Specificity and Orientation of Trigonal Carboxyl Esters and Tetrahedral Alkylphosphonyl Esters in Cholinesterases[†]

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ABSTRACT: We have examined the specificity of planar carboxyl and tetrahedral phosphonyl esters for mouse cholinesterases and have delineated the orientation of these ligands in the enzyme active center. The approach involved altering acyl pocket dimensions by site-specific mutagenesis of two phenylalanines and varying ligand size and enantiomer presentation. Substrate catalysis rates by wild type acetylcholinesterase (AChE) of acetyl-, butyryl-, and benzoylthiocholine diminished with increasing size of the acyl moiety. In contrast, substitution of the acyl pocket phenylalanines giving the mutants F295L and F297I of AChE yielded more efficient catalysis of the larger substrates and a specificity approaching that of butyrylcholinesterase. Extension from planar substrates to enantiomerically pure organophosphonates allowed for an analysis of enantiomeric selectivity. We found that AChE reactions are 200-fold faster with the S_p than the R_p enantiomer of cycloheptyl methylphosphonyl thiocholine. Upon the acyl pocket size being enlarged, the R_p enantiomer became more reactive while reaction with the S_p enantiomer was slightly reduced. In fact, the F297I mutant displayed inverted stereospecificity. A visual correlation with the kinetic data has been developed by docking the ligands in the active site. Upon placement of the phosphonyl oxygen in the oxyanion hole and the leaving group being directed out of the gorge, the R_p, but not the S_p, enantiomer engendered steric hindrance between the alkoxyl group and the acyl pocket. Replacing F297 with Ile accommodated the bulky alkoxyl group of the R_p isomer in the acyl pocket, allowing similar orientations of the phosphonyl oxygen and the leaving group to the S_p isomer. Thus, analysis of reaction rates and absolute stereochemistry enabled us to position the organophosphonates and ascertain loci of interaction of their functional groups in the active center gorge.

Acetylcholinesterases (AChEs)[1] catalyze ester hydrolysis of the neurotransmitter acetylcholine, resulting in termination of neurotransmission. As found in the serine hydrolase family of enzymes, mammalian AChE employs a catalytic triad of Glu 334, His 447, and Ser 203, where the serine residue is the proximal nucleophile in the hydrolysis mechanism (Gibney et al., 1990; Sussman et al., 1991). The butyrylcholinesterases (BuChEs), which share about 55% amino acid sequence identity (Cygler et al., 1993), also hydrolyze acetylcholine with high efficiency. However, AChE and BuChE differ in their capacity to catalyze the hydrolysis of substrates larger than acetylcholine, where BuChE can efficiently hydrolyze butyrylcholine and benzoylcholine and AChE shows diminished catalysis rates with substrates larger than propionylcholine (Augustinsson, 1948). The side chains of two phenylalanines, numbered as Phe (F) 295 and 297 in mouse, form a boundary to the site of binding of the substrate acyl moiety sterically restricting larger acyl containing substrates from optimal positioning for catalysis in the active site (Harel et al., 1992; Vellom et al., 1993; Radic′ et al., 1993; Ordentlich et al., 1993). Aliphatic residues of smaller dimensions are found at the corresponding positions in BuChE (Cygler et al., 1993), for example Leu (L) and Ile (I) in mouse, allowing larger substrates to fit into the active site in an orientation appropriate for efficient catalysis. Individual mutant cholinesterases containing F295L and F297I have enabled us to examine the basis of the divergence between AChE and BuChE inhibitor specificity and kinetics (Vellom et al., 1993).

Cholinesterases also catalyze the acylation of the enzyme by organophosphonates, ligands which react with the enzyme by rapidly phosphonylating the active site serine (Oosterbaan & Cohen, 1964; Aldridge & Reiner, 1972). However, unlike carboxyl ester substrates, the phosphonyl enzyme reacts slowly with water, and thus, these ligands behave as hemisubstrates (Froede & Wilson, 1971). Their selectivity for the cholinesterases has been a subject of continued study over the past fifty years [cf. Froede and Wilson (1971) and Aldridge and Reiner, (1972)]. Furthermore, AChE reaction with organophosphonates displays marked stereospecificity and dependence on substituent size with differences generally greater than 200-fold in reaction rates between enantiomers (Berman & Leonard, 1989). This specificity might arise from steric hindrance by the acyl pocket phenylalanines as found for carboxyl ester substrates.

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[1] Abbreviations: AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; Phe or F, phenylalanine; Leu or L, leucine; Ile or I, isoleucine; Ala or A, alanine; Tyr or Y, tyrosine; ATC, acetylthiocholine; BuTC, butyrylthiocholine; BzTC, benzoylthiocholine; OP, organophosphonates; CHMP, cycloheptyl methylphosphonyl; iPrMP, isopropyl methylphosphonyl; DMBMP, 3,3-dimethylbutyl methylphosphonyl; k_i, bimolecular rate constant in min^{-1} M^{-1}.

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Organophosphonates, by virtue of their tetrahedral configuration, afford an additional dimension in the study of structure−activity relationships and, therefore, complement and extend the analyses derived from the study of planar substrate molecules. Moreover, the availability of resolved enantiomerically pure methylphosphonates offers a unique means for analysis of configuration and spatial orientation in the active center with respect to the available three dimensional crystal coordinates of *Torpedo californica* AChE (Sussman et al., 1991). Herein, we examine the role of the acyl pocket of mouse cholinesterases in dictating stereospecificity for enzyme acylation by the organophosphonates and describe the positioning of tetrahedral and planar ligands in the active center of the enzymes. Structural models consistent with the reaction kinetics provide plausible orientations achieved by the phosphonates within the active site.

MATERIALS AND METHODS

Materials. Acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BuTC), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were products of Sigma Chemical Co, St. Louis, MO. Benzoylthiocholine iodide (BzTC) was a product from TCI-GR, Japan. (S_p)- and (R_p)-alkyl methylphosphonyl thioates were synthesized and isolated as resolved R_p and S_p enantiomers as described previously (Berman & Leonard, 1989). 7-[[(Methylethoxy)phosphinyl]-oxyl]-1-methylquinolinium (MEPQ) was a gift of Y. Ashani and B. P. Doctor (Walter Reed Army Research Center, Washington, DC; Levy & Ashani, 1986).

