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Overlapping Drug Interaction Sites of Human Butyrylcholinesterase Dissected by Site-Directed Mutagenesis

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SUMMARY

Butyrylcholinesterase [BuChE (acylcholine acyl hydrolase); EC 3.1.1.8] limits the access of drugs, including tacrine, to other proteins. The "atypical" BuChE variant, in which Asp70 at the rim of the active site gorge is substituted by glycine, displayed a more drastically weakened interaction with tacrine than with cocaine, dibucaine, succinylcholine, BW284c51 [1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide], or α-solanine. To delineate the protein domains that are responsible for this phenomenon, we mutated residues within the rim of the active site gorge, the region parallel to the peripheral site in the homologous enzyme acetylcholinesterase [AChE (acetylcholine acetyl hydrolase); EC 3.1.1.7], the oxyanion hole, and the choline-binding site. When expressed in microinjected *Xenopus laevis* oocytes, all mutant DNAs yielded comparable amounts of immunoreactive protein products. Most mutants retained catalytic activity close to that of wild-type BuChE and were capable of binding ligands. However, certain modifications in and around the oxyanion hole caused a dramatic loss in activity. The affinities for tacrine were reduced more dramatically than for all other ligands, including cocaine, in both oxyanion hole and choline-binding site mutants. Modified ligand affinities further demonstrated a peripheral site in residues homologous with those of AChE. BuChE mutations that prevented tacrine interactions also hampered its ability to bind other drugs and inhibitors, which suggests a partial overlap of the binding sites. This predicts that in addition to their genetic predisposition to adverse responses to tacrine, homozygous carriers of "atypical" BuChE will be overly sensitive to additional anticholinesterases and especially so when exposed to several anticholinesterases in combination.

The pharmacokinetics of the ChE inhibitor Alzheimer's disease drug tacrine are significantly influenced by blood ChEs because when tacrine enters the circulation, it first encounters and reacts with serum BuChE and AChE on the surface of erythrocytes. Only tacrine molecules that escape this screen can subsequently cause a physiological response by inhibiting brain AChE. Of the two ChEs in the blood, BuChE is quantitatively at least as important as AChE (1). Furthermore, it is undoubtedly replaced in the blood faster than is AChE. Although it does not degrade tacrine, 20% of the circulating BuChE can sequester 40% of the total tacrine in the blood of patients (2). This, and the large number of natural BuChE variants (3, 4), raises the questions of which BuChE residues interact with tacrine and how tacrine might modify the response of patients to other drugs or how changing the BuChE residues might modify the response to tacrine.

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The structure of AChE and the mode of binding of tacrine to it are known because of X-ray crystallography (5–7), but the interaction of tacrine with BuChE must be different because of differences in amino acid residues at the homologous binding site (Fig. 1). In particular, the common "atypical" genetic variant of BuChE is predicted to differ in its tacrine interactions. The mutated residue D70, although far from the catalytic site, was first shown to affect ligand specificity when its natural mutation to a glycine residue was identified as the basis of the failure of the variant to hydrolyze succinylcholine (8, 9). It has been suggested that D70 is positioned in such a way that its free carboxylate group faces outward, which allows it to attract positively charged inhibitors to the opening of the active site gorge (10, 11). More recently, we observed that atypical BuChE interacts with tacrine with a 100-fold weaker affinity than does the normal enzyme (2). This raised the questions of which additional BuChE residues are crucial for interactions with tacrine and what other drugs interact poorly with atypical BuChEs.

The X-ray crystallography of AChE (5) combined with site-

ABBREVIATIONS: ChE, cholinesterase; AChE, acetylcholinesterase; BTCh, butyrylthiocholine; BuChE, butyrylcholinesterase; DFP, diisopropylfluorophosphonate; ELISA, enzyme-linked immunosorbent assay.

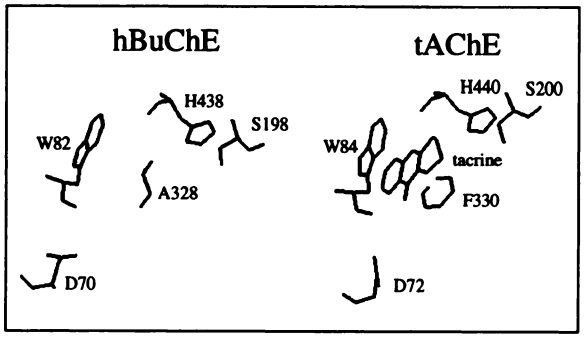

Fig. 1. Tacrine binding at the active site. The three-dimensional structure of *Torpedo* AChE crystals soaked with tacrine (*right*) is compared with the modeled homologous residues of human BuChE (*left*). The representation of human BuChE without tacrine was based on Harel *et al.* (7), and the representation of AChE with tacrine was based on Harel *et al.* (6) (Insight II, Biosym Technologies, San Diego, CA).

directed mutagenesis has been used to detail the interaction sites of AChE with drugs and inhibitors (for reviews, see Refs. 12 and 13); these include attraction of the substrate to a peripheral binding site and into a 20-Å-deep gorge and its binding to both the acyl- and choline-binding sites (Fig. 2). In the transition state, the acyl and alkoxy substrate moieties, the incoming hydroxyl group of the active site serine residue of the enzyme, and the carbonyl oxygen create a tetrahedral array around the substrate carbonyl carbon atom. The carbonyl oxygen, now bearing a negative charge, is protected by the oxyanion hole of the enzyme. The transition state collapses to the acyl-enzyme, and free choline leaves the active site (12). In a previous study, we replaced the entire region of human BuChE (residues 55–138, which includes all these sites) with the homologous region from human AChE. The resulting chimera had catalytic and ligand-binding properties that were intermediate between the two parent proteins (14). To further characterize sites of BuChE/drug interaction, we now have created a series of point mutations within this region to compare BuChE with its atypical variant and with AChE.

