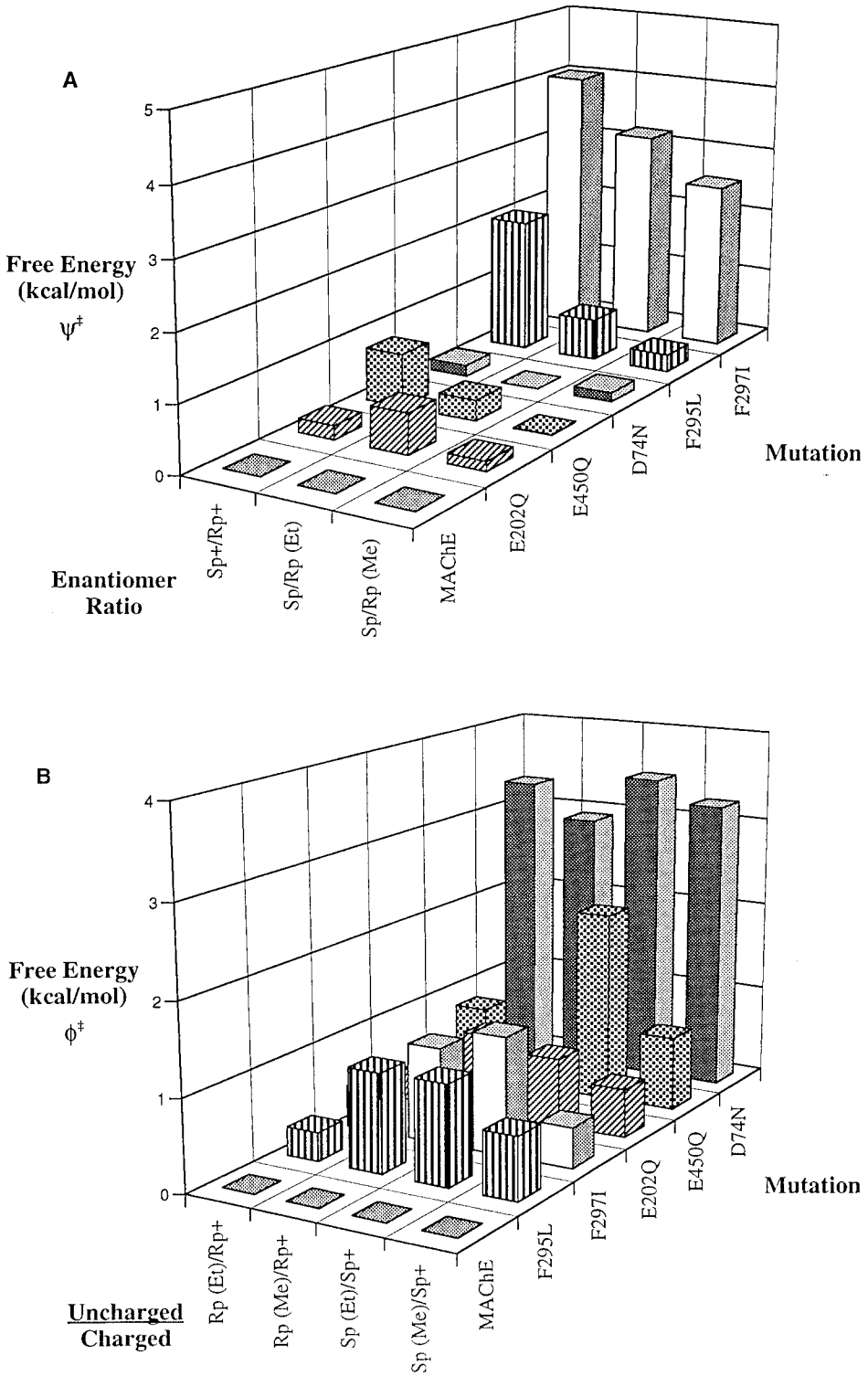


FIGURE 2: Relationship between the change in free energy of activation, selected mutations in acetylcholinesterase, and structures of cycloheptyl methylphosphonyl thioates. (A) Selectivity for the S_p and R_p enantiomers. (B) Selectivity for charged and uncharged phosphonates: +, cationic cycloheptyl methylphosphonyl thiocholine; Me, uncharged cycloheptyl methylphosphonyl thiomethane; and Et, uncharged cycloheptyl methylphosphonyl thioethane. The scheme for the analysis is shown in Scheme 1, and equations are detailed in the text.

is shown in Figure 2A. The dominant influence of the acyl pocket, and in particular residue 297, is evident. It is also apparent that the influence of residues 295 and 297 is greatest with the larger thiocholine leaving group, but the influence of the 297 residue prevails irrespective of the charge on the leaving group. The influence of mutating the charged residues or other residues in the choline binding site (not shown) is of relatively small consequence. Analysis of coupling between the charge on the leaving group and the