Production of Enzymes. Wild type AChE and BuChE and mutant AChE constructs were generated as described in Radic′ et al. (1993). Site-specific mutants not described previously were generated using the same procedures except pBluescriptKSII(-) vector and VCSM13 helper phage (Stratagene) were used to obtain a single stranded template. Mutations were conducted in cassettes flanked by BstXI sites (490 bp fragment) which were sequenced in their entirety by double stranded sequencing. The pRCCMV (Invitrogen) expression plasmids were purified by standard procedures involving poly(ethylene glycol) precipitation and centrifugation on CsCl gradients.

Human embryonic kidney (HEK-293) cells obtained from American Type Culture Collection (Atlanta, GA) were plated at 1.5×10^6 cells/10 cm of 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 constructs 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 for 48−72 h. The medium, containing a secreted form of cholinesterase, was collected, and the transfected cells were supplied fresh serum free DME. This process was continued for three to four harvests of enzyme. The medium was then concentrated using Centriprep30 Centicons (Amicon, Beverly, MA) to approximately 1−2% of original volume and stored at 4 °C.

To produce larger quantities of enzyme for detailed kinetic studies, stable transfectants were generated by selecting transiently 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 for future uses.

Enzyme Activity Measurements and Active Site Quantitation. AChE, BuChE, and mutant cholinesterase activities were measured in 0.1 M $NaPO_4$, pH 7.0, and 0.3 mM DTNB at 22 °C according to the procedure of Ellman et al. (1961) using ATC, BuTC, or BzTC as substrates. Maximum concentrations used were 100 mM for ATC and BuTC and 10 mM for BzTC due to spontaneous substrate hydrolysis and maximum solubility, respectively. Active sites were quantitated according to the procedures of Levy and Ashani (1986) and Radic′ et al. (1992) by titrating the enzyme samples with known concentrations of MEPQ.

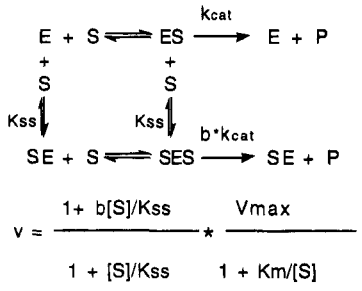
Organophosphonate Inhibition. Enzyme samples (tens of picomoles) were incubated for various amounts of time with the inhibitor in the above assay mixture in the absence of substrate; typically, four inhibitor concentrations were used. The extent of inhibition was determined by measuring the residual activity with 5 mM ATC after a designated time of incubation. 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) (Radic′ et al., 1992).

Computer Modeling. Binding of butyrylcholine, benzoylcholine, and (S_p)- & (R_p)-cycloheptyl methylphosphonyl thiocholines to AChE as reversible ligands was modeled using InsightII (version 2.3.5), Discover (Biosym Technologies, San Diego, CA), and the X-ray crystal structure of *Torpedo californica* AChE (Sussman et al., 1991). The ligands were docked manually in the active site in a position that would minimize collisional interactions between molecules and in a position appropriate for an S_N2 type reaction involving Ser 203 in mouse (200)[2] in *Torpedo*. Mutant structures were generated by replacing Phe 295 (288) with Leu and Phe 297 (290) with Ile prior to docking and energy minimization. The choline and acyl moieties of BuTC and BzTC were directed toward Trp 86 (84) and towards residues Phe 295 and 297, respectively.

The OP inhibitors were docked initially for a nucleophilic attack of the plane defined as the methylalkoxylphosphonyl oxygen (CH_3-OR-P(O)) face, or the plane opposite the leaving group, by Ser 203. This places the leaving group approximately 180° from the side chain oxygen of Ser 203 and directed out of the gorge, facilitating concomitant apical displacement with apical attack. The bulky moieties of the R_p and S_p enantiomers were directed to different domains, R_p to the acyl pocket and S_p to the choline subsite, in order to satisfy the leaving group position and to place the phosphonyl oxygen with in hydrogen-bonding distance with the oxyanion hole defined by Gly 121 (118) and 122 (119) and Ala 204 (201) (Sussman et al., 1991; Harel et al., 1991; Barak et al., 1992; Cygler et al., 1994). For all the ligands, generic constraints between serine oxygen and the carbonyl carbon of the substrates or phosphorus of the organophosphonates were set at 3.5 Å and between the carbonyl or phosphonyl oxygen and the nitrogens of the amide backbone constituting the oxyanion hole at 3.0 Å, as the upper limit with no lower limit constraint. An upper force (upr–K) limit of 1000 was used to satisfy the distance constraints with the

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

Scheme 1



lower force (lwr_*K*) set at 0. Energy minimizations were completed on the restrained molecules with the side chains proximal to the bound ligand left free to move. They were run until convergence was achieved at a maximum derivative of 0.001.

RESULTS

Acyl Pocket Dimensions and Carboxyl Ester Substrate Size. Wild type and mutant cholinesterases have been examined kinetically with acylthiocholine esters of varying chain length in order to examine the relationship between the catalytic parameters and substrate dimensions. In particular, with a wider selection of mutant enzymes, we have measured substrate catalysis as a function of substrate concentration for ATC, BuTC, and BzTC and have fitted the corresponding data to Scheme 1[3] as described in Radic' et al. (1993).

As shown in Scheme 1, the enzyme associates reversibly with carboxyl substrates in two distinct fashions, resulting in ES and SE binary complexes. These two complexes differ in their affinities and in affecting substrate catalysis. The ES complex represents substrate bound to the active site and leads to hydrolysis. The SE complex, on the other hand, results from binding of substrate to a secondary site peripheral to the active site and does not lead to direct hydrolysis. Since binding to the two sites is not mutually exclusive, a ternary complex SES can also form. The SES complex could lead to substrate hydrolysis with varying degrees of efficiency, described as a fraction of ES turnover and represented by b. Three possible scenarios are possible: when $b < 1$, the SES complex is less productive than the ES complex which indicates that binding to the secondary site leads to substrate inhibition; when $b = 1$, SES is as productive as ES and is silent in substrate catalysis measurements; and finally, when $b > 1$, the SES complex is more productive than ES and results in substrate activation. When K_{ss} approaches the maximum substrate concentration employed experimentally (100 mM for ATC and BuTC and 10 mM for BzTC), uncertainties of both K_{ss} and b increase.