Four key regions in BuChE were addressed in the current study through the use of site-directed mutagenesis: (a) The oxyanion hole is lined in *Torpedo californica* AChE by the amide groups of the peptide bonds of residues homologous with BuChE G116, G117, and A199 (12), of which we mutated G116 and G117 and the adjacent G115.[1] (b) The choline-binding site was associated with the residue W82 by photoaffinity labeling (15, 16), site-directed mutagenesis (17–19), and diffusion of quaternary ammonium inhibitors into *T. californica* AChE crystals (6). (c) A peripheral anionic site, which accounted for the allosteric inhibition of AChE by BW284c51 [1,5-bis(4-allyldimethylammoniumphenyl)pentan3-one dibromide] and propidium (20), was identified with Y70 and Y121 (6, 7, 18–23). Mutagenesis experiments in which we replaced the two AChE residues Y70 and Y72 in various combinations demonstrated decreased affinity to AChE-typical ligands (24). (d) The "back door," which was proposed by Gilson *et al.* (25) to allow the direct exit of choline through a polypeptide flap, has the residue V127 as a major feature. We used this knowledge of AChE to predict key regions of interaction between BuChE and tacrine, and we selected additional anti-ChEs.

We now report that substitution of the relevant BuChE residues (Fig. 2) reveals partially overlapping binding sites for tacrine and other ligands; this implicates other regions around BuChE residue D70 in such ligand interactions.

Materials and Methods

BW284c51, α-solanine, dibucaine hydrochloride, succinyldicholine dichloride, and tacrine hydrochloride were purchased from Sigma Chemical (St. Louis, MO). Cocaine hydrochloride was from Merck (Darmstadt, Germany). Human AChE and BuChE were from microinjected *Xenopus laevis* oocytes.

Restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were from Boehringer-Mannheim Biochemica (Mannheim, Germany). Mutagenic primer oligonucleotides, supplied by Microsynth (Windisch, Switzerland), were pTCT TGC TGT CAG GCC ATA GAT CAA AG-OH (N68A), pCT TGC TGT CAG GAC ATA GAT CAA A-OH (N68D), pT TGC TGT CAG AAG ATA GAT CAA AG-OH (N68K), pTCT TGC TGT CAG CGC ATA GAT CAA AG-OH (N68R), pCT TGC TGT CAG TAC ATA GAT CAA A-OH (N68Y), pGA TCA GAG ATG TTC AAC CCA AAC ACT-OH (W82F), pGA TCA GAG ATG TAC AAC CCA AAC ACT-OH (W82Y), pA TGG ATT TAT GCT GGT GGT TTT CA-OH (G115A), pTA TGG ATT TAT TGT GGT GGT TTT C-OH (G115C), pA TGG ATT TAT GAT GGT GGT TTT CA-OH (G115D), pTA TGG ATT TAT GAG GGT GGT TTT CAA-OH (G115E), pGG ATT TAT GGT GAG GGT TTT CAA ACT-OH (G116E), pT TAT GGT GGT GAT TTT CAA ACT GG-OH (G117D), pTT TAT GGT GGT TGT TTT CAA ACT G-OH (G117C), pTT TAT GGT GGT GAG TTT CAA ACT GGA-OH (G117E), pGGT GGT GGT TTT GCA ACT GGA ACA TC-OH (Q119A), pGT GGT GGT TTT GAA ACT GGA ACA T-OH (Q119E), pGGT GGT GGT TTT GGA ACT GGA ACA TC-OH (Q119G), pGGT GGT TTT CAC ACT GGA ACA TCA-OH (Q119H), pGT GGT GGT TTT AAA ACT GGA ACA T-OH (Q119K), pT GGT GGT TTT CGA ACT GGA ACA TC-OH (Q119R), pGGT GGT GGT TTT TAC ACT GGA ACA TC-OH (Q119Y), pGGT GGT TTT CAA GAG GGA ACA TCA TCT T-OH (T120E), pGGT GGT TTT CAA GGT GGA ACA TCA TC-OH (T120G), pGGT GGT TTT CAA CAT GGA ACA TCA TC-OH (T120H), pGT GGT TTT CAA AAG GGA ACA TCA TCT-OH (T120K), pCA TCT TTA CAT GAT TAT GAT GGC A-OH (V127D), and pTA TGG ATT TAT GGT GCT GAT TTT CAA ACT GGA AC-OH (G116A/G117D). Bases that are mismatched to wild-type BuChE cDNA (1) are underlined, and the substituted amino acids are shown in parentheses. The homologous (-) strands were also synthesized for use as mutagenic primers.

External primers for PCR mutagenesis were pCAT ACT GAA GAT GAC ATC ATA-OH (+154 to +174) and pAGC CAA CTG TTG ATC AAA TAA-OH (−701 to −681) for use with *Nco*I and *Ava*I in the mutagenesis of G115, G116, G117, G116/G117, Q119, and T120; pCAT ACT GAA GAT GAC ATC ATA-OH (+154 to +174) and pAGC CAA CTG TTG ATC AAA TAA-OH (−701 to −681) for use with *Acc*I and *Ava*I in the mutagenesis of N68 and W82; and pCAT ACT GAA GAT GAC ATC ATA-OH (+154 to +174) and pTC AGT CTC ATT CTC TCT AG-OH (−953 to −935) for use with *Nco*I and *Bam*HI in the mutagenesis of V127. *In vitro* transcription kits were from Promega (Madison, WI).

After mutagenesis, the modified regions in each of these plasmids were sequenced for verification of structure. *In vitro* transcription, microinjection into *X. laevis* oocytes, and expression of BuChE mRNA were performed as described by Gnatt *et al.* (26). BuChE activity measurements and K_m and IC_{50} determinations in multiwell plates were as described in Neville *et al.* (10). Immobilization of BuChE in the multiwell plates via adsorbed antibodies further enabled ELISA determination of BuChE (24). K_i values were calculated according to Hobbiger and Peck (27): $K_i = IC_{50}(1 + S/K_m)$. Although

[1] The residue numbers in the text are those of human BuChE (1). For these, the corresponding *T. californica* AChE residue number is given in parentheses: N68 (Y70), D70 (D72), W82 (W84), G115 (G117), G116 (G118), Q119 (Y121), T120 (T122), V127 (V129), S198 (S200), and A199 (A201).