mutations is shown in Figure 2B. We observe that, despite large influences of charge on the reactivity of the cationic and uncharged phosphonates, Asp 74 has the dominant influence on altering relative selectivity for the phosphonates containing a charged leaving group. This phenomenon prevails irrespective of whether the R_p or S_p enantiomers are employed.

Hence, analysis of the linkage between residue mutations and enantiomeric specificity in one dimension and between charge on the leaving group in a second dimension shows a distinct dependence on residue location. Moreover, the

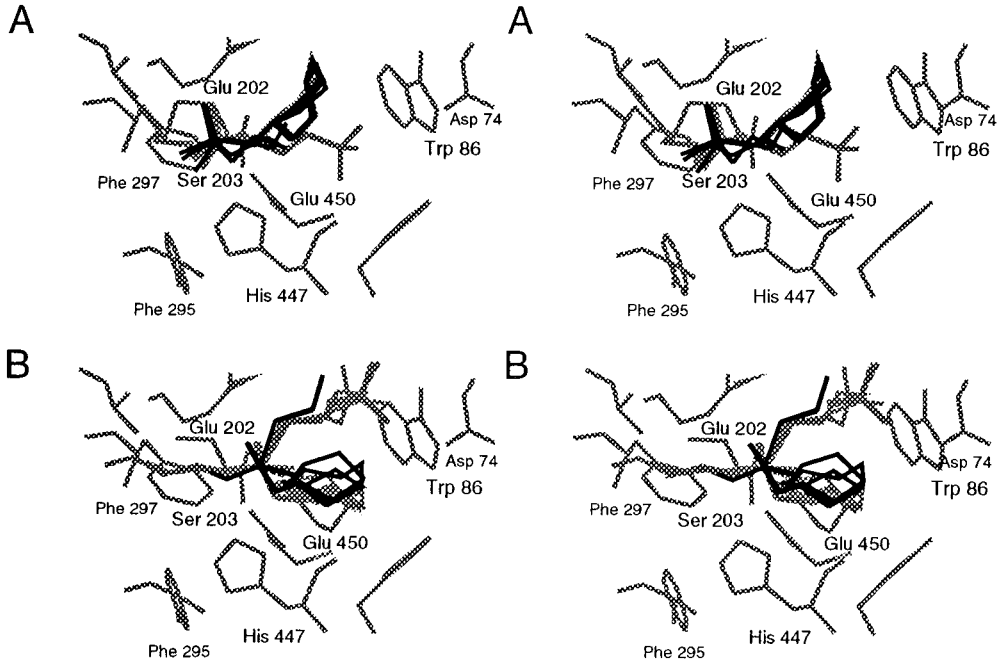


FIGURE 3: Stereoviews of the computational docking using molecular dynamics (see Materials and Methods) of (A) S_p and (B) R_p cycloheptyl methylphosphonyl thiocholines (gray) and cycloheptyl methylphosphonyl thioethylates (black) showing the spatial arrangement of the substituents about the phosphate.

proposed orientation of the organophosphonate in its transition state (Figure 3) is completely consistent with the positions of the residues on AChE dictating specificity.

