Role of Aspartate 70 and Tryptophan 82 in Binding of Succinyldithiocholine to Human Butyrylcholinesterase†

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Received October 3, 1996; Revised Manuscript Received December 26, 1996⊗

ABSTRACT: The atypical variant of human butyrylcholinesterase has Gly in place of Asp 70. Patients with this D70G mutation respond abnormally to the muscle relaxant succinyldicholine, experiencing hours of apnea rather than the intended 3 min. Asp 70 is at the rim of the active site gorge 12 Å from the active site Ser 198. An unanswered question in the literature is why the atypical variant has a 10-fold increase in K_m for compounds with a single positive charge but a 100-fold increase in K_m for compounds with two positive charges. We mutated residues Asp 70, Trp 82, Trp 231, Glu 197, and Tyr 332 and expressed mutant enzymes in mammalian cells. Steady-state kinetic parameters for hydrolysis of butyrylthiocholine, benzoylcholine, succinyldithiocholine, and *o*-nitrophenyl butyrate were determined. The wild type and the D70G mutant had identical k_{cat} values for all substrates. Molecular modeling and molecular dynamics suggested that succinyldicholine could bind in two consecutive orientations in the active site gorge; formation of one complex caused a conformational change in the omega loop involving Asp 70 and Trp 82. We propose the formation of three enzyme−substrate intermediates preceding the acyl−enzyme intermediate; kinetic data support this contention. Substrates with a single positive charge interact with Asp 70 just once, whereas substrates with two positive charges, for example succinyldithiocholine, interact with Asp 70 in two complexes, thus explaining the 10- and 100-fold increases in K_m in the D70G mutant.

The atypical variant of human butyrylcholinesterase (EC 3.1.1.8, serum cholinesterase, BuChE)[1] was first recognized by the physician Werner Kalow [reviewed by Kalow (1962), Whittaker (1986), Lockridge (1992), and Kalow and Grant (1995)]. The clinical observation leading to his discovery was the fact that some people responded abnormally to the muscle relaxant drug succinyldicholine (succinylcholine, suxamethonium). They could not breathe for up to 2 h, whereas the intended period of apnea was 3−5 min. Kalow showed that the abnormal response was due to an altered butyrylcholinesterase in serum, which he named atypical. Atypical butyrylcholinesterase had a reduced affinity for all positively charged substrates and inhibitors (Kalow & Davies, 1958; Davies et al., 1960; Valentino et al., 1981), a trait which was shown to be hereditary. The reduced affinity meant that virtually none of the injected dose of succinyldicholine was hydrolyzed, and consequently, a huge overdose reached the nerve−muscle junction. In contrast, people with usual (wild-type) butyrylcholinesterase hydrolyzed 90% of the injected succinyldicholine so that only 10% reached the nerve−muscle junction.

Kalow hypothesized that the atypical variant was due to mutation of a single negatively charged amino acid in the substrate binding site. Thirty years later, McGuire et al. (1989) validated this prediction by finding that the atypical variant had glycine in place of aspartic acid 70. Models of human BuChE (Harel et al., 1992; Millard & Broomfield, 1992) based on the crystal structure of *Torpedo* acetylcholinesterase (Sussman et al., 1991) show that aspartate 70 (*72*)[2] is located near the rim of the active site gorge where it is a component of the peripheral anionic site (Sussman & Silman, 1992; Shafferman et al., 1992; Radic et al., 1993; Barak et al., 1994; Masson et al., 1996). Before the crystal structure was available, Asp 70 was thought to be the anionic site (McGuire et al., 1989; Neville et al., 1990), defined as the site that stabilizes the positively charged choline group of choline esters in the active site. However, it is now clear

† This work was supported by the U.S. Medical Research and Materiel Command under Grant DAMD17-94-J-4005 to O.L., by National Cancer Institute Grant P30 CA 36727 to the Eppley Institute, and by DRET (94/05) to P.M. The opinions or assertions contained herein belong to the authors and should not be construed as the official views of the U.S. Army or the Department of Defense.

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⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1997.

[1] Abbreviations: AChE, acetylcholinesterase (EC 3.1.1.7); BuChE, butyrylcholinesterase (EC 3.1.1.8); BCHE, butyrylcholinesterase gene; CHO, Chinese hamster ovary cells; MEPQ, 7-[(methylethoxyphosphinyl)oxy]-1-methylquinolinium iodide.

