Three Distinct Domains in the Cholinesterase Molecule Confer Selectivity for Acetyl- and Butyrylcholinesterase Inhibitors†

Zoran Radić, Natilie A. Pickering, Daniel C. Vellom, Shelley Camp, and Palmer Taylor*

Department of Pharmacology, University of California at San Diego, La Jolla, California 92093-0636

Received June 1, 1993; Revised Manuscript Received August 23, 1993®

ABSTRACT: By examining inhibitor interactions with single and multiple site-specific mutants of mouse acetylcholinesterase, we have identified three distinct domains in the cholinesterase structure that are responsible for conferring selectivity for acetyl- and butyrylcholinesterase inhibitors. The first domain is the most obvious; it defines the constraints on the acyl pocket dimensions where the side chains of F_{295} and F_{297} primarily outline this region in acetylcholinesterase. Replacement of these phenylalanine side chains with the aliphatic residues found in butyrylcholinesterase allows for the catalysis of larger substrates and accommodates butyrylcholinesterase-selective alkyl phosphates such as isoOMPA. Also, elements of substrate activation characteristic of butyrylcholinesterase are evident in the $F_{297}I$ mutant. Substitution of tyrosines for F_{295} and F_{297} further alters the catalytic constants. The second domain is found near the lip of the active center gorge defined by two tyrosines, Y_{72} and Y_{124}, and by W_{286}; this region appears to be critical for the selectivity of bisquaternary inhibitors, such as BW284C51. The third domain defines the site of choline binding. Herein, in addition to conserved E_{202} and W_{86}, a critical tyrosine, Y_{337}, found only in the acetylcholinesterases is responsible for sterically occluding the binding site for substituted tricyclic inhibitors such as ethopropazine. Analysis of a series of substituted acridines and phenothiazines defines the groups on the ligand and amino acid side chains in this site governing binding selectivity. Each of the three domains is defined by a cluster of aromatic residues. The two domains stabilizing the quaternary ammonium moieties each contain a negative charge, which contributes to the stabilization energy of the respective complexes.

Cholinesterases of two distinct types were distinguished in the 1940s on the basis of their distinct substrate specificities and distribution in various tissues (Alles & Hawes, 1940; Augustinnson, 1948). The acetylcholinesterases (AChE) rapidly catalyze the hydrolysis of acetylcholine, show a slight diminution for the propionylcholine hydrolysis rate, and show a marked reduction for the butyrylcholine hydrolysis rate. By contrast, the butyrylcholinesterases (BuChE) show little difference in the catalytic efficiencies for the three acylcholines and will accommodate substrates with even larger acyl groups, such as benzoylcholine. Over the years, selective inhibitors for the cholinesterases, BW284C51 for AChE and ethopropazine and isoOMPA for BuChE, have enabled investigators to ascertain the tissue localization of the respective cholinesterases (Silver, 1974).

AChE is widely distributed in the nervous system; its only well-defined function is the rapid hydrolysis of the neurotransmitter, acetylcholine. While BuChE is found in muscles and in the nervous system during development, the presence of BuChE-deficient mutants in humans suggests that the enzyme at most plays only an auxiliary role in the nervous system (Silver, 1974; Whittaker, 1986; Soreq & Zakut, 1990; LaDu et al., 1991). Its presence in the liver and plasma, as well as the population distribution of human BuChE mutations that confer resistance to inhibition by indigenous plant alkaloids, suggests a function of BuChE is to catalyze the hydrolysis of dietary esters (Soreq & Zakut, 1990).

Drosophila has a single cholinesterase whose sequence characteristics and kinetic properties lie between those of mammalian AChE and BuChE (Hall & Spierer, 1986). BuChE and AChE appear to have diverged prior to the evolutionary appearance of lower vertebrates (Toutant et al., 1985). In the mammals studied thus far, AChE and BuChE are each encoded by a single gene (Rachinsky et al., 1990, 1992; Soreq et al., 1990; Getman et al., 1992; Ehrlich et al., 1992). The two proteins exhibit 51–54% amino acid identity and identical disulfide bond arrangements (MacPhee-Quigley et al., 1986; Lockridge et al., 1987; Chatonnet & Lockridge, 1990; Soreq et al., 1990). The recent elucidation of the three-dimensional structure of AChE from *Torpedo* (Sussman et al., 1991) enables one to model related cholinesterase structures. In previous studies using cholinesterase chimeras constructed from mouse AChE and BuChE sequences, we have shown that substrate and inhibitor specificities can arise from distinct regions of the molecule (Vellom et al., 1993). Herein, we examine the particular amino acid residues responsible for the inhibitor specificity of AChE and BuChE. Three distinct domains of the molecule appear to be involved, and the analysis of the residues in each domain reveals important details on the energetics of inhibitor binding and the orientations of the bound ligands.

MATERIALS AND METHODS

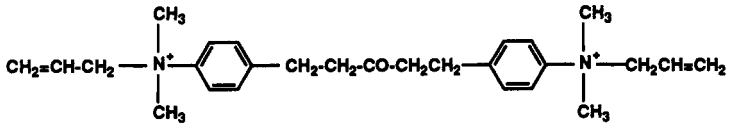
Materials. BW284C51, ethopropazine [10-[2-(diethylamino)propyl]phenothiazine], promazine [10-[3-(dimethylamino)propyl]phenothiazine], promethazine [10-[2-(dimethylamino)propyl]phenothiazine], 8-chlorpromazine, phenothiazine, tacrine (9-amino-1,2,3,4-tetrahydroacridine), and acridine were products of Sigma Chemical Co. (St. Louis, MO). 9-Aminoacridine was purchased from Aldrich Chemical Co. (Milwaukee, WI). *N*-Methylacridinium was a product of Molecular Probes, Inc. (Eugene, OR). 7-[[(Methylethoxyl)phosphinyl]oxyl]-1-methylquinolinium (MEPQ) was a gift

† Supported by DAMD17-9-C-1058 and USPHS Grant GM18360. Z.R. is a visiting Fogarty Fellow from the Institute for Medical Research and Occupational Health, University of Zagreb, Zagreb, Croatia. D.C.V. was supported by a fellowship from the Tobacco Related Disease Program of the University of California, FT-43.

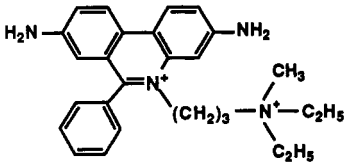
® Abstract published in *Advance ACS Abstracts,* October 15, 1993.

0006-2960/93/0432-12074$04.00/0

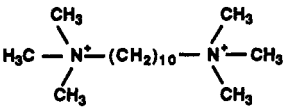
Chart I



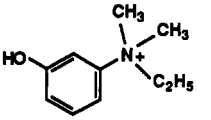
BW284C51



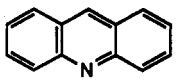
Propidium



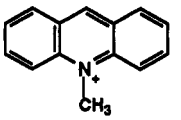
Decamethonium



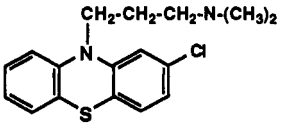
Edrophonium



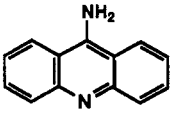
Acridine



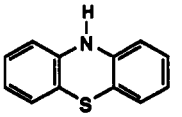
N-methylacridinium



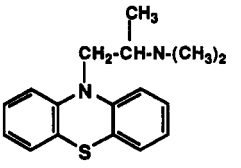
Chlorpromazine



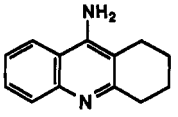
9-aminoacridine



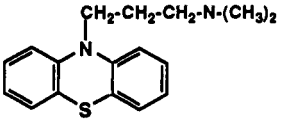
Phenothiazine



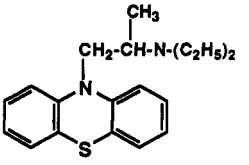
Promethazine



Tetrahydro-9-aminoacridine (Tacrine)



Promazine



Ethopropazine

of Y. Ashani and B. P. Doctor (Walter Reed Army Research Center, Washington, D.C.) (Chart I).