The three substrates, ATC, BuTC, and BzTC (Figure 1), were examined with wild type mouse AChE, wild type mouse BuChE, and acyl pocket mutants, where the Phes 295 and 297 were mutated to Leu and Ile, respectively, to Ala, or to Tyr. Table 1, which tabulates the K_m, K_{ss}, b, and k_{cat} values, shows that mutating 295 and 297 to the corresponding aliphatic groups found in mouse BuChE, Leu and Ile respectively, gives rise to a reduction in k_{cat} for ATC and an enhancement in k_{cat} for BuTC and BzTC, kinetic properties which approach BuChE's catalytic behavior. When examined in terms of efficiency of catalysis (k_{cat}/K_m, Table 1), F295L and F295A display the most dramatic increase in BuTC catalysis, and in fact, mutation at this position yields constants exceeding those of BuChE wild type. With the largest substrate BzTC, F295L and F297I show similar enhancements of catalysis; however, the constants are still diminished compared to those of BuChE. This indicates that while the acyl pocket dimensions may constrain catalytic rates of large substrates, they are not the sole determinant in the dependence of catalytic constants on substrate dimensions.

Mutating Phe 295 and Phe 297 to alanines and tyrosines gave unexpected results. F295A demonstrated kinetic parameters (k_{cat} and K_m) with all three substrates similar to those of the corresponding Leu mutation. However, F297A displayed greater catalytic turnover rates (k_{cat}) for ATC and lower rates for BzTC than seen with the corresponding Ile mutation, indicating that simply removing steric bulk does not ensure a greater capacity to hydrolyze choline substrates with large acyl groups. Examining the mutation to tyrosine further supports this contention, since enlarging the residues at 295 and 297 by the addition of a hydroxyl group to a phenyl ring does not further limit the rates of hydrolysis of the largest substrate BzTC. In fact, k_{cat}/K_m values for F295Y and F297Y with BzTC are approximately 10-fold greater than those found with AChE wild type. This phenomenon may be a consequence of altered structural integrity of the acyl pocket in the mutant enzymes. It should be noted that no native cholinesterases thus far sequenced contain Ala or Tyr at positions corresponding to 295 and 297 in mouse AChE (Cygler et al., 1993).

As initially reported by Radic' et al. (1993), and extended by the data in Table 1, AChE and BuChE show distinct behaviors when a second molecule of substrate binds to the enzyme, forming a ternary complex. The SES complex of BuChE with ATC is more active than ES, whereas this ternary complex in AChE is less active. The acyl pocket mutants F295L and F297I differ when ATC and BuTC bind at a peripheral site; while F295L resembles AChE in that this mutant species displays substrate inhibition ($b < 1$). F297I resembles BuChE in that the mutant species shows substrate activation ($b > 1$). Hydrolysis of the largest substrate BzTC by AChE wild type, F295L, and F297I occurs with substrate activation and therefore displays behavior that differs qualitatively from that of BuChE, which shows substrate inhibition with BzTC. Nonetheless, these observations signify that the residue at position 297 plays a role in linking occupation at a peripheral site with substrate hydrolysis at the base of the active center gorge. When the residue at 297 is a Phe, as in AChE wild type, the enzyme displays substrate inhibition; when the residue at 297 is Ile, as in BuChE wild type, substrate activation is evident [see also, Radic' et al., 1993)].

Influences of Acyl Pocket Dimensions on Reaction Rates with Enantiomeric Organophosphonates. Reaction of enantiomeric phosphonates with AChE is known to be dependent on the chemical nature of the leaving group and the size and stereochemical configuration of substituents about phosphorus (Berman & Leonard, 1989). The analysis of the organophosphonate (OP) reactions affords a clear kinetic

[3] Scheme 1 assumes that S has equivalent affinity for the active site of E and SE as does S for a peripheral site of E and ES. Should these affinities differ, a more complex equation would describe substrate catalysis and modulation of catalysis [cf. Webb, 1963].