[2] By convention, the italicized number in parentheses immediately following a BuChE amino acid residue refers to the corresponding amino acid residue in *Torpedo californica* acetylcholinesterase. For example, BuChE D70 (*D72*) corresponds to acetylcholinesterase D72.

S0006-2960(96)02484-1 CCC: $14.00

that the anionic site is actually Trp 82 (*84*) (Sussman et al., 1991; Ordentlich et al., 1993; Harel et al., 1993; present work). Asp 70, at the rim of the deep gorge, is too far away from the active site Ser 198 (*200*), located at the bottom of the gorge, to bind the substrate molecule while it is undergoing bond breaking. What then is the function of Asp 70, and why does the atypical variant, D70G, have reduced affinity for all positively charged compounds? In particular, why does the atypical variant have a 100-fold lower binding affinity for succinyldicholine and other bisquaternary compounds but only a 10-fold lower binding affinity for monoquaternary compounds (Kalow & Davies, 1958) such as butyrylcholine?

To address these questions, we expressed D70G, D70N, D70K, Y332F, W82A, E197G, and W231A mutants of human BuChE and determined binding affinities and catalytic constants. Modeling succinyldicholine in the active site of wild-type and D70G mutant BuChE allowed us to propose a mechanistic model for the hydrolysis of mono- and bisquaternary esters such as butyrylcholine and succinyldicholine. Our results suggest that Asp 70 orients the positive charge of the substrate, allowing the substrate to slide directly onto Trp 82. The mechanism for hydrolysis of succinylcholine and butyrylcholine appears to have three enzyme−substrate intermediates preceding the formation of the acyl−enzyme intermediate. Differences in the interaction at Asp 70 for the first two intermediates explain why bisquaternary compounds have a 100-fold decrease in binding affinity, whereas monoquaternary compounds have a 10-fold decrease in binding affinity for the D70G mutant.

METHODS

Expression of Recombinant Human BuChE. Oligonucleotide-directed *in vitro* mutagenesis of BCHE in M13 template was performed with the Amersham kit (code RPN.1523). Alternatively, mutagenesis was performed with the polymerase chain reaction and Pfu polymerase (Stratagene). Oligonucleotides for mutagenesis were synthesized by the University of Nebraska Molecular Biology Core Facility. The cDNA of human BCHE (McTiernan et al., 1987) was modified to give the optimal ATG start site context (Kozak, 1991) GCCACCATGG for the ATG of the 28-amino acid leader sequence. Mutagenesis at Asp 70 replaced GAT with either GGT (Gly), AAT (Asn), or AAG (Lys). Other codon replacements were TTT (Phe) for Tyr 332, GCG (Ala) for Trp 82, GCG (Ala) for Trp 231, and GGA (Gly) for Glu 197. The 1.8 kb cDNA was cloned into expression plasmid pRc/CMV (Invitrogen Corp., San Diego, CA) or pGS (T. White, Scios Nova, Mountainview, CA). The entire cDNA was resequenced to ensure the absence of unwanted mutations. Plasmid DNA (10−20 μg), purified with the Qiagen plasmid kit (Qiagen catalog number 12162), was transfected by calcium phosphate coprecipitation.

Human embryonal kidney 293 cells (ATCC CRL 1573) were chosen because of the success of this cell line in expressing human AChE (Kronman et al., 1992). Cells (293) transfected with pRc/CMV plasmids were selected with 0.8 mg/mL Geneticin (Gibco catalog number 066-1811). Colonies with the highest activity were expanded in T75 flasks and grown to confluence in the presence of 10% fetal calf serum. For the period when rBuChE was collected for analysis, culture medium was free of fetal calf serum. Cells (293) were maintained in serum-free medium for 2−4 days. The serum-free medium for 293 cells was Dulbecco's modified Eagle's medium (DMEM, Gibco 11995-016).