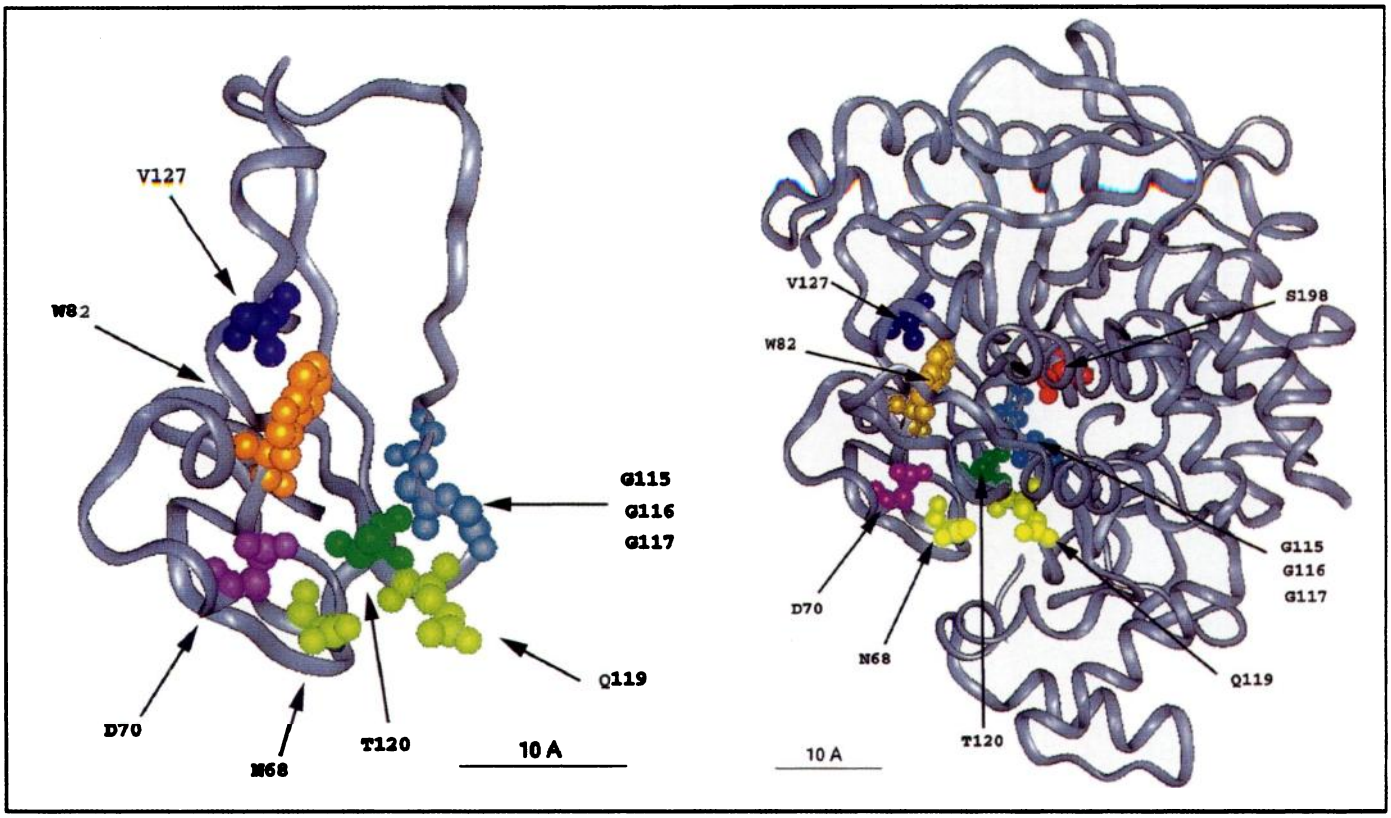

Fig. 2. Sites of mutagenesis of human BuChE. Ribbon diagrams of (*left*) a cut-away view of human BuChE residues 55 to 138, and (*right*) the entire enzyme. Both are viewed from above the opening of the active site gorge, toward the catalytic site. Shown are the peripheral binding site (N68, Q119 and T120) in *yellow* and *green*, the oxyanion hole (G115, G116 and G117) in *light blue*, the choline-binding site (W82) in amber, and the "back door" (V127) in *dark blue*. Also shown are the site of natural variance (D70) in *violet* and the catalytic site serine (S198) in *red*. The model of human BuChE (7) based on the three-dimensional structure of *T. californica* AChE (5) was analyzed using the Insight II program (Biosym Technologies, San Diego, CA) on a Silicon Graphics Computer.

steady state hydrolysis of BTCh by BuChE probably does not obey the assumptions of Michaelis-Menten kinetics, we used K_m, K_i, and k_{cat} values derived from the experimental data (as others do) on the assumption that they are sufficiently close to justify this analysis. The substrate concentration, S, was 5 mM.

To test the integrity of the active site in oxyanion hole mutants that showed no catalytic activity (G117E, G117C, and G117D), we incubated 100 ml of 1:10 (w/v) oocyte homogenate for 5 min at 21° in phosphate-buffered saline with 2 mCi of [^{3}H]DFP (DuPont-New England Nuclear, Bad Homburg, Germany), electrophoresed the product with commercial BuChE as a mobility standard, and autoradiographed the gel for 2 months under an intensifying screen.

Results

Compared with normal BuChE, the affinity of the atypical BuChE variant for tacrine was reduced 100-fold, but for cocaine, succinylcholine, BW284c51, α-solanine, and dibucaine, the affinity was reduced only 30-fold. Furthermore, atypical BuChEs frequently displayed lower affinity than AChEs for many of these drugs (Table 1). Therefore, the properties of D70 alone could not account for the ligand-binding differences between atypical and normal BuChEs. The involvement of additional residues was expected because D70 is conserved in AChE and BuChE, yet their drug interactions are drastically different. We previously examined the chimeric ChE formed by the replacement of residues 55–138 of BuChE with the corresponding peptide from AChE and found its affinities for other anti-ChEs to be generally intermediate between those of BuChE and AChE (14). To more precisely locate interaction sites, we prepared a series of point mutations at selected residues of the binding sites included in the chimeric peptide from BuChE (Fig. 2).

To evaluate changes that occurred in their catalytic properties, V_{max} and K_m values for BTCh were compared for oocyte-produced mutant BuChEs and atypical and normal BuChEs and AChEs. All mutants were detectable by the antibody to native human BuChEs. Comparison with an ELISA standard curve constructed with BuChEs from human serum allowed evaluation of the amount of enzyme being assayed and enabled the determination of several k_{cat} values of key oxyanion hole mutants (Table 2). The ELISA assay also revealed that nearly equal amounts of each mutant human BuChE were produced in the *X. laevis* oocytes, regardless of its intrinsic activity, suggesting that none of these mutations hampered appropriate protein folding.