Site-Specific Mutagenesis. Wild-type AChE and BuChE cDNAs isolated from mouse libraries (Rachinsky et al., 1990) were subcloned into M13 and into CMV-based expression vectors (Andersson et al., 1989; pRC/CMV, Invitrogen). The presence of the neomycin resistance gene in the CMV vectors enabled the selection of stable transfectants. The cDNAs included the initiation methionine, the leader sequence, and the full coding region for the hydrophilic enzyme form (exon 4–6 splice). The AChE constructs included a 3′ noncoding sequence and a polyadenylation signal from the cDNA. Oligonucleotide-directed site-specific mutagenesis, according to Kunkel et al. (1987), in M13 employed convenient, unique restriction sites, which allowed subcloning of mutagenized sequence cassettes from M13 directly back into the CMV expression vector. Mutations in amino acids 1–250 were done in a cassette that extended from the 5′-end of the cDNA insert to the first *Bst*XI site, 845 bp downstream. Amino acids 251–415 were covered in a 490-bp *Bst*XI fragment. All cassettes were sequenced in their entirety in M13 before insertion into the expression vector. Expression plasmids were purified by standard procedures involving polyethylene glycol precipitation and centrifugation on CsCl gradients.

Cell Transfections. HEK-293 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells (10^6/10-cm plate) were transfected 16–24 h after plating with 10 μg of a plasmid DNA–$Ca_3(PO_4)_2$ coprecipitate at pH 7.05 (Gibney et al., 1990). After 24 h, cells were rinsed and placed in serum-free medium. Medium and cells were examined for activity 48–72 h after transfection. Supernatants were concentrated to 1–2% of the original volume by ultrafiltration in Centricon concentrators (Amicon, Beverly, MA). Clones of stable transfectants were selected by treatment with G-418 for 2 weeks and amplification of the surviving colonies.

Measurements of AChE Activity, Active Sites, and Inhibition; Structural Analysis. AChE and BuChE activities were

Table I: Kinetic Parameters for Cholinesterase Wild-Type and Mutant Enzymes[a]

enzyme	K_m (μM)	K_{ss} (mM)	b	k_{cat} (10^5/min)	k_{cat}/K_m (10^9/min·M)
AChE wt[b]	46 ± 3	15 ± 2	0.23 ± 0.01	1.4 ± 0.1	3.0
BuChE wt[b]	35 ± 2	1.3 ± 0.5	3.6 ± 0.2	0.40 ± 0.07	1.1
$Y_{72}N$	110 ± 20	35 ± 18	0.18 ± 0.13	1.3 ± 0.2	1.2
$D_{74}N$	1300 ± 140	530 ± 170	0	0.84 ± 0.11	0.063
$Y_{124}Q$	120 ± 21	25 ± 13	0.35 ± 0.09	1.1 ± 0.3	0.94
$W_{286}R$	420 ± 60	23 ± 8	0.24 ± 0.06	1.6 ± 0.3	0.38
$Y_{72}N/Y_{124}Q$	95 ± 11	21 ± 13	0.65 ± 0.05	0.94 ± 0.20	0.99
$Y_{72}N/W_{286}R$	670 ± 200	13 ± 8	0.26 ± 0.05	1.9 ± 0.3	0.28
$Y_{124}Q/W_{286}R$	700 ± 170	66 ± 17	0	1.6 ± 0.3	0.23
$Y_{72}N/Y_{124}Q/W_{286}R$	850 ± 130	10 ± 3	0.24 ± 0.02	2.1 ± 0.6	0.25
$Y_{72}N/Y_{124}Q/W_{286}A$	230 ± 43	14 ± 6	0.27 ± 0.05	1.5 ± 0.3	0.67
$Y_{72}N/D_{74}N/Y_{124}Q/W_{286}R$	6100 ± 730	75 ± 10	0	1.4 ± 0.2	0.023
$Y_{72}N/D_{74}N/Y_{124}Q/W_{286}A$	2100 ● 300	82 ± 14	0	1.5 ± 0.1	0.071
$F_{295}Y$	17 ± 3	27 ± 9	0	0.061 ± 0.020	0.36
$F_{297}Y$	58 ± 6	460 ± 120	0	0.39 ± 0.13	0.67
$Y_{337}A$	110 ± 16	29 ± 21	0.59 ± 0.08	0.52 ± 0.16	0.46
$Y_{337}F$	53 ± 5	10 ± 2	0.42 ± 0.03	0.68 ± 0.26	1.3
$F_{338}G$	75 ± 9	1300 ± 870	0	0.23 ± 0.10	0.31
$B_{5-174}A_{175-575}$[b]	160 ± 2	27 ± 10	0.29 ± 0.06	1.7 ● 0.2	1.0
$B_{5-174}A_{175-487}B_{488-575}$[b]	160 ± 15	18 ± 8	0.39 ± 0.08	1.2 ± 0.4	0.77
$F_{295}L$[b]	49 ± 4	62 ± 8	0	0.44 ± 0.07	0.90
$F_{297}I$[b]	172 ± 64	43 ± 46	1.8 ± 0.3	0.15 ± 0.02	0.087
$R_{296}S$[b]	50 ± 8	15 ± 4	0.20 ± 0.16	0.92 ± 0.08	1.8
$V_{300}G$[b]	50 ± 4	46 ± 6	0	1.6 ± 0.2	3.2
$F_{295}L/F_{297}I$[b]	200 ± 93	83 ± 87	0.67 ± 0.08	0.12 ± 0.01	0.059

[a] Substrate was acetylthiocholine. Values for K_m, K_{ss}, and b were calculated using nonlinear computer fitting according to eq 2. k_{cat} was evaluated using MEPQ titrations as shown in Figure 2. The means and standard errors from 3–5 experiments and 2 different transfections are given. [b] Data of Vellom et al. (1993) calculated according to eq 2.

measured in 0.1 M $NaPO_4$ (pH 7.0) at 22 °C by the assay of Ellman et al. (1961) using acetylthiocholine (ATCh) as a substrate. Active sites were quantitated by titration with a high-affinity phosphorylating agent, MEPQ, to ascertain the minimal concentration required for complete inhibition (Levy & Ashani, 1986; Radić et al., 1992). Inhibition by the reversible inhibitors was measured in the same buffer at 4–5 substrate and 3 inhibitor concentrations to establish the components of competitive and noncompetitive inhibition. For studies with inhibitors, substrate concentrations were selected to be below the range for substrate inhibition and activation. Kinetic parameters were analyzed from a full triplicate analysis of reciprocal and regression plots using enzyme preparations from at least two separate transfections.

Inhibitor and substrate binding to AChE and BuChE was modeled by an energy minimization docking program (Discover-Insight 2.2.0, Biosym Technologies, San Diego, CA) on a Silicon Graphics Indigo Elan. Bound ligand was initially energy-minimized by keeping the side chains of the amino acids in AChE fixed. A second minimization was then conducted by allowing flexibility in designated side chains proximal to the bound ligand.

RESULTS

Selection of Mutants. Previous studies with AChE–BuChE chimeras and with six site-specific mutant permutations in the region of residues 295–300 revealed that, of the inhibitors and substrates tested, only BW284C51 specificity is influenced appreciably by the first 174 amino acid residues (Vellom et al., 1993). Replacement of this amino-terminal sequence in AChE with the corresponding BuChE sequence substantially reduces BW284C51 affinity. A comparison of residues common to the mammalian AChEs and BuChEs, but diverging between the two gene products, reveals that two tyrosines at positions 72 and 124 are conserved in the AChEs but are substituted with asparagine and glutamine in BuChE. The residues are found near the lip of the active center gorge (Sussman et al., 1991) and are likely candidates for contribution to BW284C51 affinity. W_{286} is found in close proximity to Y_{72} and Y_{124}; it, too, is not conserved in BuChE. Finally, D_{74} is also found in the immediate region. Even though this residue is conserved in both the AChEs and BuChEs, $D_{74}\rightarrow G$ is found as a naturally occurring mutation of BuChE. The mutant lacking the negative charge has a greatly reduced capacity to catalyze the hydrolysis of succinylcholine, a bisquaternary substrate with structural resemblance to BW284C51 (La Du et al., 1990, 1991; Soreq & Zakut, 1990).

To delineate the residues responsible for the binding of a bisquaternary inhibitor selective for AChE (BW284C51) and those responsible for a nonselective bisquaternary inhibitor (decamethonium), we have examined the influence of various permutations of the Y_{72}, Y_{124}, W_{286}, and D_{74} tetrad on inhibitor binding. This region is also a prime candidate for influencing the specificity of peripheral site inhibitors (Taylor & Lappi, 1975; Shafferman et al., 1992a). Since W_{286} is replaced with arginine in mouse BuChE and with alanine in human BuChE, the cationic side chain of arginine may have dramatic effects on bisquaternary inhibitor association; therefore, in some cases we have also substituted alanine at this position.