CHO K1 (ATCC CCL 61) cells are the cell line of first choice for the synthesis of recombinant human therapeutic glycoproteins (Goochee et al., 1991; Jenkins & Curling, 1994). CHO cells transfected with pGS plasmids were selected with 0.05 mM methionine sulfoximine in Ultraculture (BioWhittaker Inc. 12-725B), a medium free of serum and free of glutamine. Colonies were picked with a sterile cotton-tipped stick for transfer to 24-well cluster plates. Colonies with the highest activity were expanded in T150 flasks and grown to confluence in Ultraculture in the absence of serum, in the presence of 0.025 mM methionine sulfoximine.

Purification of Recombinant BuChE. Mutants W82A and W231A and wild-type BuChE in 2−5 L of serumless medium (Ultraculture or DMEM/F12) were centrifuged to remove particulates. The ionic strength was reduced by adding 2 volumes of water. The diluted medium was loaded onto a 6 mL procainamide Sepharose affinity column (Lockridge, 1992). BuChE was eluted with a NaCl gradient made with 500 mL of 20 mM potassium phosphate and 1 mM EDTA (pH 7) versus 500 mL of 1.5 M NaCl in buffer. A second passage through a procainamide Sepharose column achieved a purification of approximately 50-fold. Mutants D70G, D70N, D70K, Y332F, and E197G were collected into protein-free medium (DMEM/F12), centrifuged, filtered, concentrated in an Amicon stirred cell with a PM10 membrane, and used without further purification. The activity of the concentrated enzymes was 3−5 u/mL measured under standard conditions (50 μM benzoylcholine at 25 °C and 0.067 M Na/K phosphate buffer at pH 7.4).

Kinetics of Substrate Hydrolysis. The buffer was 0.1 M potassium phosphate at pH 7.0 and 25 °C. Hydrolysis of butyrylthiocholine iodide was measured by the Ellman method (Ellman et al., 1961) at concentrations ranging from 0.01 to 100 mM. Stock solutions of 0.2 M butyrylthiocholine iodide were prepared in water and frozen in 0.5 mL aliquots. Hydrolysis of succinyldithiocholine was measured by the Ellman method at concentrations ranging from 1 to 500 μM. The D70K mutant was assayed at succinyldithiocholine concentrations up to 4 mM. Succinyldithiocholine dihydroiodide was custom synthesized by Molecular Probes Inc. (Eugene, OR). Since this compound is unstable in solution, a 10 mM stock solution in pH 7.0 buffer was frozen in 0.5 mL aliquots. Hydrolysis of benzoylcholine chloride was followed by recording the decrease in absorbance at 240 nm where the difference in the absorptivity constant between substrate and product was 6700 M^{-1} cm^{-1}; substrate concentrations ranged from 12.5 to 200 μM. Hydrolysis of *o*-nitrophenyl butyrate was in buffer containing 5.5% methanol with substrate concentrations from 0.03 to 0.8 mM; the rate of appearance of *o*-nitrophenol was monitored at 371 nm where the extinction coefficient was 2600 M^{-1} cm^{-1}. A wavelength of 371 nm was chosen over the usual 414 nm (Main et al., 1961) because absorbance at 371 nm is independent of pH. Autohydrolysis rates of butyrylthiocholine, succinyldithiocholine, and *o*-nitrophenyl butyrate were subtracted from enzyme-catalyzed rates.

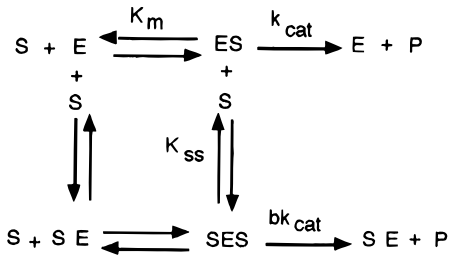
Determination of Catalytic Parameters. When the data conformed to Michaelian behavior, K_m and V_{max} were determined by simple weighted nonlinear regression of the

Michaelis−Menten equation (eq 1) using Sigma Plot (Jandel Scientific, San Rafael, CA).