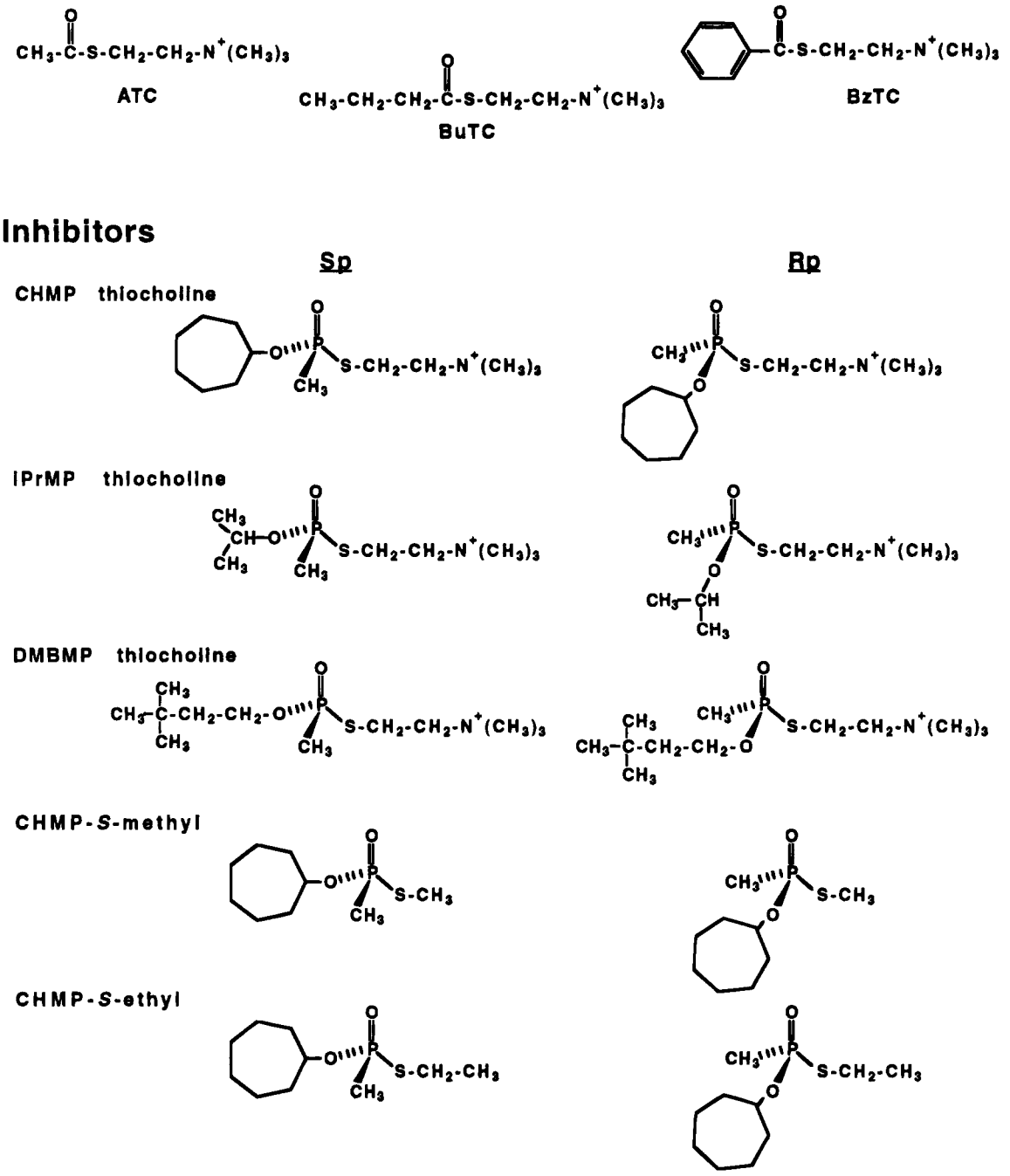


FIGURE 1: Structures of substrates and inhibitors employed. ATC, BuTC, and BzTC refer to acetyl-, butyryl-, and benzoylthiocholine substrates, respectively. CHMP, iPrMP, and DMBMP refer to cycloheptyl-, isopropyl-, and 3,3-dimethylbutyl methylphosphonyl, respectively. CHMP *S*-methyl and CHMP *S*-ethyl refer to cycloheptyl methyl *S*-methylphosphonyl thioate or cycloheptyl methyl *S*-methyl phosphonyl thioate.

separation of the acylation step (Froede & Wilson, 1971), and presents two additional features that complement analysis of planar carboxyl substrates. First, the tetrahedral configuration of organophosphonates provides a structural dimension outside the plane of the sp^2 trigonal geometry of substrate molecules. Second, the availability of enantiomerically pure phosphonates allows one to assess the spatial orientations of individual residues within the dissymmetric active center.

The series of compounds shown in Figure 1 have been analyzed with mouse AChE and mouse BuChE and the acyl pocket mutants, F295L, F295A, F297I, and F297A. Bimolecular rate constants (*ki*) for inhibition by enantiomers of cycloheptyl methylphosphonyl, isopropyl methylphosphonyl, and 3,3-dimethylbutyl methylphosphonyl thiocholine are shown in Table 2.

The S_p enantiomer is the most reactive for both CHMP and iPrMP thiocholine with a selectivity difference of more than 2 orders of magnitude for AChE; this ratio decreases significantly for BuChE. More specifically, the R_p enantiomers of CHMP and iPrMP thiocholine show a 10−20-fold increase in the reaction rate for BuChE over AChE, whereas the S_p enantiomers show little difference between the wild types. Hence, the (S_p)-OPs are binding and phosphonylating

Table 1: Kinetic Constants[a] Calculated for the Catalysis of Acetylthiocholine, Butyrylthiocholine, and Benzoylthiocholine by Recombinat DNA-Derived Cholinesterases

	acetylcholine					butyrylthiocholine					benzoylthiocholine				
enzyme	K_m (μM)	K_{ss} (mM)	b	k_{cat}[b] (10^3 min^{-1})	k_{cat}/K_m (10^8 min^{-1} M^{-1})	K_m (μM)	K_{ss} (mM)	b	k_{cat} (10^3 min^{-1})	k_{cat}/K_m (10^8 min^{-1} M^{-1})	K_m (μM)	K_{ss} (mM)	b	k_{cat} (10^3min^{-1})	k_{cat}/K_m (10^8min^{-1} M^{-1})
AChE	46	13	0.21	140	30	93	7.1	0.48	1.1	0.12	46	3.3	13	0.03	0.0063
BuChE	23	1.0	3.9	40	17	55	1.7	2.0	37	6.7	27	3.7	<0.2	3.8	1.4
F295A	64	75	<0.2[c]	73	11	3.6	>100	ID	8.0	22	16	4.2	<0.2	0.39	0.24
F295L	52	67	<0.2	44	8.5	10	23	0.40	12	12	10	1.0	2.4	0.24	0.24
F295Y	17[d]	27	<0.2	6.1	3.6	84	1.4	0.74	0.8	0.10	5.1	0.44	6.2	0.02	0.031
F297A	61	1.6	1.7	61	10	86	2.5	2.0	17	2.0	4.8	0.54	1.5	0.13	0.27
F297I	170	43	1.8	15	0.9	82	2.4	1.7	60	7.3	19	>4	ID	0.45	0.24
F297Y	58[d]	>100	ID[e]	39	6.7	45	0.96	2.7	1.8	0.40	13	1.1	3.1	0.08	0.062

[a] Values come from three measurements. Standard errors are typically within 20% of the mean, except where K_{ss} approached the maximal substrate concentrations employed (see text). Then K_{ss} and b incurred greater uncertainties. [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 computer fit according to $v = [V_{max}(1 + b[S]/K_{ss})]/[(1 + K_m/[S])(1 + [S]/K_{ss})]$ using Sigma Plot. [c] When substrate inhibition is evident, resulting b values less than 0.2 cannot be distinguished from zero. [d] Data of Radic' et al. (1933). [e] When K_{ss} was greater than the highest substrate concentration employed, the b values were indeterminable (ID).