Second, mutations in the acyl pocket have been shown to influence isoOMPA specificity (Harel et al., 1992; Vellom et al., 1993). Replacements of either F_{295} or F_{297} by aliphatic residues increase isoOMPA reactivity by 2 orders of magnitude (Vellom et al., 1993). Simultaneous mutation of both residues produces only a slight additional increase in reactivity (Vellom et al., 1993). To further define the acyl pocket, we have replaced the phenylalanines with tyrosines in order to constrain the dimensions of the acyl pocket.

Third, studies with the chimeric enzymes indicated that ethopropazine specificity arises from residues in the middle portion of the amino acid sequence; ethopropazine's low affinity for AChE was not enhanced in the $B_{5-174}A_{175-575}$ or $B_{5-174}A_{175-487}B_{488-575}$ chimeras. A region likely to affect

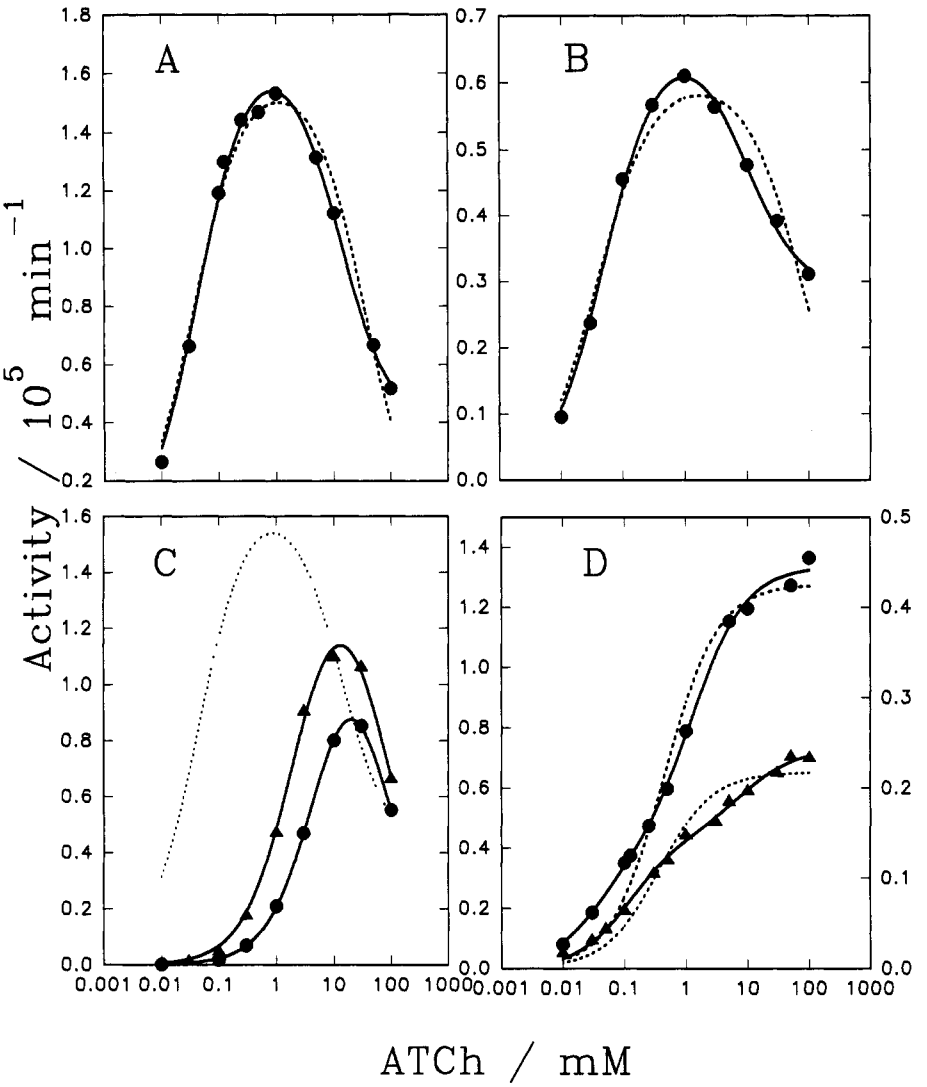


FIGURE 1: Concentration dependencies for acetylcholine hydrolysis by recombinant DNA-derived acetylcholinesterases. Data for wild-type AChE and BuChE are from previously determined concentration dependencies (Vellom et al., 1993). Activities have been normalized to their respective k_{cat} values. (A) Wild-type AChE: - - -, eq 1 (K_m = 39 μM, K_{ss} = 38 mM); —, eq 2 (K_m = 46 μM, K_{ss} = 15 mM, b = 0.23). (B) Y_{337}→F mutation: - - -, eq 1 (K_m = 40 μM, K_{ss} = 73 mM); —, eq 2 (K_m = 53 μM, K_{ss} = 10 mM, b = 0.42). (C) Substrate concentration dependencies for mutations substantially diverging from that of wild-type AChE (- - -); Y_{72}N, D_{74}N, Y_{124}Q, W_{286}A (▲); Y_{72}N, D_{74}N, Y_{124}Q, W_{286}R (●). Values for K_m, K_{ss}, and b, which determine the solid lines, are given in Table I. Since K_m and K_{ss} approach each other, note that k_{cat} is greater than the apparent V_{max}. (D) Substrate concentration dependence for BuChE (●): - - -, eq 1 (K_m = 451 μM, K_{ss} = ∞); —, eq 2 (K_m = 35 μM, K_{ss} = 1.3 mM, b = 3.6). F_{297}I mutation (right-hand ordinate scale) (▲): - - -, eq 1 (K_m = 357 μM, K_{ss} = ∞); —, eq 2 (K_m = 172 μM, K_{ss} = 43 mM, b = 1.8).

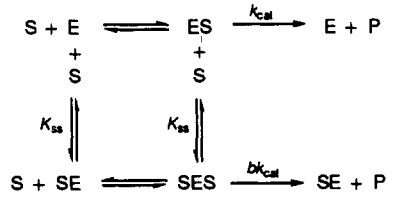
ethopropazine specificity is the choline-binding subsite where two aromatic residues, W_{86} and Y_{337}, are important for stabilizing the choline moiety of the substrate as well as several ligands that bind within the active center. Phenylalanine or tyrosine is present at the 337 position in AChE instead of the alanine in BuChE; steric occlusion with the aromatic side chain might form the basis of ethopropazine specificity. By contrast, W_{86} is conserved in the cholinesterases. Therefore, we have mutated the mouse Y_{337} to A, as found in BuChE, and to F, which is found in *Torpedo* AChE. Inhibition of these mutant enzymes was studied with a congeneric series of tricyclic ligands containing either acridine or phenothiazine ring systems.

Analysis of the Kinetic Parameters of the Mutants. In order to determine the catalytic parameters of each mutant, we have measured catalysis as a function of substrate concentration (Table I). Previously, we modeled the data to the Haldane equation, where the concentration dependence is described in terms of the Michaelis–Menten constant, K_m, and a substrate inhibition constant, K_{ss}:

$$v = V_{max}/(1 + K_m/[S] + [S]/K_{ss}) \quad (1)$$

However, examination of a larger number of mutants suggests that a more general scheme yields an improved fit to the collection of data (Scheme I).

Scheme I



In Scheme I, S combines at two discrete sites, forming two binary complexes, ES and SE, only one of which, ES, results in substrate hydrolysis. We have assumed, for simplification,

that S combines equally well with E and ES. The parameter, b, reflects the efficiency of hydrolysis of the ternary complex, SES, relative to ES [cf. Webb (1963) and Aldridge and Reiner (1972)]. Scheme I is described by a slightly more complex equation:

$$v = \left(\frac{1 + b[S]/K_{ss}}{1 + [S]/K_{ss}}\right)\left(\frac{V_{max}}{1 + K_m/[S]}\right) \quad (2)$$

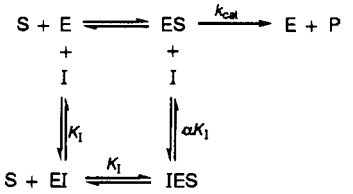
The use of this scheme not only eliminates systematic deviations in the substrate inhibition curves found at high substrate concentrations (up to 100 mM in Figure 1A–C) but also produces a convergence of K_{ss} values for a large number of mutants (Table I). Moreover, the scheme can account for the apparent substrate activation that has been observed for BuChE (Figure 1D) [cf. Augustinsson et al. (1974) and Vellom et al. (1993)]. For BuChE, we find that the data are fit best with b values of 3.6 for acetylthiocholine and 1.7 for butyrylthiocholine. Particularly noteworthy is the $F_{297}I$ mutation, since this single residue change in AChE not only eliminates substrate inhibition (Figure 1D) but confers elements of substrate activation seen for BuChE (b = 1.8 for acetylthiocholine).