Inhibition of BTCh hydrolysis was observed over a wide range of inhibitor concentrations (Fig. 3). Succinylcholine, cocaine, and dibucaine are substrates of BuChE, and as such compete with BTCh, yet have no sulfhydryl moiety that may be detected in the colorimetric assay that we use. Whatever hydrolysis may have occurred did not materially change their concentrations because the enzymatic rates that we determined were linear over the course of the 20–30-min period for which the average rate of BTCh hydrolysis was calculated. In many cases, a mutation was innocuous (Table 1); in other cases, inhibition of the mutants fell into one of only a few patterns, of which representative inhibition curves are

TABLE 1

Kinetic properties of variant BuChEs: K_m and K_i values

The first column identifies the natural or site-directed mutant. Assays were performed in 0.1–25 mM BTCh (in the case of AChE, acetylthiocholine), pH 7.4, and K_m values were extracted using GraFit 3.0 (Erithacus Software, Staines, UK). Note that the assays were all conducted at 22°, which may account for differences in the kinetic constants from data collected by other laboratories. IC_{50} values were determined in 1 mM substrate over the indicated range of inhibitor concentrations (GraFit); from them, K_i values were calculated (27). Standard deviations are shown for two or three determinations; where no standard deviation is shown, only one determination was performed. The "atypical" natural BuChE mutant is also included. Mutants G115C, G115D, G115E, and G116A/G117D are not included because they had drastically reduced or no detectable activity.

Variant/mutation		K_m	K_i					
			Tacrine	Succinylcholine	Dibucaine	α-Solanine	BW284c51	Cocaine
		mM	μM	mM	μM	μM	μM	mM
Normal	BuChE	2.8 ± 0.7	0.06 ± 0.03	4.0 ± 2.6	6.9 ± 4.6	3.3 ± 2.8	480 ± 110	0.39 ± 0.03
"Atypical"	D70G	3.8 ± 0.4	9.1 ± 2.4	52 ± 13	182 ± 100	78.0 ± 2.5	2540	3.2 ± 0.1
Peripheral site	N68D	1.1 ± 0.4	0.03 ± 0.01	0.26 ± 0.05	10.0 ± 0.2	1.26 ± 0.05	18	N.D.
	N68K	2.5 ± 1.1	0.01 ± 0.005	4.3 ± 0.6	17 ± 6	3.7 ± 1.3	>700	0.43 ± 0.0
	N68R	4.6 ± 1.0	0.06 ± 0.03	11.2 ± 0.7	4.3 ± 0.9	6.5 ± 3.3	>800	0.20 ± 0.07
	N68Y	3.4 ± 2.0	0.04 ± 0.02	2.9 ± 0.9	39 ± 2	0.5 ± 0.3	112 ± 50	N.D.
	N68A	1.1 ± 0.6	0.04 ± 0.03	0.7 ± 0.3	10 ± 6	0.7 ± 0.2	179 ± 91	N.D.
	Q119A	1.2 ± 0.6	0.02 ± 0.01	0.6 ± 0.2	20 ± 9	0.4 ± 0.1	68 ± 10	N.D.
	Q119E	0.6 ± 0.2	0.03 ± 0.03	0.12 ± 0.06	28.5 ± 5.0	0.28 ± 0.11	25.9 ± 4.6	N.D.
	Q119G	1.0 ± 0.1	0.04 ± 0.03	0.7 ± 0.3	26.2 ± 2.3	0.23 ± 0.18	95 ± 16	N.D.
	Q119H	0.7 ± 0.1	0.01 ± 0.003	0.5 ± 0.1	11.5 ± 4.7	0.3 ± 0.1	65	N.D.
	Q119K	0.8 ± 0.1	0.02 ± 0.004	30 ± 5.6	54.6 ± 5.7	9.0 ± 7.1	>450	0.20 ± 0.08
	Q119R	1.5 ± 0.6	0.05 ± 0.006	42 ± 16	115 ± 6	6.5 ± 3.7	>600	0.24 ± 0.16
	Q119Y	5.9 ± 0.6	0.09 ± 0.01	5.1 ± 0.3	107 ± 12	1.8 ± 0.9	78 ± 5	N.D.
	T120E	2.2 ± 0.4	0.12 ± 0.07	1.5 ± 0.1	1.1 ± 0.8	5.6 ± 1.3	128 ± 49	N.D.
	T120G	2.6 ± 0.6	0.15 ± 0.08	4.6 ± 1.6	32 ± 5	0.65 ± 0.0	>400	N.D.
	T120H	2.6 ± 0.3	1.20 ± 0.05	19 ± 3	80 ± 46	17 ± 6	>700	N.D.
	T120K	6.1 ± 1.1	>85	78 ± 24	>900	>200	>800	12.6
Choline site	W82Y	76 ± 28	3.2 ± 3.0	61 ± 14	140 ± 6	78 ± 37	>1000	3.6 ± 0.1
	W82F	31 ± 20	1.7 ± 1.1	86 ± 4	149 ± 39	77 ± 18	>1000	1.7 ± 1.3
Oxyanion hole	G115A	1.7 ± 0.6	9.5 ± 1.6	33 ± 10	33 ± 9	157	460	0.9 ± 0.1
	G115S	0.9 ± 0.1	0.83	24.3	74 ± 36	27	>500	1.1
	G117E	1.5 ± 0.3	0.2 ± 0.2	0.09 ± 0.05	19 ± 3	12.3 ± 9.9	330 ± 140	8.7 ± 5.3
Back door	V127D	4.9 ± 1.0	0.06 ± 0.03	2.5 ± 0.8	18 ± 5	4.6 ± 0.7	644 ± 32	N.D.
AChE		2.5	0.21	1.0	710	N.D.	0.13	3.9

N.D., not determined.

TABLE 2

Catalytic properties of variant BuChEs: k_{cat} and k_{cat}/K_m values

k_{cat} values were calculated from activity measurements against BTCh and enzyme determinations with the use of ELISA; K_m values were taken from Table 1.