Table 2: Bimolecular Rate Constants (min^{-1} M^{-1}) Determined for the Inhibition of Recombinant DNA-Derived Mouse Cholinesterases by Alkyl Methylphosphonyl Thiocholine Enantiomers[a]

	cycloheptyl methylphosphonyl thiocholine			isopropyl methylphosphonyl thiocholine			3,3-dimethylbutyl methylphosphyl thiocholine		
enzyme	$S_p \times 10^6$	$R_p \times 10^6$	S_p/R_p	$S_p \times 10^6$	$R_p \times 10^6$	S_p/R_p	$S_p \times 10^6$	$R_p \times 10^6$	S_p/R_p
AChE	190 ± 30	0.81 ± 0.09	230	16 ± 1	0.14 ± 0.03	110	360 ± 10	19 ± 9	19
BuChE	470 ± 90	6.7 ± 0.7	70	10 ± 1	3.3 ± 0.1	3	500 ± 150	32 ± 16	16
F295A	290 ± 50	7.1 ± 0.7	37	16 ± 1	1.2 ± 0.1	14	530 ± 40	15 ± 0	35
F295L	66 ± 9	8.7 ± 1.1	7.6	3.4 ± 0.1	1.2 ± 0.1	3	140 ± 10	10 ± 5	14
F297A	17 ± 4	2.4 ± 0.3	7.1	1.5 ± 0.3	0.098 ± 0.012	15	56 ± 1	1.8 ± 0.1	31
F297I	16 ± 3	62 ± 3	0.3	0.95 ± 0.46	1.2 ± 0.1	0.8	56 ± 4	12 ± 4	5

[a] Data shown as means ± standard deviations.

the two enzymes at similar rates, while the amino acid differences presumably in the acyl pocket with BuChE confer a selective increase in rate of reaction of the R_p enantiomers. Examination of the acyl pocket mutants supports this conclusion since replacing either F295 or F297 with smaller aliphatic residues results in a 10−100-fold increase in the k_i values for (R_p)-CHMP and (R_p)-iPrMP thiocholine. The F297 mutation also decreases the reaction rate for the S_p enantiomers, resulting in an inversion in chiral specificity for F297I where the reaction rate of the R_p enantiomer exceeds that of the S_p enantiomer.

The third asymmetric OP, DMBMP thiocholine, shows reaction rates similar to those of (S_p)-CHMP thiocholine but is distinctive in lacking the enantiomeric selectivity of the other two inhibitors. Moreover, little difference in reaction rate for AChE and BuChE is seen for the two DMBMP thiocholine enantiomers. Mutations to smaller residues in the acyl pocket region do not affect the bimolecular rate constants for (R_p)-DMBMP thiocholine, unlike the other two asymmetric OPs, although, as with the other inhibitors, some reduction in rate is seen with F297A.

Influence of Thioalkyl and Thiocholine Leaving Groups on Reaction Kinetics. The second set of compounds for kinetic analysis of enantiomers is made up of two neutral cycloheptyl methylphosphonyl organophosphonates, shown in Figure 1. Table 3 displays the inhibition kinetics for two CHMP thioate congeners with thiomethyl and thioethyl leaving groups. These CHMP compounds, with neutral leaving groups of diminished molecular dimensions, show stereospecificity of a magnitude comparable to that of the corresponding methylphosphonyl thiocholines, although the reaction rates are 2−3 orders of magnitude slower for the uncharged CHMP OPs.

Table 3: Bimolecular Rate Constants (k_i in min^{-1} M^{-1}) for the Inhibition of Recombinant DNA-Derived Cholinesterases by Neutral (S_p)- and (R_p)-Cycloheptyl Methylphosphonyl Thioates[a]

	leaving group					
	S-methyl			*S*-ethyl		
enzyme	$S_p \times 10^4$	$R_p \times 10^4$	S_p/R_p	$S_p \times 10^4$	$R_p \times 10^4$	S_p/R_p
AChE	31 ± 4	0.18 ± 0.07	170	7.6 ± 0.5	0.018 ± 0.002	420
BuChE	23 ± 6	0.25 ± 0.07	92	13 ± 1	0.17 ± 0.02	76
F295L	34 ± 2	0.29 ± 0.11	120	16 ± 2	0.100 ± 0.03	160
F297I	5.5 ± 0.1	2.2 ± 0.4	2.5	5.5 ± 1.3	2.6 ± 0.6	2.1

[a] Data shown as means ± standard deviations.

Also, as shown in Table 3, the R_p enantiomers react with F297I more rapidly than with AChE; the S_p enantiomer agents display a slight reduction in rate. Consequently, substitution for Phe at position 297 with the Ile found in BuChE virtually eliminates the enantiomeric preference. The similarity in S_p/R_p chiral specificity seen for the cationic and uncharged CHMP compounds suggests that the charge on the leaving group does not dictate enantiomeric preference.

DISCUSSION

Analysis of substrate specificity reveals that differences between AChE and BuChE substrate specificity are largely due to the phenylalanines at 295 and 297. Clearly, replacement of these residues by Leu and Ile results in an enhancement in catalysis of larger substrates that can be attributable to enlarging the acyl pocket region (Figure 2A,B). With their sp^2 planar geometry, the substrates dock for catalysis presumably by placing the carbonyl oxygen toward the oxyanion hole. With the choline moiety toward Trp 86, and the acyl moiety toward F295 and F297 (Sussman et al., 1991), only one of the two faces of the planar compound is