Figure 3 shows the docking orientation achieved for the two enantiomers using molecular dynamics. With the phosphonyl oxygen directed toward the oxyanion hole (amide backbone nitrogens of Gly 121, Gly 122, and Ala 204 as hydrogen bond donors) and the leaving groups of both R_p and S_p isomers directed toward Asp 74, the cycloheptyl and methyl substituents remain fixed in space with respect to the enzyme coordinates. Previous studies showed that the bimolecular inhibition constants for uncharged and cationic OPs differ by up to 3 orders of magnitude. Analysis of the kinetics shows that 2 orders of magnitude are attributable to affinity and 1 order of magnitude is attributable to unimolecular phosphonylation (Berman & Leonard, 1989). The present study indicates a role for D74 in establishing the orientation of the cationic OPs prior to reaction and further suggests that the interaction of the thiocholine moiety with D74 further stabilizes the transition state. The uncharged phosphonothioates, since they cannot derive electrostatic stabilization energy through ion-pair formation with D74, bind with lower affinity than the cationic OPs. Consequently, they are less likely to be oriented by D74, and free diffusion of these agents into the gorge has a higher probability of resulting in nonproductive orientations (Berman & Leonard, 1989).

Reductions in inhibition constants with increasing chain length of substituted alkylphosphonylthiocholines have been shown to result mostly from reduction in phosphorylation rate while with identically substituted alkyl phosphonylfluoridates to result mostly from reduction in affinity (greater K_d) (Berman, 1995). This phenomenon is thought to arise from constraints imposed on the alkylphosphonylthiocholines, but not the fluoridates, which impede rotation for optimal reaction of phosphonylation, allowing one to conclude that the fate of the orientation for the cationic alkyl phosphonates is determined near the rim of the gorge. Our data on the D74N mutation suggest that this Asp residue at this location likely serves to dock the phosphonate inhibitor prior to and during formation of the transition state, thus dictating the orientation of cationic ligands in the active center.

Results of ligand docking of the reversible complex of AChE with CHMP congeners using molecular dynamics show that initial docking of the S_p enantiomer occurs with the leaving group thiocholine approximating an apical position 180° from the apical position of the γ-oxygen of Ser 203. At the same time, the phosphonyl oxygen resides within hydrogen-bonding distance (3−4 Å) of the hydrogen bond donors of the oxyanion hole (Figure 4). This orientation, in that the attacking nucleophile and the leaving group adapt apical positions and the P═O bond is polarized through hydrogen bonding in the oxyanion hole, represents a productive configuration allowing for facile direct in-line attack by the γ-oxygen of Ser 203 and facile displacement of the thioate moiety. The dynamics for the less reactive, R_p enantiomers, however, show that either positioning of the P═O bond or apical positioning of the leaving group is possible; but one cannot obtain the two positions simultaneously (Figure 4). The primary reason for such exclusion is that the positioning of the thiocholine moiety toward D74 requires positioning of the cycloheptyl moiety in the acyl pocket, where it encounters steric hindrance with Phe 295 and 297 (Hosea et al., 1995). Thus, the reduced reaction rate constants for the R_p enantiomers in comparison with the S_p enantiomers are due to steric hindrance within the acyl pocket, as shown previously (Hosea et al., 1995).