Variant/mutation		k_{cat}	k_{cat}/K_m
		× 10^{-3} (min^{-1})	× 10^6 ($M^{-1}min^{-1}$)
Normal BuChE		98 ± 33	35
"Atypical"	D70G	−30	−8
Oxyanion hole	G115A	3	2
	G115S	2	2
	G115C	0.71	N.D.
	G115D	0.094	N.D.
	G115E	0	N.D.
	G116E	0	N.D.
	G117E	17 ± 2	11
	G117D	0.016	N.D.
	G116A/G117D	0	N.D.
AChE		70	28

N.D., not determined.

shown (Fig. 3). It is evident from the figure and from additional curves (not shown) that the inhibition profile of tacrine was largely similar to that of α-solanine, whereas cocaine and dibucaine constituted another group. Despite the low affinity of mutant BuChEs for succinylcholine, it may also fit this latter group. From curves such as those in Fig. 3 and the K_m values, K_i values for each mutation were also calculated (Table 1). To assist visualization of the results, we calculated the ratios between the K_i values of each of the mutants to the corresponding K_i value for the normal enzyme (Fig. 4). There was no mutation with any detectable activity that had decreased affinity for all ligands, further excluding the possibility that a generalized failure prevented folding into an active conformation. Even the T120K mutation, which had much lower affinity for all of the inhibitors, had a normal K_m value (Table 1 and Figs. 3 and 4).

The changes observed in inhibition parameters indicated involvement of several sites in ligand/BuChE interactions yet excluded involvement of other sites. Thus, both choline-binding site mutations W82Y and W82F caused a significant increase in K_m and K_i values for the set of inhibitors (>100-fold for tacrine, which is greater for these mutations than for any other inhibitor) (Table 1 and Fig. 4). This implied that the interaction between the quaternary ammonium moiety of the substrate and the indol groups of the tryptophan residue is quite specific because other aromatic groups, the phenyl of phenylalanine (W82F) or the hydroxyphenyl of tyrosine (W82Y), conferred lower affinity. However, mutation of V127 into aspartate resulted in no dramatic change in the K_m or K_i values for the examined substrate and inhibitors, which excludes the involvement of this back door residue with ligand interactions of BuChE.

Of the oxyanion hole mutations, the glycine residues at positions 115, 116, and 117 have drawn special attention because they are conserved throughout the ChE family and are found in the sequence of related α/β hydrolase-fold proteins (28). These glycine residues were suggested by Sussman *et al.* (5) to participate in the formation of the oxyanion

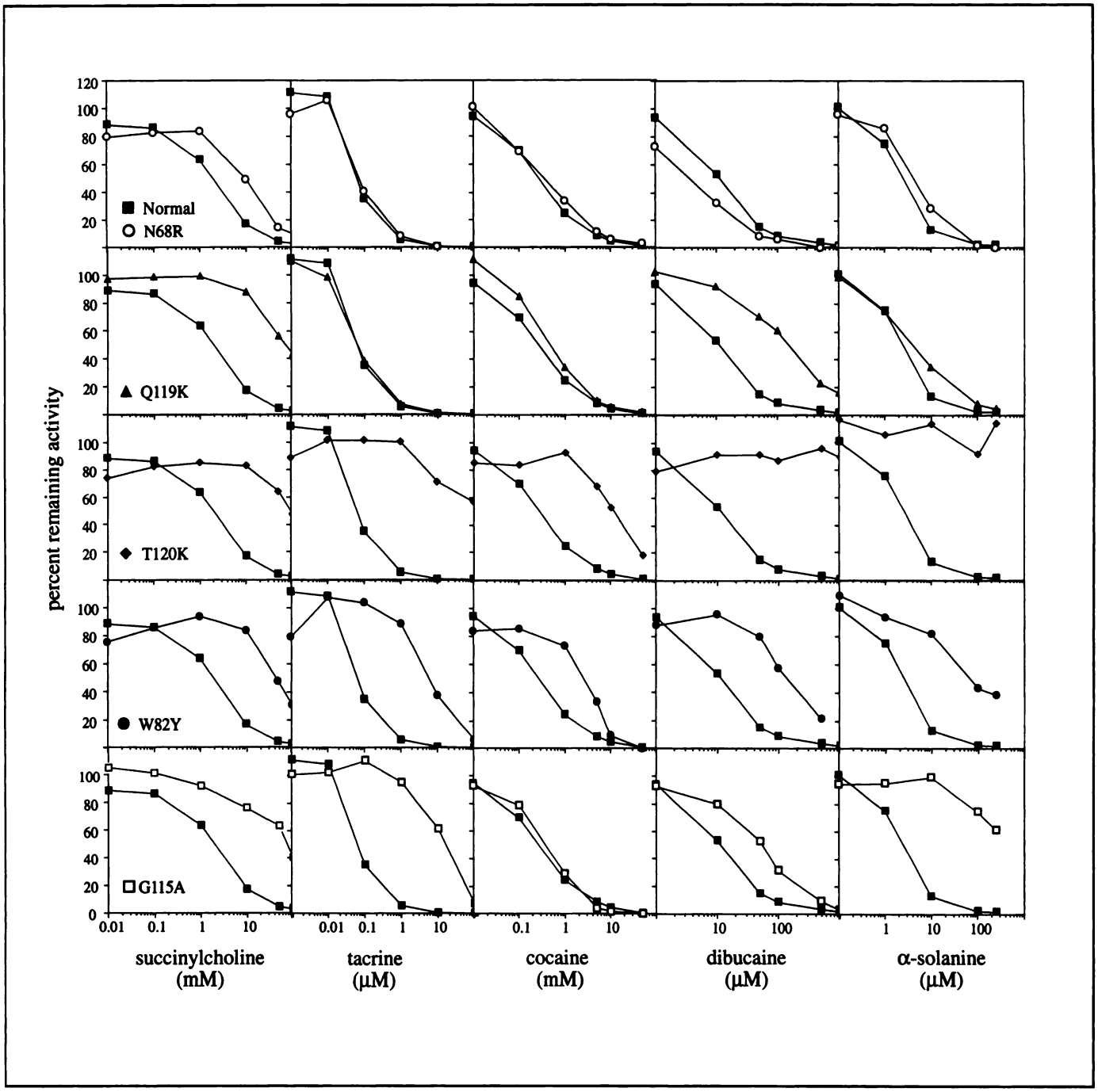

Fig. 3. Representative inhibition profiles of variant BuChEs. Inhibition profiles for a series of ChE inhibitors are shown for three peripheral site mutants (N68R, Q119K, and T120K), a choline-binding site mutant (W82Y), and an oxyanion hole mutant (G115A) compared with the inhibition profile of normal BuChE for each inhibitor. Note the different concentration units for each inhibitor.