In Figure 1C, we show substrate concentration profiles for the mutants where larger increases in K_m relative to the wild-type enzyme values are evident. Several mutations increase K_m, while only the $F_{295}Y$ mutation decreases K_m (Table I). When K_m converges toward K_{ss}, V_{max} can be greatly underestimated if the maximum rate in the concentration dependence is used rather than a procedure of computer fitting to eq 2. Scheme I is a minimal scheme which provides an accurate fit of the data.

To determine k_{cat}, we compared V_{max} to the number of titratable active center serines, determined with the high-affinity phosphorylating agent, MEPQ. MEPQ reactions are rapid and irreversible at sub-nanomolar concentrations of inhibitor and native enzyme (Levy & Ashani, 1986). It is possible that mutations affecting active center conformations diminish the reactivity of the alkyl phosphates with AChE. Our titrations using enzyme concentrations between 5 and 50 nM revealed a rapid rate of inhibition with no evidence of a slow phase of reaction. Although MEPQ is an enantiomeric pair, it seems unlikely that enantiomeric specificity is large since the ethoxy and methyl moieties attached to the phosphorus in MEPQ are not sufficiently large to drive a stereospecific preference (Berman & Leonard, 1989). If stereospecificity were absolute, the estimate of normality would be overestimated by a factor of 2. The titrations shown in Figure 2, as well as those for the other mutations, exhibit a linear concentration dependence, reflecting a stoichiometric titration.

Analysis of Inhibition. In Scheme II, an inhibitor, I, may associate with the free enzyme, E, or any of the intermediate species shown:

Scheme II



S is the substrate, acetylthiocholine, ES is the composite of all enzyme–substrate intermediates, and P is the products

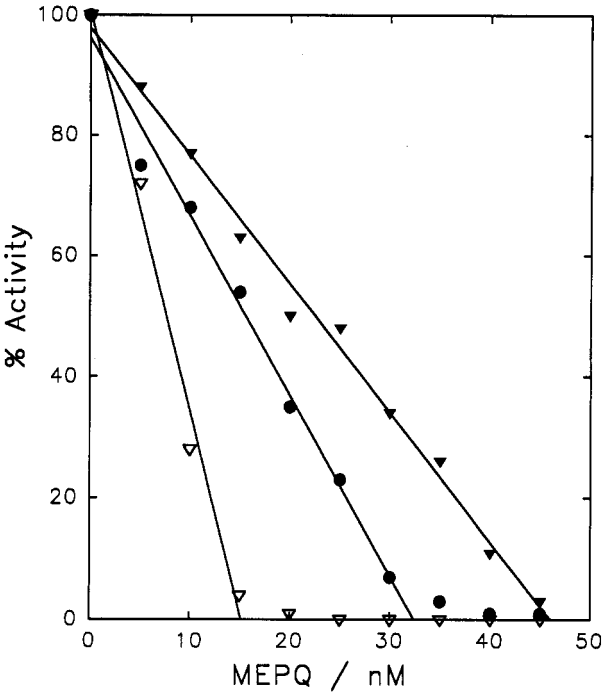


FIGURE 2: Titration of acetylcholinesterase activity as a function of MEPQ concentration. Shown are three titrations where MEPQ in the specified amount is added to a concentrated solution of the recombinant AChE mutant enzyme. Typical solution concentrations of AChE ranged between 5 and 50 nM in active sites (▼, $F_{297}Y$; ●, $Y_{72}N,Y_{124}Q,W_{286}R$; ▽, $Y_{72}N,W_{286}R$. After 30 and 60 min of reaction, aliquots were taken and diluted 1:100, and the activity was measured by the Ellman assay. Samples were taken at two time intervals to ensure that the inhibition reaction was complete. In the case of $Y_{72}N,W_{286}R$, a subsequent titration was performed at a higher concentration of enzyme, leading to a more precise stoichiometry.

thiocholine and acetate. In Scheme II, K_I designates only the inhibitor association with free enzyme, whereas αK_I reflects a composite of constants for inhibitor binding to the various substrate-bound intermediates and the acylated enzyme. This scheme applies to low concentrations of substrate where substrate inhibition can be ignored. A plot of reciprocal velocities versus reciprocal substrate concentrations (Lineweaver–Burk analysis) at a series of inhibitor concentrations yields a family of slopes and y-intercepts. Replots of these values versus inhibitor concentrations yields K_I and αK_I, respectively (Figure 3).

Since a mutation can be expected to influence the enzyme species (i.e., E, ES, and acylated E) with which the inhibitor preferentially interacts, an analysis of mutant enzymes should be based on considerations of the same enzyme species. In this regard, analysis of IC_{50} is inadequate, and the mechanism of inhibition should be sought. Accordingly, we have compared K_I's for inhibitor interactions with the free enzyme.

In both Schemes I and II, we have not distinguished the individual enzyme–substrate intermediates with which inhibitors or excess substrate interact. Such an analysis requires the introduction of more complicated schemes [cf. Krupka and Laidler (1961)] and the use of non-steady-state methods to resolve individual species and kinetic constants.

Free Energy of Interaction. We define the following reactions:

$$E—X^W + L^W \rightleftharpoons E—X\cdots L + WW \quad (3)$$

$$E—Y^W + L^W \rightleftharpoons E—Y\cdots L + WW \quad (4)$$

where X and Y represent the amino acid side chains responsible

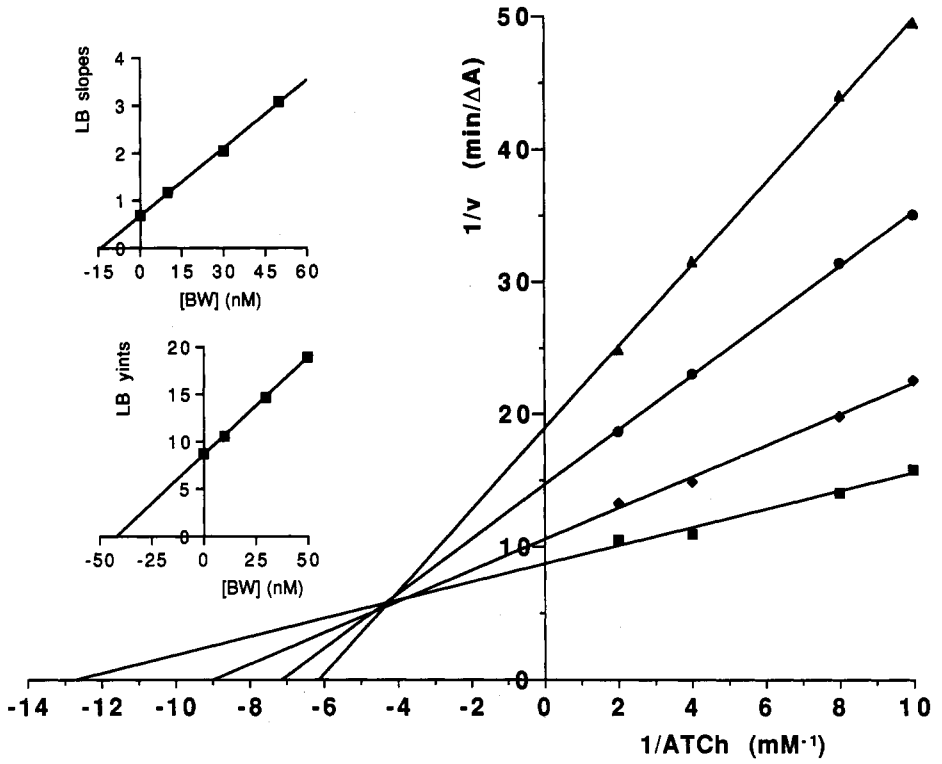


FIGURE 3: Representative analyses of inhibition of a mutant acetylcholinesterase by reversible inhibitors. BW284C51 inhibition of the $Y_{124}Q$ AChE mutant is shown. The Lineweaver–Burk plots for this case of mixed inhibition yield a series of slopes and intercepts. The insets show plots of the slopes and y-intercepts as a function of inhibitor concentration. These values yield K_I and αK_I, respectively: ■, no inhibitor; ◆, 10 nM BW284C51; ●, 30 nM BW284C51; ▲, 50 nM BW284C51.