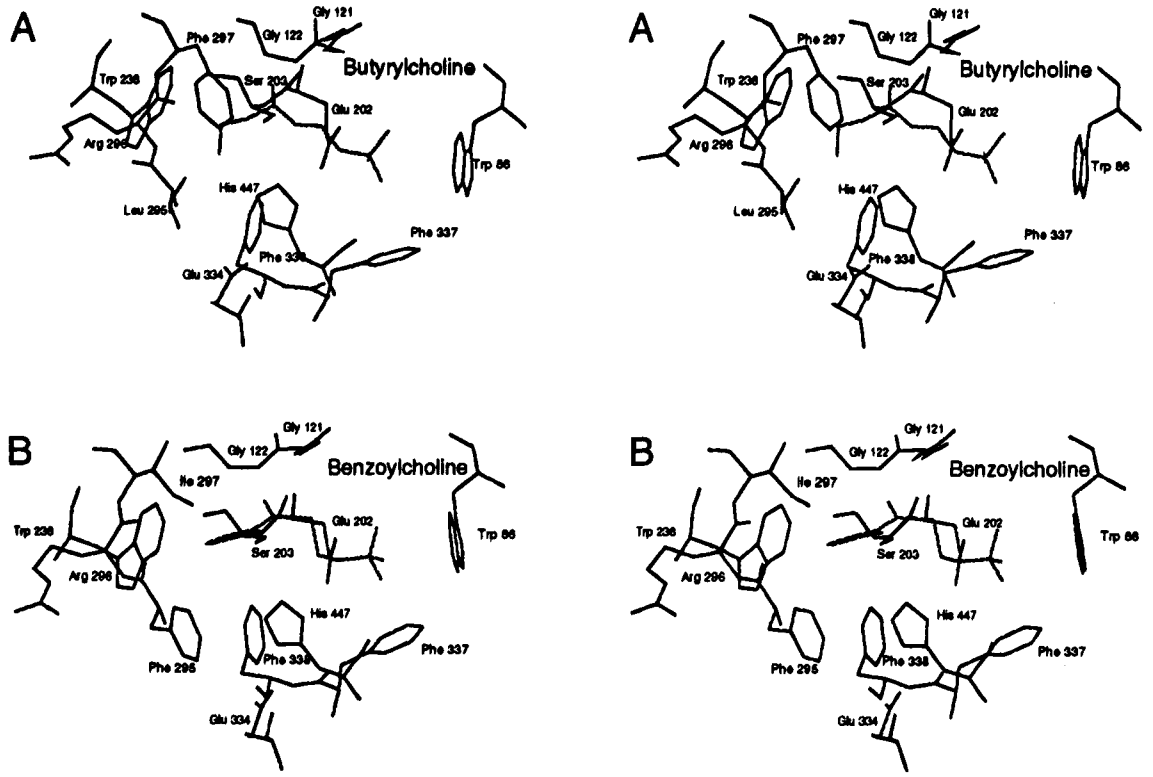


FIGURE 2: Energy-minimized stereoviews of carboxyl choline substrates in the active center of mutant acetylcholinesterase structures as described in the Materials and Methods: (A) F295L with butyrylcholine and (B) F297I with benzoylcholine. In parts A and B of this figure, the carbonyl oxygen resides within the oxyanion hole defined by the hydrogen bonds (<3 Å) with amide backbone hydrogens of Gly 121, Gly 122, and Ala 204. The residue numbers correspond to mouse sequence.

Table 4: Summary of $\Delta\Delta G$ Values[a] (Kilocalories per Mole) Obtained from Free Energy (ΔG) Calculations of k_{cat}/K_m for the Catalysis of Acetylcholine (ATC), Butyrylthiocholine (BuTC), and Benzoylthiocholine (BzTC) and from the Bimolecular Rate Constants (k_i) for (S_p)- and (R_p)-Cycloheptyl Methylphosphonyl, Isopropyl Methylphosphonyl, and 3,3-Dimethylbutyl Methylphosphonyl Thiocholines

	substrates			CHMPTCH		IMPTCH		DMBMPTCH	
enzyme	ATC	BuTC	BzTC	S_p	R_p	S_p	R_p	S_p	R_p
BuChE	−0.34	+2.4	+3.2	+0.54	+1.30	−0.28	+1.90	+0.19	+0.31
F295A	−0.59	+3.1	+2.2	+0.25	+1.30	0	+1.30	+0.23	−0.14
F295L	−0.75	+2.7	+2.2	−0.63	+1.40	−0.92	+1.30	−0.56	−0.38
F297A	−0.65	+1.7	+2.2	−1.40	+0.64	−1.40	−0.21	−1.10	−1.40
F297I	−2.1	+2.4	+2.2	−1.50	+2.60	−1.67	+1.30	−1.10	−0.27

[a] $\Delta\Delta G$ values obtained using values in Tables 1 and 2 and the equation $\Delta\Delta G = \Delta G_{AChE} - \Delta G_{BuChE/mutant} = RT \ln (k_i'/k_i)$, where k_i = the bimolecular rate constant for the organophosphonates and is equal to k_{cat}/K_m for carboxyl ester substrates (Fersht, 1988). The ′ refers to either BuChE or mutant AChE; $R = 1.99 \times 10^{-3}$ kcal/mol K, and $T = 298$ K.

available for nucleophilic attack by the active center serine. Upon such attack of the carbonyl carbon, the bond angles change with formation of a tetrahedral intermediate. The change in bond angles might move the thiocholine slightly in the direction of the gorge exit, a position well-suited for leaving the active site where no hindrance to exit would be encountered by other substituents on the substrate. Furthermore, with the planar configuration and the acyl moiety directed toward the residues F295 and F297, changes in the acyl chain length will be reflected only in the acyl pocket direction.

The OP compounds have an additional substituent due to their tetrahedral configuration with 109° bond angles, and thus, the substituent groups will project differently in the active site than for the planar (trigonal) substrates with 120°-bond angles. This becomes evident with AChE wild type which most efficiently catalyzes the hydrolysis of carboxyl choline substrates with small acyl group size while the same enzyme is acylated by organophosphonates with bulkier substituents (Tables 1−3). Table 4 tabulates the relative free energies of reaction for the various substrates, where a positive $\Delta\Delta G$ value indicates a more energetically favorable reaction relative to AChE wild type. The direction of the free energy changes between AChE and mutant cholinesterases with the S_p enantiomers reflects that of acetylthiocholine, while the R_p compounds parallel that of the larger acyl containing substrates.

The CHMP and iPrMP thiocholine compounds display a similar enantiomeric selectivity with AChE even though iPrMP thiocholine is approximately 10-fold less reactive. From the analysis of the individual enantiomers, the diminished inhibition rates of (R_p)-CHMP and iPrMP thiocholine with AChE also can be attributed to the phenylalanines at 295 and 297 since their replacement with smaller residues gives a substantial enhancement in the bimolecular rate constant, approaching or exceeding that with BuChE.

Therefore, the respective alkoxyl substituents of these two R_p compounds are restricted from optimal positioning in the active site of AChE due to occlusion by F295 and F297 in a manner similar to restricting large carboxyl ester substrates.