Orientation of Tetrahedral Phosphonates Compared to That of Trigonal Carboxyl Esters. With the position of the leaving group directed out of the gorge and the phosphonyl oxygen in the oxyanion hole, the more energetically favorable in-line attack by γ-oxygen of Ser 203 on one face of the tetrahedron of the S_p and R_p isomers would then give rise to choline subsite occupation by the alkoxyl substituent of the S_p isomer and the methyl substituent of the R_p isomer (Hosea et al., 1995). Evidence for such a spatial arrangement derives from kinetic analysis of the aging profiles of the final

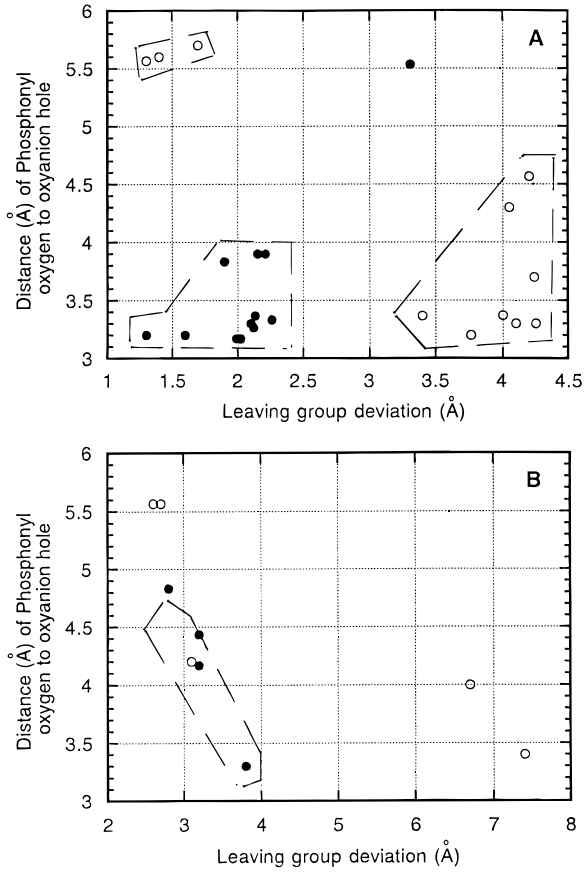


FIGURE 4: Relationship between phosphonyl oxygen location and leaving group orientation for (A) S_p (closed circles) and R_p (open circles) cycloheptyl methylphosphonyl thiocholines and for (B) S_p (closed circles) and R_p (open circles) cycloheptyl methylphosphonyl thioethylates in reversible complexes with TAChE. Each circle represents one conformer. Productivity is assumed to require insertion of the phosphonyl oxygen of CHMP congeners into the oxyanion hole (formed by the amide backbone hydrogens of Gly 121, Gly 122, and Ala 204), where the phosphonyl oxygen mean distance is measured from the three potential hydrogen bond donors. Leaving group deviation refers to the distance of the quaternary nitrogen (for the charged thiocholine complexes) or the β-carbon (for the neutral thioethylate complexes) of the leaving group from that of a conformer in the pentavalent complex which most closely resembles the ideal leaving group position of 180° from the γ-oxygen of Ser 203, for an S_N2 concerted mechanism which would give apical positions of the γ-oxygen and leaving group (Westheimer, 1968). The ideal conformer was approximated by S_p CHMP-thiocholine, which kinetically is most reactive.

cycloheptyl methylphosphonyl (CHMP) AChE conjugates. Upon dealkylation during aging of the conjugate of S_p CHMP-AChE (reaction with S_p CHMP thiocholine), the fluorescent bisquaternary inhibitor decidium exhibits enhanced affinity with the conjugated enzyme, a phenomenon not present with conjugates formed by reactions with the R_p isomer (Berman & Decker, 1986, 1989). In that decidium likely occupies the choline subsite with one of the quaternary ammonium groups in the nonacylated enzyme, the differences observed between the reactions with S_p and R_p enantiomers provide evidence for choline subsite occupation by the alkoxyl group of the S_p enantiomer which departs through dealkylation during aging. Furthermore, the reduced aging rates with the conjugates formed through reactions with the R_p enantiomer suggest that the alkoxyl substituent resides elsewhere while positioning the methyl substituent in the choline subsite. Molecular modeling further supports this spatial arrangement of the substituents and shows, during the pentavalent intermediate, occupation of the choline subsite by the alkoxyl moiety of the S_p isomer and the methyl substituent of the R_p isomer for both the uncharged and cationic CHMP congeners (Figure 3).