hole. Several mutations were introduced in these residues: G115C, G115D, G115E, G115A, G115S, G116E, G117D, G117E, and the double mutation G116A/G117D, all of which resulted in a significant reduction in activity but not in the level of protein expression, which is in complete agreement with the predictions of others (5). The most significant influence of the substitution was noted for mutations G115D, G116E, and G116A/G117D, all of which displayed no detectable catalytic activity. However, G115C BuChE reacted with [^{3}H]DFP, although it had lost almost all of its activity (Fig. 5). Covalent labeling with [^{3}H]DFP, like measures of enzymatic activity, relies on the structural integrity of catalytic site elements, but unlike enzymatic tests, the labeling is unrelated to substrate turnover rate. Thus, an oxyanion hole mutant might still react, even if very slowly, with DFP; labeling by DFP would therefore indicate that the protein has folded properly, although there may be poor substrate binding or product release, which destroys its catalytic efficiency. Because the covalent reaction of this organophosphate agent with the active site serine of normal ChEs is quite fast, this

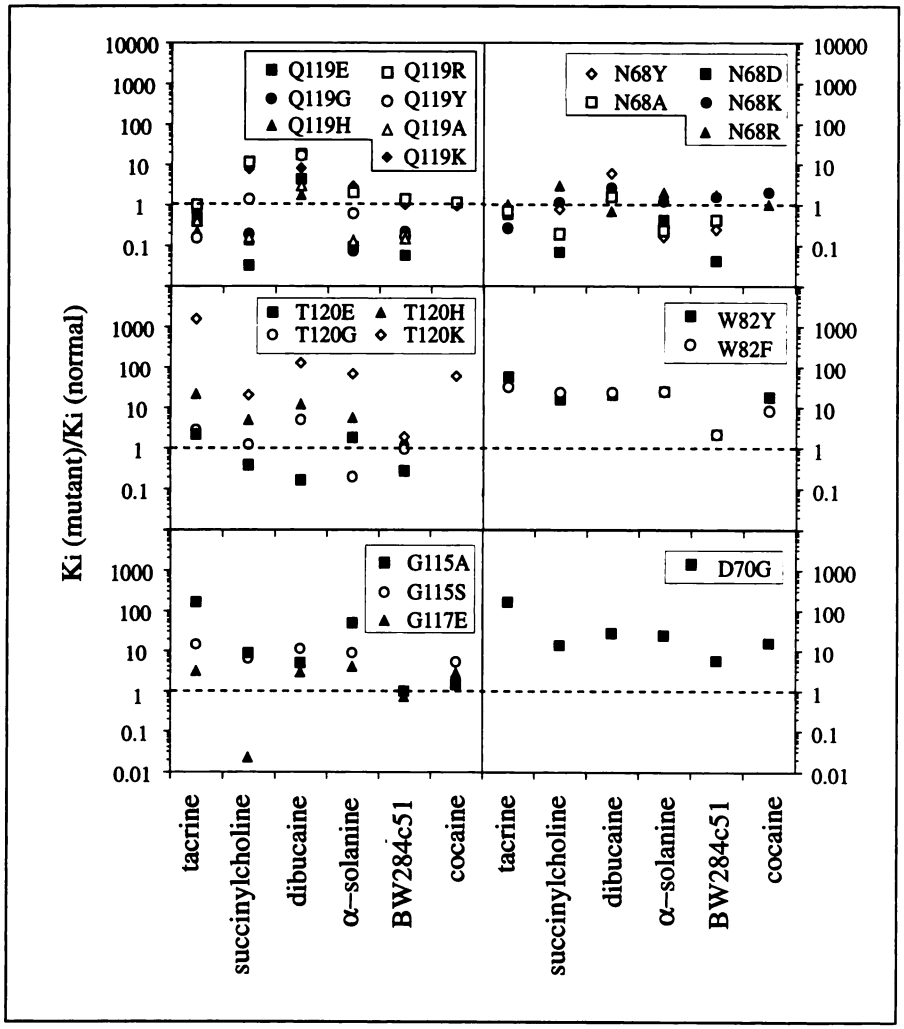

Fig. 4. Relative inhibition constants of BuChE mutants. The K_i values of inhibitors for the mutant enzymes (from Table 1) are shown relative to normal BuChE for mutations of three residues at the peripheral site (N68, Q119 and T120), the choline-binding site (W82), two at the oxyanion hole (G115 and G117), and the atypical variant (D70G). Where only a minimum value is known, that value has been used in calculating the relative constant.

is a very sensitive test for the integrity of the active site conformation. Slightly higher catalytic activity could be detected for mutants G115A and G115S, but k_{cat} and k_{cat}/K_m values were considerably lower than that of normal BuChE (Table 2). In contrast, a milder reduction of ≤10-fold was found for k_{cat} values for G115A and G115S. The introduction of a glutamate at position 117 was better tolerated: only a slight decrease of ~5-fold was noted in k_{cat}. Furthermore, the K_m value was not altered in oxyanion hole mutants in which moderate activities could be detected. When interactions with the group of reversible inhibitors were examined for these mutations, significant decreases in the interactions with tacrine, α-solanine, and succinylcholine were found for G115A (≤100-fold compared with normal BuChE). Thus, the oxyanion hole participates in both substrate and inhibitor interactions of BuChE.

To further identify sites that determine the specificity of BuChE, we created several mutants in the region homologous with the AChE peripheral site: N68D, N68K, N68R, N68Y, N68A, Q119G, Q119H, Q119E, Q119R, Q119K, Q119Y, and Q119A. All of these mutants were catalytically active, and they displayed no significant change in their k_{cat} or K_m value compared with normal BuChE (Tables 1 and 2). The inhibitor interactions of the various peripheral site mutant BuChEs were examined using tacrine and a group of other reversible inhibitors. None of the mutations in N68 displayed any significant alteration in inhibition toward the set of reversible inhibitors (Table 1 and Fig. 4). However, mutations in Q119 caused changes in sensitivity to inhibitors, with the greatest occurring when basic residues replaced the glutamine. A significant loss of sensitivity to dibucaine and succinylcholine, but not for other ligands such as tacrine, α-solanine, and cocaine, was found for Q119R and Q119K.

Interestingly, when inhibition by the AChE-specific inhibitor BW284c51 was examined, a significant increase in sensitivity was noted for most of the peripheral site mutants except when basic residues were introduced. The highest increase in sensitivity was found when acidic residues (glutamate and aspartate) replaced Q119 and N68, respectively, but increases were also seen when tyrosine residues, as in AChE, were introduced (Table 1 and Fig. 4). Peripheral site interactions thus seemed to depend primarily on charge.