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

Non-Michaelian behavior with either inhibition or activation at high substrate concentration may be described by Scheme 1 in which the substrate molecule (S) binds to two different sites (Radic et al., 1993):

Scheme 1



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

where K_{ss} is the dissociation constant of the SES and ES complexes. The dissociation constant for SE is K_{ss} and for SES is αK_{ss} where α is equal to 1. Assuming that S binds to both E and ES, the parameter b reflects the efficiency with which the ternary complex SES forms product. If $0 < b < 1$, there is substrate inhibition; if $b > 1$, there is substrate activation, and if $b = 1$, the enzyme is said to have Michaelian behavior. This model is similar to those previously used for describing substrate activation of BuChE (Ericksson & Augustinsson, 1979; Cauet et al., 1987). K_m, K_{ss}, V_{max}, and b were calculated by nonlinear computer fitting of eq 2 using Sigma Plot (Jandel Scientific).

Active Site Titration. MEPQ, 7-[(methylethoxyphosphinyl)oxy]-1-methylquinolinium iodide, a specific inhibitor of cholinesterases, was a gift from Y. Ashani (Levy & Ashani, 1986). A 1 mM stock solution was prepared in acetone containing 0.2% glacial acetic acid. The concentration of BuChE active sites was calculated from plots of percent residual activity versus volume of MEPQ following overnight incubation of rBuChE and MEPQ. The number of titratable active sites allowed determination of catalytic constants (k_{cat}).

Reversible Inhibition by Dibucaine and Pralidoxime. Inhibition of butyrylthiocholine hydrolysis by dibucaine hydrochloride or pralidoxime iodide (2-PAM) was carried out in 0.1 M phosphate at pH 7.0 and 25 °C. Types of inhibition and inhibition constants were determined according to Cornish-Bowden (1974) by using Dixon plots (reciprocal of velocity against inhibitor concentration) and plots of substrate concentration/velocity against inhibitor concentration.

Molecular Modeling. The aim of the modeling work was to produce models for the enzyme−substrate complexes that could help to interpret kinetic results. Molecular dynamics and minimization calculations were carried out *in vacuo* with the program CHARMM (Brooks et al., 1983) on a Hewlett-Packard 9000/730 workstation. The topological and energy parameters for the protein were adapted from CHARMM 23 All Hydrogen Parameter Set, Version 6.0 (A. D. MacKerell, J. Wiorkiewicz, and M. Karplus, unpublished) except for Ser 198, for which the Oγ partial charge was set to −0.8 because of its high nucleophilicity. The parameters used to represent succinyldicholine were adapted from this parameter set and semiempirical quantum mechanical calculations. These calculations were performed using the AM1 Hamiltonian, as implemented in MOPAC V6.0 (Dewar et al., 1985). To let the succinyldicholine molecule move freely in the active site, no covalent bond was assigned between the Ser 198 Oγ and succinyldicholine, in contrast to previous modeling (Masson et al., 1993). Electrostatic energies were calculated with a dielectric constant of 3.5. The cutoff criteria were 9.0−10.0 Å for electrostatic interactions and 4.5−5.0 Å for H-bonding interactions. All atoms (polypeptide backbone of BuChE and side chains, as well as substrate) were included in molecular dynamics simulations with no fixed coordinates or angles.

The starting three-dimensional model of human BuChE was the Harel model (Harel et al., 1992). The missing amino acid segment (483−487) of the AChE X-ray structure was added from the Millard−Broomfield human BuChE model (Millard & Broomfield, 1992). Succinyldicholine was docked into the BuChE model in the position previously described (Masson et al., 1993). A model was obtained by docking followed by a short energy minimization using X-PLOR. This model was a representation attempt to describe the structure of the intermediate preceding the acyl−enzyme intermediate. Protonation states for all amino acids at neutral pH were assigned (Gilson et al., 1994) on the basis of atomic solvent accessibility, salt bridging and H bonding possibilities, and the putative catalytic mechanism. This led to the protonated form of glutamic acids 325 and 441 and neutral histidines 207 and 438 with a proton exchanged from Nδ1 to Nϵ2. The construction of the D70G mutant model was achieved by replacing the Cβ atom of the Asp 70 residue with a hydrogen atom and removing all lateral chain atoms.

Models were first subjected to 800 steps of conjugate gradient Powell (CGP) minimization. Molecular dynamics was used to prepare the system. The molecule was slowly heated from 100 to 300 K in 10 ps, by increments of 20 K per picosecond, followed by 10 ps of equilibration at 300 K. To produce a relaxed minimized structure, a slow cooling molecular dynamics calculation was performed from 300 to 0 K with increments of −10 K for each picosecond, by Gaussian reassignment of velocity. The last of these coordinates was submitted to 800 steps of CGP minimization. This procedure has no expectation of the final model and thus contrasts with docking.