Table II: Constants (μM) for Reversible Inhibition of Wild-Type AChE and Peripheral Site Mutant Cholinesterases[a]

	inhibitor							
	BW284C51		propidium		decamethonium		edrophonium	
enzyme	K_I	αK_I	K_I	αK_I	K_I	αK_I	K_I	αK_I
AChE wt	0.0028 ± 0.0010	0.0048 ± 0.0018	2.2 ± 0.7	5.3 ± 1.8	3.5 ± 1.6	31 ± 16	0.30 ± 0.10	6.4 ± 0.6
BuChE wt	11.3 ± 0.4	42 ± 5	5.3 ± 1.8	26 ± 18	10 ± 1.1	42 ± 25	110 ± 37	1400 ± 700
$Y_{72}N$	0.024 ± 0.009	0.029 ± 0.003	18 ± 5	170 ± 33	22 ± 6	95 ± 22	ND[b]	
$D_{74}N$	1.9 ± 0.4	23 ± 6	8.7 ± 2.1	34 ± 11	830 ± 130	3000 ± 670	ND	
$Y_{124}Q$	0.016 ± 0.002	0.060 ● 0.031	13 ± 6	66 ± 18	26 ± 6	68 ± 2	ND	
$W_{286}R$	0.24 ± 0.08	0.49 ± 0.09	620 ± 170[c]	≥960	390 ± 90	1300 ± 900	ND	
$Y_{72}N/Y_{124}Q$	0.19 ± 0.08	0.46 ± 0.13	35 ± 7	120 ± 44	130 ± 14	510 ± 180	ND	
$Y_{72}N/W_{286}R$	1.6 ± 0.4	2.9 ± 1.1	600 ± 54[c]	1700 ± 1300	1600 ± 500	7700 ± 2500	ND	
$Y_{124}Q/W_{286}R$	3.1 ± 0.1	12 ± 5	1500 ± 360	≥2200	2000 ± 210	6600 ± 2200	ND	
$Y_{72}N/Y_{124}Q/W_{286}R$	16 ± 2	52 ± 20	230 ± 84[c]	470 ± 210	4000 ± 1000	10000 ± 7000	2.1 ± 0.8	12 ± 6
$Y_{72}N/Y_{124}Q/W_{286}A$	4.1 ± 1.8	12 ± 3	230 ± 0	520 ± 190	1300 ± 640	5300 ± 470	1.6 ± 0.3	
$Y_{72}N/D_{74}N/Y_{124}Q/W_{286}R$	780 ± 28	1000 ± 240	1900 ± 760	3200 ± 1600	50000 ± 18000	40000 ± 35000	28 ± 6	
$Y_{72}N/D_{74}N/Y_{124}Q/W_{286}A$	170 ± 6	690 ± 18	1100 ± 430	2700 ± 1200	16000 ± 1000	55000 ± 35000	17 ± 3	≥120

[a] Constants were evaluated as shown in Figure 3. Means and standard errors from 3–5 experiments are given. [b] ND = not determined. [c] Denotes moderately nonlinear replots approximated by a straight line.

for stabilization of the ligand, L, with the wild-type and mutant enzymes. The W superscripts represent the hydration of side chain and ligand, and WW is bulk water. Then,

$$\Delta G = G_{E-X\cdots L} + G_{WW} - G_{E-X}{}^{W} - G_{L}{}^{W} \quad (5)$$

$$\Delta G' = G_{E-Y\cdots L} + G_{WW} - G_{E-Y}{}^{W} - G_{L}{}^{W} \quad (6)$$

$$\Delta\Delta G = \Delta G - \Delta G' = RT \ln K_I'/K_I \quad (7)$$

where K_I and K_I' are the dissociation constants for inhibitor binding to the free enzyme species of mutant and wild-type AChE [cf. Fersht (1982)].

Bisquaternary Inhibitor Specificity. A comparison of the mutants Y_{72} and Y_{124} reveals that both tyrosine residues contribute to the stabilization energy for the BW284C51 complex (Table II). In fact, the double mutant shows a dissociation constant remarkably similar to that found for the $B_{5-174}A_{175-575}$ chimera (Vellom et al., 1993). The tyrosines have a similar influence on decamethonium binding (Table II). The enhanced affinity of BW284C51 over decamethonium likely reflects hydrophobic interactions of the aromatic side chains with the phenyl moieties and the allyl groups on BW284C51. Also, decamethonium shows virtually no selectivity for inhibition of AChE over BuChE.

Other residues in the distal quaternary ammonium site also play an important role in the association of these bisquaternary inhibitors with the enzyme. The likely candidates, by virtue of their proximity, are D_{74} and W_{286}. In fact, studies with

Table III: Ratio of Inhibition Constants and Resulting Differences in ΔG for Inhibition by Peripheral Site Inhibitors[a]

	inhibitor					
	BW284C51		deca-methonium		propidium	
enzyme	K_I'/K_I	$\Delta\Delta G$ (kcal)	K_I'/K_I	$\Delta\Delta G$ (kcal)	K_I'/K_I	$\Delta\Delta G$ (kcal)
$Y_{72}N$	8.6	1.3	6.2	1.1	8.2	1.3
$Y_{124}Q$	5.7	1.0	7.4	1.2	5.9	1.1
$W_{286}R$	86	2.7	110	2.8	280	3.4
$Y_{72}N/Y_{124}Q$	68	2.5	37	2.2	16	1.7
$Y_{72}N/W_{286}R$	570	3.8	460	3.7	270	3.4
$Y_{124}Q/W_{286}R$	1100	4.2	570	3.8	680	3.9
$B_{5-174}A_{175-575}$	100	2.8	ND[b]	ND	ND	ND
$B_{5-174}A_{175-487}A_{488-575}$	250	3.3	ND	ND	ND	ND
$Y_{72}N/Y_{124}Q/W_{286}R$	5700	5.2	1100	4.2	110	2.8
$Y_{72}N/Y_{124}Q/W_{286}A$	1500	4.4	360	3.5	110	2.8
BuChE	3900	5.0	3.0	0.66	2.4	0.52
$D_{74}N$	680	3.9	240	3.3	4.0	0.83
$Y_{72}N/D_{74}N/Y_{124}N/W_{286}R$	280000	7.5	14000	5.7	860	4.0
$Y_{72}N/D_{74}N/Y_{124}Q/W_{286}A$	59000	6.6	4600	5.1	490	3.7
$Y_{337}A$	5.7	1.0	1.0	0	2.2	0.47

[a] Inhibition constants (K_I') were compared to AChE wt (K_I). Values were derived from the data in Table II and, in the case of the chimeras, from Vellom et al. (1993). [b] ND = not determined.

BW284C51 and succinylcholine interactions with mutant AChEs and BuChEs point to this possibility (Shafferman et al., 1992a,b; Neville et al., 1992). To delineate the relative contributions of these four residues positioned near the lip of the gorge, we systematically analyzed the corresponding double, triple and quadruple mutants and then estimated the binding forces by a partitioning of binding free energy (Tables II and III).

In the case of BW284C51, the contributions of Y_{72}, Y_{124}, and W_{286} to the binding energy partition in a remarkably consistent fashion (Table III). This summation of $\Delta\Delta G$ values for each incremental change in amino acid residues probably reflects the involvement of several moieties in the stabilization of the high-affinity complex and the lack of appreciable interactions between binding loci. The Y_{72} and Y_{124} mutations remove 1.0–1.3 kcal of stabilization energy, while the W_{286} mutation eliminates 2.7 kcal. The double mutations show simple additive properties in ΔG over the single mutations, and the Y_{72},Y_{124} mutation yields values remarkably close to that of the chimera of $B_{5-174}A_{175-575}$ (Table III). Hence, the loss of affinity seen in the chimera can be accounted for by only two residues, Y_{72} and Y_{124}.

Mutation of the three residues yields an enzyme with a BW284C51 dissociation constant equivalent to that in BuChE (Table II). A comparison of $W_{286}R$ (mouse BuChE) with $W_{286}A$ (human BuChE) shows that the additional positive charge near the lip of the gorge reduces the binding energy by 0.8 kcal. D_{74} is found in both AChE and wild-type BuChE. The elimination of this charge in $D_{74}N$ has a dramatic effect on the stabilization of BW284C51 and the decamethonium complexes. In the quadruple mutant, D_{74},Y_{72},Y_{124},W_{286}, the influence of D_{74} is no longer additive, but we may already have reached a ceiling where the binding domain defined by these four residues is insufficient for contributing to the stabilization of the complex.

The influence of this sequence of mutations on decamethonium binding is similar, but far less dramatic. Of particular interest is the comparison of the Y_{72},Y_{124},W_{286} mutant with BuChE. Despite substitution of the three residues of BuChE at these positions, this mutant binds decamethonium with a far lower affinity than does wild-type BuChE (Tables II and III). This implies that BuChE may use different residues than AChE for stabilizing the quaternary ammonium moiety in decamethonium distal to the choline site.