T120, which is adjacent to Q119 of the peripheral site, is conserved in BuChE from several organisms; in AChE, a serine residue occupies this position. It was important, therefore, to examine whether this residue plays a role in inhibitor interactions. The following changes were introduced: T120G, T120H, T120N, and T120K, none of which displayed a significant change in k_{cat} or K_m compared with normal BuChE. When the inhibition properties were examined for the set of reversible inhibitors, a striking loss of sensitivity was ob-

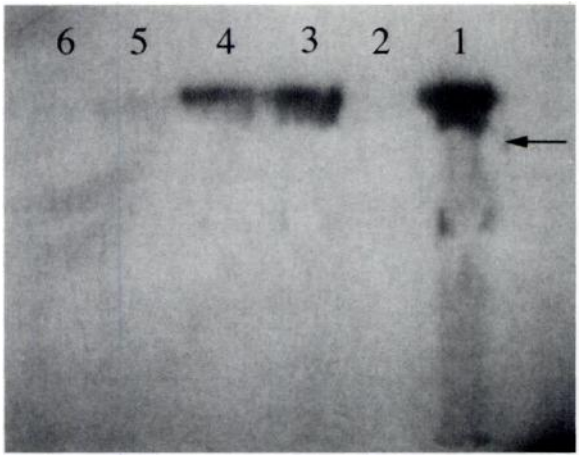

Fig. 5. Examples of [^{3}H]DFP binding to inactive BuChE mutations. Oocyte homogenates (100 μl) were reacted with 2 mCi of [^{3}H]DFP, and the product was separated by polyacrylamide gel electrophoresis and autoradiographed 2 months. *Lane 1*, normal BuChE; *lane 2*, uninjected oocyte homogenate; *lane 3*, G117E; *lane 4*, G115C; *lane 5*, G115D; *lane 6*, G116A/G117D. *Arrow*, somewhat lower position of commercial human BuChE (9).

served when a basic residue was introduced (T120K). This mutant was practically insensitive to all of the inhibitors examined, although its K_m value for BTCh remained unaltered. The introduction of a positive charge has therefore confirmed that this particular region in BuChE is important for charge-dependent ligand interactions. Alternatively, or additionally, the effect of this mutation may be due to structural changes. Thus, of all of the examined regions, it was in choline-binding site and oxyanion hole mutants, but not in the peripheral site, that affinity for tacrine was reduced.

When the logarithms of the tacrine K_i values were plotted against the logarithms of each of the other K_i values (logarithms were used to more evenly distribute points and give appropriate weight to all mutations) and a linear regression analysis was performed, there was a positive correlation with each of the other inhibitors. Representative plots are shown in Fig. 6. The correlation coefficients were $r = 0.66$ for tacrine/succinylcholine, $r = 0.58$ for tacrine/dibucaine, $r = 0.90$ for tacrine/α-solanine, $r = 0.57$ for tacrine/BW284c51, and $r = 0.81$ for tacrine/cocaine. In all cases, for the sample sizes, these correlation coefficients were higher than those for the 95% confidence limit (29). This indicates overlaps between the tacrine-binding site and the binding site of each of the other inhibitors.

Discussion

We used site-directed mutagenesis and *X. laevis* oocyte expression of resultant human BuChE variants to demonstrate the involvement of the peripheral and choline-binding sites as well as the oxyanion hole in the binding of tacrine to human BuChE. A comparison of novel mutants with AChE and the natural atypical BuChE variant revealed that normal serum BuChE but not mutants in the choline-binding site or oxyanion hole or the atypical enzyme can effectively compete with AChE in the binding of tacrine and several other drugs. Furthermore, our findings predict that BuChE/tacrine interactions compete with the interaction of BuChE with other drugs such as the muscle relaxant succinylcholine and dibucaine-like local anesthetics. Paralleling its wide sub-

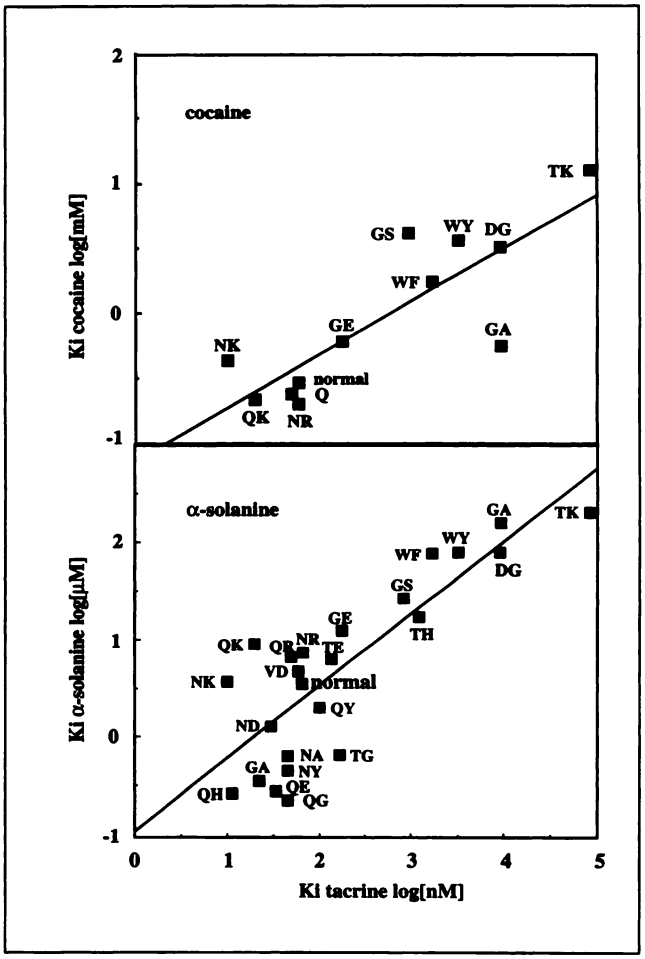

Fig. 6. Correlation of the affinity of tacrine for BuChE variants with affinities for cocaine and α-solanine. *Top*, plot of the logarithms of the dissociation constants for tacrine versus those for cocaine and the least-squares line. *Bottom*, corresponding plot for tacrine versus α-solanine. Data are from Table 1, and the identification of the mutations is the same as it is in the table, except the sequence number of each residue is not shown.

strate specificity, BuChE mutations generally caused only minor changes in ligand-binding properties, sometimes even increasing affinity. This emphasizes the plasticity of the enzyme, a property that is well suited to its role as a scavenger. Our current findings thus demonstrate that the scavenging capacity of BuChE extends across a wide array of ligands, suggesting that tacrine treatment in patients exposed to other anti-ChEs may result in a different effective dose. An important corollary of this finding is that the actions of other anti-ChEs (e.g., those that are used therapeutically) will be differentially affected by the presence of tacrine, depending on blood BuChE concentration and properties. Another possibility is that mutations at the tacrine site effected changes in the binding site of another inhibitor, or vice versa. An example of such a transmission of an effect through the protein molecule seems to be the allosteric effect of binding of fasciculin to the peripheral anionic site of AChE (30), which lowers but does not abolish the activity of the enzyme.