The third charged inhibitor, DMBMP thiocholine, shows diminished stereoselectivity for AChE which seems to be a consequence of an enhanced rate of inhibition by the R_p enantiomer, with little to no change by the S_p enantiomer when compared to (S_p)-CHMP thiocholine. Therefore, (R_p)-DMBMP thiocholine with a primary instead of secondary alkoxyl substituent probably orients slightly differently in AChE than do the other two congeners. In addition, the inhibitory efficiencies of reaction with (R_p)-DMBMP thiocholine for the acyl pocket mutants are similar to those of AChE wild type, indicating that the two phenylalanines play a diminished role in dictating the rate of reaction with (R_p)-DMBMP thiocholine and in sterically occluding the DMB substituent. Part of this may arise from steric restrictions of a secondary alkoxyl constituent, rather than solely being a consequence of molecular volumes.

The S_p enantiomers of all three thiocholine inhibitors show equivalent or slower rates of inhibition with the acyl pocket mutants where F297I and F297A show the largest reduction. Removing the aromatic group from the side chain reduces the potency of the S_p thiocholine inhibitors which may be attributed to increased degrees of freedom of the organophosphonate in the active site region. BuChE, lacking the large aromatic side chains at 295 and 297, reacts more efficiently with the S_p compounds, supplying evidence that the mutant AChEs do not precisely mimic BuChE and that other groups close to the substrate, such as Tyr 337 in mouse AChE (Phe in *T. californica*), might indirectly influence substrate positions. In addition, the reduced reaction rates with (S_p)-CHMP and iPrMP enantiomers along with the concomitant increased reaction rates with the R_p enantiomers give rise to the dramatic inversion in enantiomeric preference for the F297I mutant relative to AChE wild type.

Interestingly, the F297A mutation does not display the same kinetics as F297I with the (R_p)-alkyl methylphosphonyl thiocholine inhibitors, where the Ala mutant gives a 10-fold lower k_i for common inhibitors than for the Ile mutant. A similar lack of reactivity was noted for the carboxyl esters (k_{cat} values, Table 1). Replacement of the large Phe residue with Ala can be expected to yield a void area which should be filled with water or which may result in collapse of the α-carbon chain. For the F297I substitution, the volume reduction is smaller and would require a smaller structural perturbation than would the equivalent Ala mutant.

A final factor in determining the rate of reaction of organophosphonates with AChE is the chemical nature of the leaving group. The uncharged cycloheptyl methylphosphonyl thioates display a chiral selectivity similar to that of the cationic cycloheptyl methylphosphonyl thiocholines. Since the uncharged phosphonyl thioates react at rates that are 3 orders of magnitude slower than the corresponding charged compounds, the large difference in reaction rates depends on the nature of the leaving group. As analyzed by Kitz et al. (1967), the reaction rates reflect the interaction energy gained upon formation of the phosphonate−enzyme complex rather than p*K*a differences between the leaving groups. As discussed by Berman and Leonard (1989), the slower reaction rate typical of the uncharged methylphosphonyl thioates reflects diminished electrostatic attraction for the enzyme. Therefore, stabilization of the quaternary choline group enhances the reaction of the charged compounds but carries little influence in enantiomeric preference. These findings are consistent with the thiocholine or thioalkyl leaving group residing in a common position for the individual enantiomers.

Substrate and Organophosphonate Orientation. As a model for the orientation of substrates and organophosphonates begins to emerge, it is first instructive to compare the tetrahedral phosphonyl esters with the planar carboxyl esters. The phenylalanines in the acyl pocket of AChE hinder noncovalent association of reactive substrates and phosphonates containing large bulky substituents. The phenylalanine residues also serve to enhance association of reactive substrates and phosphonates containing small, less bulky substituents, as seen for ATC and (S_p)-methylphosphonates. In Figure 2, the large carboxyl substrates are modeled in the active site region of F295L and F297I and are positioned with their acyl moieties occupying the region of the acyl pocket where butyryl is directed toward 295 (Figure 2A) and where benzoyl is closer to the 297 side chain (Figure 2B). By comparison, the F297I mutant modeled with (R_p)-CHMP thiocholine (Figure 3B) shows that the mutation at 297 to the smaller residue, Ile, allows the bulky cycloheptyl group to be accommodated in a manner similar to BzTC. Therefore, steric occlusion by the phenylalanines within the acyl pocket of AChE, in particular Phe 297, represents a primary determinant of substrate selectivity and enantiomeric preference. Such steric constraints can be relieved by replacing the phenylalanines with dimensionally smaller aliphatic residues.

A comparison of AChE covalent reactivity with respect to large substrates, such as BuTC and BzTC, and large phosphonates, such as the most reactive (S_p)-CHMP and (S_p)-DMBMP thiocholines, indicates that reaction efficiency is governed in part by the spatial orientation achieved at the base of the active center gorge by the substrate molecules and the organophosphonates. This conclusion is supported by the observations that AChE reacts 10-fold more rapidly with large alkoxyl (S_p)-methylphosphonates than the large acylcholine substrates (k_i in Table 2 versus k_{cat}/K_m in Table 1; Table 4). Furthermore, mutations of the two Phes to smaller aliphatic residues give rise to enzymes with reduced efficiency in catalyzing the hydrolysis of small ligands such as ATC and the reactions with ligands, such as (S_p)-CHMP thiocholine, whose binding of the large alkoxyl moiety does not necessitate interaction with the acyl pocket. From the modeling of AChE with (S_p)-CHMP thiocholine (Figure 3A), it can be inferred that removal of aromatic groups at 295 and 297 permits increased degrees of freedom for binding, association of nonproductive complexes, and resulting slower reaction rates. Thus, a systematic study of a series of congeneric compounds with a graded diminution of side chain volume in the acyl pocket reveals an optimal volume or dimension, a feature unresolvable from substrate structure−activity relationships alone. This demonstrates that the Phes in wild type AChE, in addition to occluding substrates exceeding a critical dimension, may serve to stabilize and orient substrates of smaller dimensions for efficient catalysis. Opening the acyl pocket creating a larger dimension may lead to additional orientations of bound substrate, whose alignment may not be optimal for catalysis.