RESULTS

Kinetic Analysis of Mutants

We selected five amino acid residues which appeared to play important roles in the binding of succinyldicholine to BuChE: Asp 70, Tyr 332, Trp 82, Glu 197, and Trp 231. We mutated BuChE at each of these residues and expressed the mutant BuChE. Steady-state kinetic analysis of the recombinant enzymes with neutral and positively charged substrates was carried out in order to determine the extent to which each residue contributes to the catalytic properties of BuChE. The majority of these results are shown in Table 1.

Table 1: Kinetic Constants of Human BChE Mutants Determined in 0.1 M Potassium Phosphate at pH 7.0 and 25 °C

substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} M^{-1})	K_{ss} (mM)	b
		D70G			
butyrylthiocholine	150 ± 50	24 000 ± 10 000	0.1×10^9	1.1	1.2 ± 0.2
succinyldithiocholine	330 ± 114	350 ± 35	0.001×10^9	−	1
benzoylcholine	27 ± 4	15 000 ± 700	0.6×10^9	−	1
o-nitrophenyl butyrate	120 ± 20	36 000 ± 2000	0.3×10^9	−	1
		D70N			
butyrylthiocholine	180 ± 40	7300 ± 1000	0.04×10^9	14 ± 1	10 ± 1
succinyldithiocholine	530 ± 50	140 ± 20	0.0002×10^9	−	1
benzoylcholine	54 ± 4	6000 ± 1000	0.1×10^9	−	1
o-nitrophenyl butyrate	300 ± 100	27 000 ± 9000	0.1×10^9	−	1
		D70K			
butyrylthiocholine	1000 ± 125	20 000 ± 5000	0.02×10^9	−	1
succinyldithiocholine	750 ± 320	40 ± 11	0.00005×10^9	−	1
benzoylcholine	170 ± 20	14 300 ± 700	0.07×10^9	−	1
o-nitrophenyl butyrate	125 ± 50	23 000 ± 4000	0.2×10^9	−	1
		Y332F			
butyrylthiocholine	27 ± 4	30 000 ± 1000	1.1×10^9	1.8 ± 0.2	3.0 ± 0.1
succinyldithiocholine	25 ± 3	1040 ± 110	0.04×10^9	−	1
benzoylcholine	10 ± 1	14 000 ± 1000	1.4×10^9	−	1
o-nitrophenyl butyrate	175 ± 50	40 000 ± 5000	0.2×10^9	−	1
		W82A			
butyrylthiocholine	44 000 ± 5000	2000 ± 200	0.4×10^5	−	1
succinyldithiocholine		0	0		
benzoylcholine	700 ± 300	100 ± 10	1.4×10^5		
o-nitrophenyl butyrate	1400 ± 200	25 000 ± 2500	180×10^5		
		W231A			
butyrylthiocholine	34 ± 4	750 ± 75	0.02×10^9	80 ± 20	3.3 ± 0.2
succinyldithiocholine	0.8	3	0.004×10^9		
benzoylcholine	20 ± 2	1600 ± 160	0.1×10^9		
o-nitrophenyl butyrate	390 ± 20	3800 ± 400	0.01×10^9		
		wild type			
butyrylthiocholine	18 ± 5	24 000 ± 10 000	1.3×10^9	1.0 ± 0.1	3.2 ± 0.1
succinyldithiocholine	3.0 ± 0.05	350 ± 35	0.1×10^9	−	1
benzoylcholine	5.4 ± 0.3	15 000 ± 700	2.8×10^9	0.21 ± 0.04	0.46 ± 0.06
o-nitrophenyl butyrate	125 ± 20	36 000 ± 5000	0.3×10^9	−	1

Asp 70 Mutants. The D70G mutant had normal kinetics with the neutral substrate *o*-nitrophenyl butyrate but had reduced affinity for positively charged substrates without affecting k_{cat} (Table 1). These results suggested that the function of Asp 70 is to form a transient charge−charge complex with positively charged substrates at the top of the gorge. This interpretation was bolstered by the D70K mutant which replaced the negatively charged aspartate with a positively charged lysine. The affinity of D70K for positive substrates was weaker than that of D70G. k_{cat} for succinyldithiocholine was reduced nearly 10-fold, while k_{cat} values for butyrylthiocholine and benzoylcholine were unaffected.