X-ray crystallography was used to locate tacrine at the bottom of the active site gorge of AChE that extends to the choline-binding site, ~5 Å from D70 (6). Not surprisingly, therefore, inhibitors that stretch from the catalytic site to the

peripheral site, such as succinylcholine and BW284c51, overlap with tacrine and show approximately the same pattern of inhibition of the mutant BuChEs as does tacrine. The aglycone of α-solanine, solanidine also inhibits ChEs; presumably, it is the shared bulky steroidal alkaloid moiety that blocks the entrance to the active site. The bulkiness of α-solanine with its three glycoside residues requires that it inevitably overlap the tacrine-binding site. Although the atomic details of their bindings to BuChE are unknown, cocaine and dibucaine have binding sites that overlap that of tacrine.

In BuChE, the residues homologous with glutamine and asparagine of the peripheral anionic site of AChE are N68 and Q119 (2, 13). Mutagenesis of N68 and Q119 in BuChE was performed to examine whether in BuChE these residues participate in interactions with AChE-specific inhibitors or with less specific ligands. Neither of these mutations caused a significant change in the K_m or k_{cat} value, which indicates that this region does not participate directly in substrate interactions. We examined the inhibition of these mutants by the set of reversible inhibitors. The mutation of N68 did not cause a significant change in the sensitivity of BuChE to any of these inhibitors. In contrast, the mutation of residue 119 resulted in changes of sensitivity that were most striking when a basic residue was introduced. Interestingly, significant reduction in sensitivity was also found for inhibition by succinylcholine and dibucaine but not by tacrine, cocaine, or α-solanine.

The results of our current mutagenesis study demonstrate that peripheral site residue Q119 but not N68 participates in the primary interactions with some reversible inhibitors. The fact that these interactions were more drastically altered when a basic residue was introduced further strengthens the conclusion that these primary interactions involve the positive charge on the inhibitor and are disrupted as a result of electrostatic repulsion between the inhibitor and the peripheral site. These findings extend the findings of Masson *et al.* (31) that the peripheral site is not unique to AChE and also exists in BuChE, in which it plays a role in interactions with reversible inhibitors. The increase in sensitivity to the AChE-specific inhibitor BW284c51 that was seen when the AChE-conserved tyrosine residue was introduced in either position 68 or 119 of BuChE is in accord with reciprocal experiments that were performed in AChE (19, 32). Interestingly, an increase in sensitivity to BW284c51 was also found when acidic residues were introduced at positions 68 or 119. This might be accounted for by stabilizing electrostatic interactions that occurred between these negatively charged residues and the positively charged inhibitor. The relatively low affinity of the peripheral site makes it unlikely that scavenging of drugs by serum BuChE will act via this site.

Mutagenesis of T120, adjacent to Q119, further emphasizes the importance of this region in ligand binding in BuChE. Although mutations in this residue caused no change in the catalytic properties, a significant decrease was detected in the sensitivity toward the examined reversible inhibitors. A striking decrease of sensitivity toward all inhibitors, including tacrine, was found on substitution of a basic residue. This prominent change may be the result of a combination of structural alteration and the introduction of charge. Because the spectrum of inhibitors that we chose includes representative natural anti-ChEs to which humans are exposed, the peripheral site of normal BuChE seems to be well adapted to the role of attracting these xenobiotics to high affinity binding sites on the protein.

When W82 was replaced at the choline-binding site with two smaller but still aromatic residues, tyrosine and phenylalanine, a significant reduction occurred in both K_m and k_{cat} values and led to significant increases in K_i values for the set of reversible inhibitors. This extended to BuChE the importance of the tryptophan residues at the choline-binding site and indicated the importance of this site in the binding of various other ligands. That the observed changes were similar to those found in parallel human AChE mutations (33) demonstrates the conservation between AChE and BuChE active sites.

The mutations of oxyanion hole glycine residues 116 and 117 and the adjacent residue 115 in BuChE caused quite dramatic reductions in catalytic activity. In some cases, BTCh-hydrolyzing activity was so low that residual enzyme function was detectable only through the use of DFP reaction. The loss of catalytic activity may be attributed to distortions in the fine structure of this region that are caused by the introduction of residues that are larger than glycine, which prevent the formation of the oxyanion hole and stabilization of the catalytic transition state. Another possible reason could be the introduction of a charge that might have disturbed the formation of a stabilizing hydrogen bond. The fact that changes in G115 led to activity loss further implies that the effect of mutagenesis in this case was the result of a structural impairment. Also, the introduction of alanine at position 115 was tolerated better than the introduction of a larger residue. Interestingly, the mutation that was best tolerated was the introduction of a glutamate residue at position 117, which led to only a small reduction in the catalytic activity, whereas the introduction of an aspartate at residue 115 caused the total loss of detectable BTCh-hydrolyzing activity. The substitution of histidine for G117 is also reported to have little effect on catalytic rate (34). When the inhibition properties of the catalytically active mutants were examined, it was reassuring to find that the mutations not only affected catalysis but also caused a decrease in sensitivity to several reversible inhibitors that, like substrates, interact with this region of BuChE. Taken together, these findings provide experimental evidence for the importance of the putative oxyanion hole region to catalytic functioning and ligand interactions of BuChE.

The mutation of V127 into an aspartate residue resulted in little change in *k*cat (data not shown) or K_m, complementing the work of Kronman *et al.* (35), who had mutated this residue of AChE to alanine and arginine and also found no major effect on catalysis. Thus, neither an acidic, a neutral, nor a basic residue at this position affects catalysis. These data make the back door theory less appealing, despite its elegance. It therefore remains to be proved whether the choline moiety is regurgitated back through the gorge of BuChE and against the direction of the electrostatic field or via an alternative pathway, and the mechanism that accounts for the extremely high turnover rate for both ChEs remains to be determined.

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