Inhibition by Peripheral Site Inhibitors. Since early studies showed that the binding of the above bisquaternary inhibitors is mutually exclusive with inhibitors selective for both the active center and the peripheral site (Taylor & Lappi, 1975), we have also examined the interaction of the set of replacements of Y_{72}, Y_{124}, and W_{286} with the peripheral site inhibitor, propidium. As is the case for the bisquaternary inhibitors, these mutations exhibit dramatic effects on both the binding affinity and the apparent mode of propidium inhibition. One can eliminate much of the binding energy for propidium by the same set of mutations. This is consistent with the finding that one end of the bisquaternary molecule occupies an overlaping position with propidium. As with decamethonium, the mutations forming the triple mutants produce a greater difference in propidium affinity than is observed between AChE and BuChE (Table II). Hence, propidium also may bind at a different locus in AChE and in BuChE.

Inhibition by Tricyclic Amine-Containing Compounds. A large family of polycyclic compounds is known to inhibit AChE, and several studies point to the subsite of choline association within the active center to be critical in the binding of these ligands. Early studies showed that *N*-methylacridinium binding (Mooser & Sigman, 1974) was competitive with the binding of edrophonium but not that of propidium (Taylor & Lappi, 1975). Also, the position of tacrine ring stacking with W_{86} was established by crystallography (Sussman et al., 1992). The movement of the Y_{337} side chain also contributes to the stabilization of the tacrine complex. Studies with chimeras indicated that the critical residues in ethopropazine binding were located in the central region of the sequence (Vellom et al., 1993). We have also found that the triple mutation of Y_{72},Y_{124},W_{286} to the residues in BuChE had little influence on the enhancement of ethopropazine binding (data not shown). Following these leads, we have modified Y_{337} to the alternative residues, A and F, found in BuChE and *Torpedo* AChE.

The most striking specificity difference in the substituted phenothiazines is found for ethopropazine, where the K_I's for AChE and BuChE differ by 1800-fold (Table IV). This 4.5-kcal increase in binding free energy for BuChE is achieved primarily by the substitution of A for Y_{337}. By contrast, substitution of F for Y_{337} or G for F_{338} (not shown) or modification of residues outlining the acyl pocket (not shown) has little influence on ethopropazine binding. The specificity difference of AChE and BuChE for promethazine with its smaller (dimethylamino)-2-propyl side chain is reduced by 1.5 kcal (Table IV). The Y_{337} to A substitution also has a far more modest influence on promethazine affinity when compared to ethopropazine. A further diminution of the free energy differences between the AChE and BuChE complexes is evident with the (dimethylamino)-*n*-propyl analogue, promazine. Also, the Y_{337} to A substitution is largely without effect on promazine binding energy.

Binding of the phenothiazine ring itself is difficult to analyze because of its low solubility; however, the basic ring structure has a low affinity for native and mutant AChEs ($K_I > 100$ μM). Little difference in the binding of phenothiazines between AChE and BuChE was detected (data not shown).

An examination of the inhibition capacity of acridine shows little difference in the dissociation constants for AChE and

Table IV: Dissociation Constants[a] (μM) and Changes in Free Energy (ΔΔG) for Reversible Inhibition of Mouse Wild-Type and AChE Y_{337} Mutant Cholinesterases

	enzyme									
	AChE wt		BuChE wt		$Y_{337}A$		$Y_{337}F$			
inhibitor	K_I	αK_I	K_I	αK_I	K_I	αK_I	K_I	αK_I	$\Delta\Delta G_{AChE-BuChE}$ (kcal)	$\Delta\Delta G_{AChE-Y_{337}A}$ (kcal)
edrophonium	0.30 ± 0.10	6.4 ± 0.6	110 ± 37	1400 ± 700	5.6 ± 1.3	26 ± 14	0.29 ± 0.06	7.8 ± 0.8	−3.5	−1.8
acridine	6.1 ± 2.2	26 ± 7	1.6 ± 0.9	5.4 ± 3.6	0.87 ± 0.15	3.4 ± 0.8	20 ± 4	210 ± 22	0.80	1.2
N-methyl-acridininium	0.73 ± 0.19	1.2 ± 0.3	0.19 ± 0.05	0.29 ± 0.08	0.049 ± 0.010	0.13 ± 0.03	1.3 ± 0.3	7.8 ± 2.3	0.80	1.6
9-aminoacridine	0.040 ± 0.010	0.11 ± 0.03	0.022 ± 0.010	0.034 ± 0.013	0.0023 ± 0.0007	0.0059 ± 0.0010	0.171 ± 0.055	0.71 ± 0.15	0.36	1.7
tacrine	0.040 ± 0.010	0.13 ± 0.05	0.017 ± 0.004	0.047 ± 0.022	0.0015 ± 0.0005	0.005 ± 0.001	0.15 ± 0.03	0.68 ± 0.18	0.52	2.0
promazine	50 ± 10	790 ± 230	1.8 ± 0.7	7.1 ± 3.9	37 ± 11	150 ± 27	51 ± 12[b]	850 ± 21	2.0	0.18
chlorpromazine	26 ± 8	410 ± 290	2.1 ± 0.9	8.5 ± 4.0	17 ± 7	91 ± 38	22 ± 11[b]	540 ± 191	1.5	0.25
promethazine	230 ± 64	4100 ± 4100	1.4 ± 0.7	3.3 ± 0.2	29 ± 11	130 ± 29	110 ± 20	1700 ± 440	3.0	1.2
ethopropazine	110 ± 51	1100 ± 160	0.061 ± 0.027	0.130 ± 0.076	0.041 ± 0.003	0.13 ± 0.002	34 ± 6	330 ± 86	4.5	4.7

[a] Constants were evaluated as in Figure 3. Means and standard errors are from 3–6 experiments. [b] Denotes moderately nonlinear replots approximated by a straight line.

BuChE (Table IV). The affinities of acridine analogues for both enzymes are enhanced by *N*-methyl and 9-amino substitutions by 1 and 2 orders of magnitude, respectively. The differences in binding energy between the $Y_{337}A$ mutant and AChE for tacrine, 9-aminoacridine, and *N*-methylacridinium are even larger than the BuChE to AChE difference (Table IV).

DISCUSSION

Substrate Inhibition. The use of Scheme II to describe the kinetics of acetylthiocholine hydrolysis yields a markedly improved fit to the kinetic data in the substrate inhibition range for a series of AChEs. Deviations from Scheme I are less evident for wild-type AChE (Figure 1A) than for some of the mutant enzymes (Figure 1B). The difference in mechanism is simply attributable to not restricting hydrolysis to the binary ES complex and acknowledging that the ternary complex SES will generate product, albeit at a different rate. Hence, mutations might influence either the dissociation constant (K_{ss}) or the hydrolysis capacity (bk_{cat}) of the ternary complex. Inspection of the data in Table I reveals that the majority of mutations do not influence K_{ss}. Only the $F_{338}G$, $F_{297}Y$, and $D_{74}N$ mutations and some of their permutations produce a significant increase in K_{ss}. The *b* value, reflecting the relative commitment of the ternary complex to proceed to product, varies with the individual mutations. Two caveats should be mentioned here. First, in cases where there is little substrate inhibition, we cannot distinguish between a large K_{ss} and a value of *b* which approaches 1.0. This situation would apply for the F_{338} mutation. Second, the estimation of these parameters depends on measurements at high substrate concentration, conditions where the ratio of general base relative to enzyme-catalyzed hydrolysis increases. Although we have measured concentration profiles to 100 mM acetylthiocholine, it is difficult to establish whether *b* values between 0 and 0.20 differ from zero. The low rate of catalysis also precludes obtaining an accurate K_{ss} value for $F_{295}L/F_{297}I$ and $F_{297}I$. It is noteworthy that butyrylcholinesterase kinetics is best fit by a *b* value greater than 1.0, which reflects substrate activation. Unusual activation profiles for BuChEs have been noted in previous studies (Augustinsson et al., 1974; Vellom et al., 1993); one BuChE residue substitution in AChE ($F_{297}I$) partially mimics this activation behavior.