Examination of the crystal structure of TAChE conjugated with TFK^+ allows for a comparison of distances between critical anionic and aromatic residues in AChE and the quaternary ammonium groups of TFK^+ (Harel et al., 1996) and the two organophosphonate enantiomers (Figure 3). The cationic, quaternary ammonium moiety in the tetrahedral conjugate of TFK^+ with AChE is 3.7 Å from Trp 86 $(84)^2$ and nearly 9 Å away from Asp 74 $(72)^2$. Therefore, the decrease in the association rate constant of TFK^+ with the D74N mutated enzyme (Table 3) likely arises through diminishing the diffusion rate of the ligand into the gorge. By contrast, in the pentavalent intermediate formed from the tetrahedral phosphonates, the quaternary ammonium moiety appears equidistant from Asp 74 and Trp 86. The type of interaction that can be expected from the Trp 86 is cation$-\pi$, whereas that from Asp 74 is ion-pair, both of which are suggested to be Coulombic in nature where the energy of the interaction is inversely proportional to the distance between the charges (Dougherty, 1996). That Trp 86 contributes negative charge from the center above and below the face of the ring and possesses a positive charge on the perimeter of the ring [cf. Creighton (1993) and Dougherty (1996)] suggests Coulombic interactions involving cation$-\pi$ interactions would be strongest when the cation resides in-line with the quadrupole moment perpendicular to the face of the aromatic ring (Dougherty, 1996). On the other hand, the strength of interaction between the cationic quaternary ammonium moiety and Asp 74, possessing a negative charge, should not be dependent on the angle of the substituent orientation. Thus, the leaving group position directed toward D74 and along side W86 represented in Figure 3 indicates that Asp 74 would contribute more to electrostatic stabilization of the OP$-$AChE complex than Trp 86 and is consistent with D74N having a more dramatic affect on rates with cationic phosphonates than Trp 86.

Distinct Activation Barriers for the Organophosphonates and Trifluoromethyl Acetophenones. The trifluoromethyl acetophenones and organophosphonates can be expected to have distinct activation barriers for their reactions with AChE. Hence, Asp 74 will affect different steps in the two overall reactions. In the case of TFK^+, the activation barrier arises from diffusion of the ligand through the restricted gorge dimensions. Accordingly, the D74N mutation exerts its influence on the diffusional step of TFK^+ entry. At the base of the gorge, Trp 86 stabilizes the cationic moiety (Harel et al., 1996), and the diffusion-limited reaction rate constant (1.8×10^{11} M^{-1} min^{-1}) suggests that TFK^+ conjugation with the active site serine occurs without traversing through an additional, limiting activation barrier. Association of TFK^+ with the W86A mutant enzyme has a decreased association rate constant and increased dissociation rate constant (Radić et al., 1995). This reflects not only formation of a less stable conjugate but also the imposition of a second activation barrier for association. The latter may arise from the multiple orientations of TFK^+ in the mutant AChE gorge when the indole ring is removed, many of which may be unproductive.

The alkyl phosphonates exhibit reaction rates below the diffusion limitation, which suggest that an activation barrier is encountered with acylation by the phosphonate. Whether this arises in the formation of a pentavalent intermediate or in the dissociation of the leaving group is not resolved.

However, it is likely that the charge on Asp 74 influences this activation barrier either through facilitating the positioning of the charged organophosphonate with the thiocholine directed out of the gorge or in facilitating the loss of the thiocholine leaving group. The dissection of bimolecular rate constants using stopped-flow methods into components of affinity and phosphonylation should further elucidate determinants which dictate productive versus nonproductive binding.

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