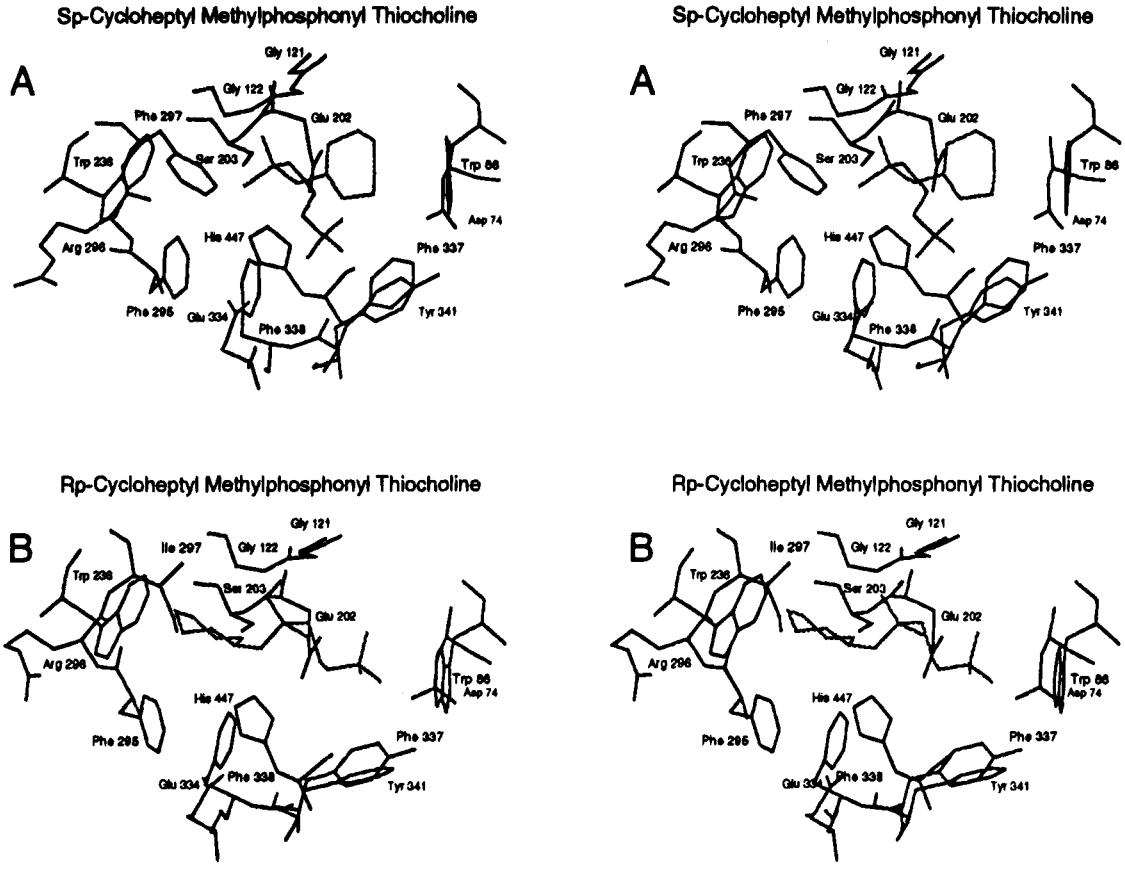


FIGURE 3: Energy-minimized stereoviews of the reversible complex in the active center of (A) acetylcholinesterase with (S_p)-cycloheptyl methylphosphonyl thiocholine and (B) F297I mutant acetylcholinesterase with (R_p)-cycloheptyl methylphosphonyl thiocholine as described in the Materials and Methods. In parts A and B of this figure, the phosphonyl oxygen resides within the oxyanion hole defined by the hydrogen bonds (<3 Å) with amide backbone hydrogens of Gly 121, Gly 122, and Ala 204. The residue numbers correspond to mouse sequence.

Enantiomeric Selectivity. The information to be gained from the analysis of enantiomeric selectivity requires the absolute stereochemical assignment of the R_p and S_p enantiomers along with the positioning of a primary functional group on the dissymmetric macromolecular surface. Formation of the alkylphosphonyl conjugate in addition to the obvious nucleophilic attack by the serine requires polarization of the phosphonyl bond through hydrogen bonding to the phosphonyl oxygen in the oxyanion hole. The region in AChE is likely created by hydrogen bond donors of the backbone amide hydrogens from Gly 121, Gly 122, and Ala 204 (Sussman et al., 1991). Evidence for the obligate phosphonyl oxygen−oxyanion hole interactions stems from the rapid rates of conjugation by the cholinesterases and the X-ray crystal structure of a phosphonyl enzyme conjugate in *Candida rugosa* lipase, a structure homologous to cholinesterase (Cygler et al., 1993, 1994).

With this initial substituent placement, orientation of the leaving group in the direction of the mouth of the active center gorge allows for preferential nucleophilic attack by the γ-oxygen of Ser 203 at the one face of the tetrahedron, comprising CH_3-OR-P(O), not containing the leaving group (Figure 3A). Such attack at this face opposite the leaving group results in formation of a trigonal bipyramidal intermediate in which both the attacking nucleophile and the potential leaving group assume apical positions. In this case, displacement of the leaving group can occur without a requirement for pseudorotation of the trigonal bipyramidal enzyme conjugate (Berman & Decker, 1989). In the case of docking (S_p)-CHMP thiocholine, these initial residue placements find the cycloheptyl group oriented toward the more space-accommodating choline subsite (Figure 3A).

By contrast, these requirements enforce an orientation of (R_p)-CHMP thiocholine in which the cycloheptyl moiety encounters obvious steric hindrance from the Phes 295 and 297, precluding either optimal positioning of the phosphonyl oxygen in the oxyanion hole or displacement of the leaving group from an optimal position for in-line attack by Ser 203. The former case would lead to loss of stabilization energy which would arise from polarization of the phosphonyl−oxygen bond; the latter would result in equatorial positioning of the leaving group and thus would require pseudorotation of the intermediate prior to apical displacement (Berman & Decker, 1989). Since both cases require an expenditure of additional energy (Berman & Decker, 1989; Cygler et al., 1994; Ashani et al., 1995), steric interference engendered upon docking (R_p)-enantiomers is expected to reduce the rates of reaction. Such steric constraints are relieved in the F297I mutation, as shown for association of (R_p)-CHMP thiocholine in Figure 3B, leading to the increased reaction rates observed for the R_p methylphosphonates.

This study clearly shows that substrate selectivity and enantiomeric preference of AChE as well as rates of covalent reaction are all governed in large part through steric limitations imposed with the acyl pocket. One component of the acyl pocket that plays a prominent role is identified

as F297. This postulate can be subjected to further analysis through modification of the gorge dimensions and charge distribution of the residues within the gorge.

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