Three Inhibitor-Distinguishing Domains in the Cholinesterases. Our initial studies of inhibition with AChE–BuChE chimeras, when coupled with the site-specific mutants reported here, enable us to define three distinct domains responsible for the specificity of inhibitor interactions. Each domain is defined by a cluster of aromatic residues that appear uniquely disposed to handle the particular specificity requirements of the substrate or inhibitor. The acyl pocket of AChE is defined primarily by two phenylalanines, F_{295} and F_{297}, which not only outline the shape of the pocket (Sussman et al., 1991) but also, by restricting the degree of freedom of the bound substrate, enhance the catalysis of acetylthiocholine (Vellom et al., 1993). *Drosophila* cholinesterase contains only a single phenylalanine in this region, and its conversion to tyrosine confers an apparent resistance of the insect enzyme to certain organophosphates (Mutero & Fournier, 1992). We have previously shown in mouse AChE that mutation of either F_{295} or F_{297} to L or I, respectively, reduces catalytic efficiency for acetylthiocholine, while these mutations enhance catalytic efficiency for butyrylthiocholine. Of the two residues, F_{295} is the most restrictive to butyrylthiocholine hydrolysis (Vellom et al., 1993). We observe here that the F_{295} mutation to Y

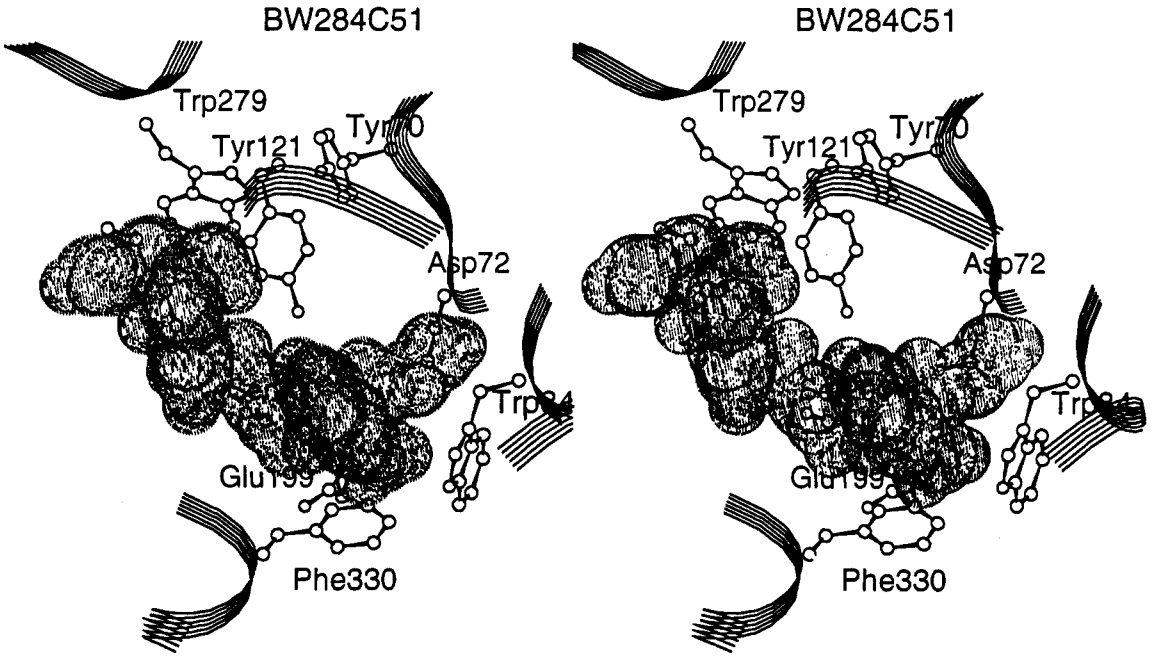


FIGURE 4: Energy-minimized structure of BW284C51 bound to *Torpedo* acetylcholinesterase. The initial orientation fixed one quaternary moiety near $W_{86(84)}$ and $F_{337(330)}$ (Harel et al., 1993). By allowing the flexible groups in the ligand and the $Y_{72(70)}$, $W_{86(84)}$, $Y_{124(121)}$, $E_{202(199)}$, $W_{286(279)}$, and $F_{297(290)}$ to rotate, a minimized structure was achieved where the second quaternary moiety extends out of the gorge, residing in the vicinity of the $W_{286(279)}$,$Y_{124(121)}$,$Y_{72(70)}$ aromatic cluster. The numbers in parentheses denote the *Torpedo* sequence and correspond to the residues in the figure.

influences acetylthiocholine kinetics by decreasing K_m and markedly decreasing k_{cat}. Examination of these mutants in relation to the size of the acylating agent for carbamoylating and phosphorylating agents should be of interest in finely mapping how this domain affects substrate specificity.

A second cluster of aromatic residues, Y_{72}, Y_{124}, and W_{286}, is found near the lip of the gorge. This cluster is found in the vicinity of D_{74}, which may contribute longer range Coulombic forces in the stabilization of substrate or inhibitor complexes. Shafferman and colleagues (1992a) have shown that W_{286} and D_{74} mutations diminish the apparent binding of BW284C51, decamethonium, and propidium, as reflected in changes in IC_{50} values. Neville et al. (1992) have examined inhibitor binding to D_{74} position mutants in BuChE. Finally, Harel et al. (1992) have observed that W_{286} also influences propidium binding. However, these mutations also markedly affect the K_m for acetylthiocholine. Reporting the inhibition constants as IC_{50}'s without ascertaining competitive and noncompetitive components makes it difficult to assess the true contribution of these residues to inhibitor binding. By analyzing interactions with the same species of enzyme, the free energy contributions can be partitioned between individual side chains.

There is a remarkably close correspondence in the $\Delta\Delta G$ of binding for BW284C51 between the $B_{5-175}A_{175-575}$ chimera and the Y_{72},Y_{124} mutations and between butyrylcholinesterase and the Y_{72},Y_{124},W_{286} mutation. This observation, coupled with the linear summation of free energy values in the single, double, and triple mutations in this cluster, points to directly additive energy contributions in the stabilization of this structurally rather rigid inhibitor. Energy minimization of BW284C51 docked within the active center of AChE shows virtual contact with all of these residues (Figure 4). Moreover, the additional interactions from the π-orbitals of the allyl group and the phenyl group in BW284C51 should be enhanced by the close apposition of the aromatic residues (Figure 4).

In the complex with another prototype bisquaternary compound, decamethonium, mutagenesis studies reveal differences in contributions from the various side chains. First, the mutations show that the aromatic side chains, Y_{72}, Y_{124}, and W_{286}, have a slightly diminished role, as might be expected for a compound devoid of aromatic groups with only methylenes connecting the quaternary nitrogens. More importantly, the complex with this triple mutation has a much lower affinity for decamethonium when compared to the BuChE–decamethonium complex. The latter observation, in fact, suggests different orientations of bound decamethonium in this mutant and in BuChE. The crystal structure of the *Torpedo* AChE–decamethonium complex shows the two trimethylammonio groups spanning between $W_{86(84)}$ and $W_{286(279)}$ (Harel et al., 1993). The numbers in parentheses designate corresponding positions in the *Torpedo* sequence. By removing W_{286} and the aromatic environs contributed by $Y_{72(70)}$ and $Y_{124(121)}$, the site for association of one of the trimethylammonio groups is obliterated. Accordingly, one trimethylammonio group is free to seek a different region of AChE with which to associate. This region is limited by the length and flexibility of the interquaternary chain. In BuChE, such a site may be available by virtue of the opening of the acyl pocket and the stabilization of the two trimethylammonino groups by $W_{236(233)}$ and $W_{86(84)}$ (Figure 5). While this configuration is possible for decamethonium, the longer and more rigid BW284C51 can only be positioned between $W_{86(84)}$ and the entry into the gorge.

Similar arguments can be raised for propidium as it can be accommodated deep in the BuChE gorge, while this location for propidium binding is occluded sterically by virtue of the additional aromatic side chains in the case of AChE. Hence, the characteristics of propidium binding (i.e., not being influenced by phosphorylation of the serine by DFP or by the binding of active center ligands such as edrophonium) (Taylor & Lappi, 1975) would not be evident in the BuChE–propidium complex. Analysis of ΔG's for the ligand–AChE complexes shows another distinctive feature of propidium (Table III). When W_{286} is substituted, Y_{72} and Y_{124} no longer appear to contribute to the stabilizing energy of the complex.

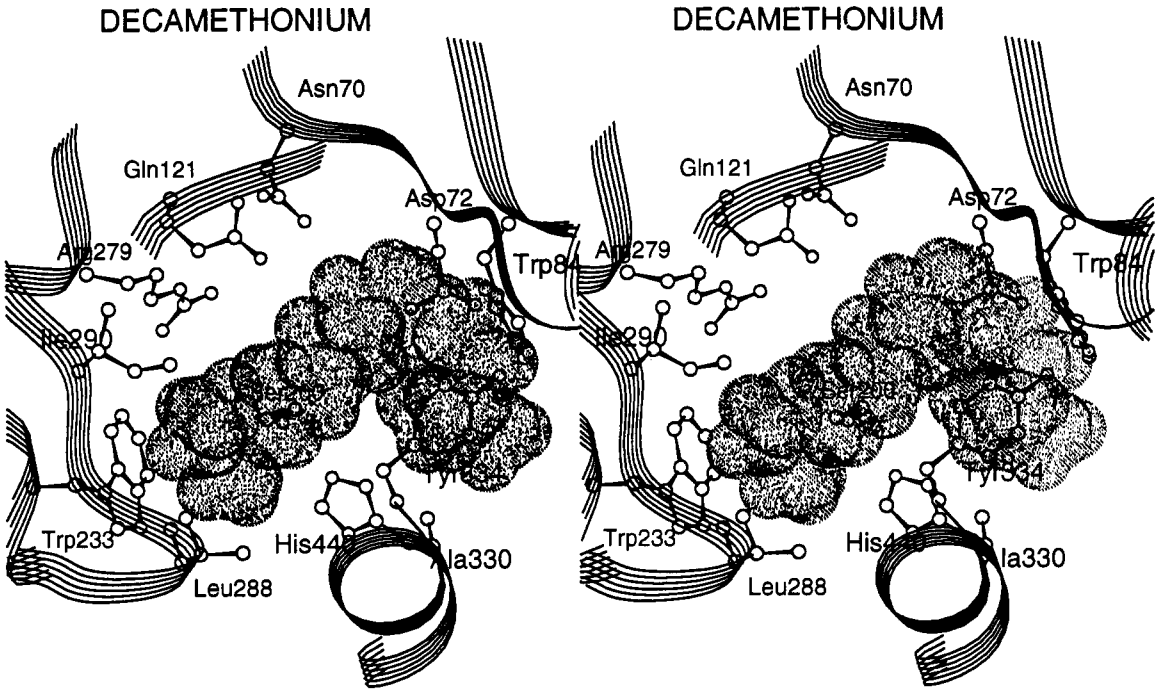


FIGURE 5: Proposed binding orientation of decamethonium associated with acetylcholinesterase containing the following mutations: $Y_{72(70)}N$, $Y_{124(121)}Q$, $W_{286(279)}R$, $F_{295(288)}I$, $F_{297(290)}L$, and $Y_{337(330)}A$. The quaternary ammonium group at one end of the molecule was fixed near $W_{86(84)}$ and $A_{337(330)}$ (Sussman et al., 1992). Energy minimization was conducted allowing the bonds in decamethonium and the side chains of residues $N_{72(70)}$, $S_{203(200)}$, $W_{236(233)}$, $Y_{341(334)}$, and $W_{86(84)}$ to rotate freely. The numbering in parentheses donotes positions in the *Torpedo* sequence and corresponds to the figure. It is proposed that that the additional space in the acyl pocket region of the active center gorge enables decamethonium to span between $W_{86(84)}$ and $W_{236(233)}$.

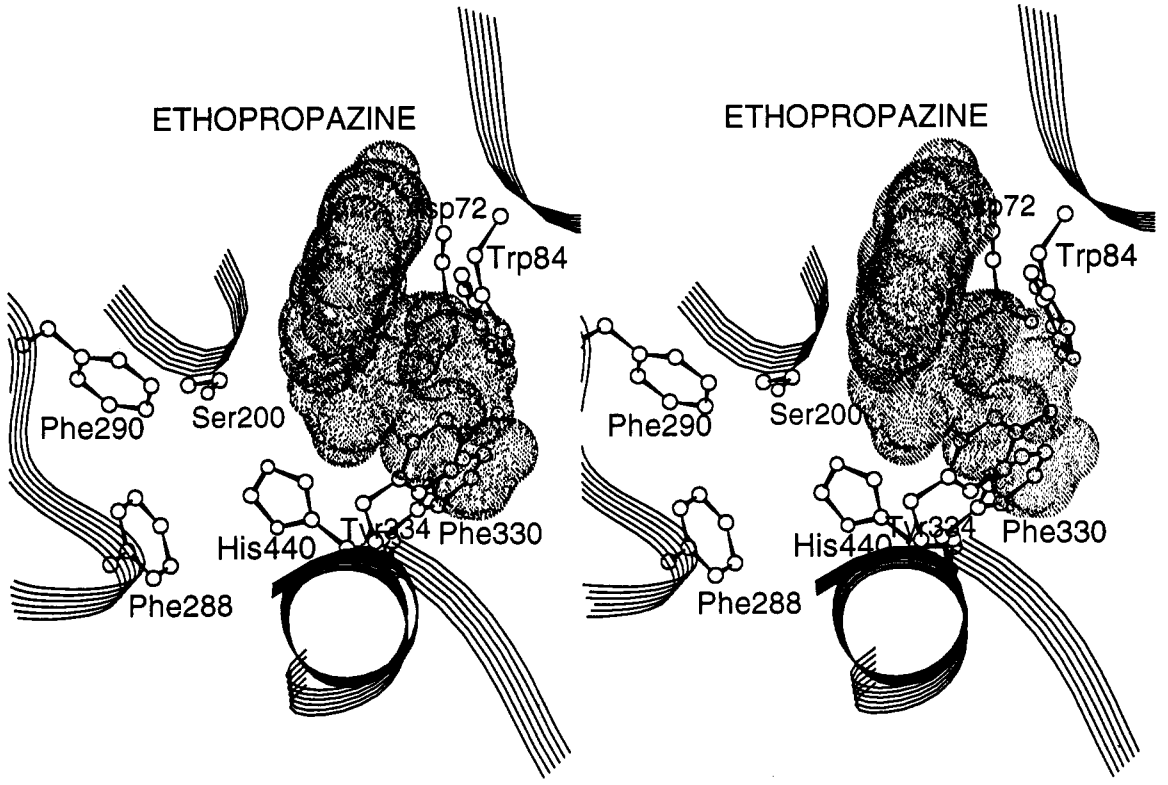


FIGURE 6: Stereoview of the energy-minimized orientation of ethopropazine bound to *Torpedo* acetylcholinesterase. The initial orientation of the phenothiazine ring was based on the orientation of the acridine ring of tacrine (Harel et al., 1993). In the minimization, the flexible bands of the ligand [i.e., the (diethylamino)-2-propyl side chain] and the amino acid side chains $Y_{72(70)}$, $D_{74(72)}$, $W_{86(84)}$ $Y_{124(121)}$, $A_{337(330)}$, and $Y_{341(334)}$ were allowed to rotate. The residue numbering in parentheses is from the *Torpedo* sequence and corresponds to the figure. Energy minimization was conducted for the $F_{337(330)}A$ mutation. By then substituting F for $A_{337(330)}$, the overlap of the ethopropazine side chain with the phenylalanine is evident.

A third aromatic cluster is found in the region where the choline moiety associates during catalysis. Similar to the site at the lip of the gorge, but in contrast with the acyl pocket site, this site contains a charged residue, E_{202}, which contributes to the stabilization energy of the complexes. In the acridine series, *N*-methylacridinium, 9-aminoacridine, and tacrine show

higher affinities for the cholinesterases than does the parent acridine ring. Moreover, they show higher affinities for BuChE than for AChE. The higher affinities of these ligands for BuChE correlate with alanine being substituted for Y_{337} in mammalian AChE or for the homologous F_{330} residue in *Torpedo* AchE (Figure 6). Thus, the aromatic ring at position $Y_{337(330)}$A does not contribute to stabilization of the complexes of the substituted acridines. By contrast, Y_{337} (or F_{330} in *Torpedo*) appears to play a role in the stabilization of the edrophonium complex (Table IV).

In the case of the phenothiazines, the longer side chain substitutions, as in ethopropazine, markedly reduce the binding affinity for AChE. Since the affinity differences are large, this must apply to both ethopropazine enantiomers. A qualitatively similar situation of reduced magnitude is seen for promethazine [the (*dimethyl*amino)-2-propyl congener]. Removal of the aromatic side chain in the 337 position also produces dissociation constants for the phenothiazines resembling that of BuChE (Table IV).

Hence, by using the AChE crystal structure template and sequence comparisons among the cholinesterases, it is possible to delineate the critical side chains giving rise to the AChE–BuChE specificity differences. Initial studies with AChE–BuChE chimeras from mouse and then with site-specific mutations enabled us to analyze these differences by partitioning of free energy and to attribute specificity differences to three distinct domains of the cholinesterase molecule.

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