Replacing aspartate with asparagine led to a breakdown of the simple interpretation developed for D70G. The affinity of the positively charged substrates was reduced as expected, but the affinity of the neutral *o*-nitrophenyl butyrate was also weakened, though not to the same degree. In addition, the k_{cat} values for all the substrates tested was reduced. These results strongly suggest that factors in addition to charge−charge interactions can be altered by replacing aspartate at position 70. The nature of these other factors is not clear, but protein conformational changes must be considered.

Tyr 332 Phe Mutant. The X-ray structure of *Torpedo* AChE and the structure of modeled human BuChE show that Tyr 332 forms a hydrogen bond with Asp 70 on unliganded cholinesterases (Sussman et al., 1991; Harel et al., 1992). Mutating tyrosine to phenylalanine gave a recombinant protein which behaved normally with all substrates except succinyldithiocholine. The affinity for succinyldithiocholine was reduced about 10-fold. Weakening of the affinity is opposite to the effect expected for removing the competition between Tyr 332 and positively charged substrates for Asp 70. The Y332F mutation had no effect on k_{cat} except for an unexpected 3-fold increase for succinyldithiocholine. These results support the interpretation taken from D70N, that interactions other than simple charge−charge interactions, probably involving conformational changes, are important to the binding and k_{cat} of positively charged succinyldithiocholine.

Trp 82 Ala Mutant. The role of the catalytic anionic site has been given to Trp 82 (Sussman et al., 1991; Ordentlich et al., 1993; Harel et al., 1993). Trp 82 has been proposed to be critical for the proper binding and alignment of positively charged substrates in the active site. As such, its removal should prove devastating to the activity of positively charged substrates. As shown in Table 1, changing Trp 82 into alanine did in fact disrupt the hydrolysis of positively charged substrates. The affinity was weakened 100−2500-fold for benzoylcholine and butyrylthiocholine, substrates with a single quaternary amine. The W82A mutant was totally unable to hydrolyze the bisquaternary succinyldithiocholine in the range of 0.005−4 mM so that using K_m as a measure of affinity was impossible. An attempt to measure binding affinity by testing inhibition potency of succinyldithiocholine showed that concentrations as high as 0.1

Table 2: Difference in Binding Energy to Wild-Type BuChE and D70G (Atypical) Depends on the Number of Positive Charges in the Ligand[a]

substrate or inhibitor	wild-type binding energy ΔG (kcal/mol)	D70G (atypical) binding energy ΔG (kcal/mol)	difference in binding energy $\Delta\Delta G$ (kcal/mol)	reference
one positive charge				
acetylthiocholine	5.98	4.10	1.78	Wetherell & French, 1986
butyrylthiocholine iodide	6.40	5.00	1.40	present work
benzoylcholine chloride	7.15	6.29	0.86	present work
heroin	5.38	4.55	0.83	Lockridge et al., 1980
dibucaine	8.40	6.42	1.98	present work
procaine hydrochloride	5.93	4.39	1.54	Kalow & Davies, 1958
chlorpromazine	7.29	5.61	1.68	Kalow & Davies, 1958
tetracaine	8.92	7.07	1.85	Kalow & Davies, 1958
monoquaternary analog of pancuronium	8.81	7.83	0.98	Whittaker & Britten, 1980
(−)-cocaine	5.94	4.24	1.70	Schwartz & Johnson, 1996
pralidoxime iodide	5.02	3.41	1.61	present work
two positive charges				
succinyldithiocholine iodide	7.50	4.72	2.78	present work
decamethonium bromide	6.63	3.72	2.91	Kalow & Davies, 1958
pancuronium bromide	9.28	6.32	2.96	Whittaker & Britten, 1980
dibutyryloxy analog of pancuronium	10.64	7.31	3.33	Whittaker & Britten, 1980
17-desoxy analog of pancuronium	9.36	6.29	3.07	Whittaker & Britten, 1980
neutral				
o-nitrophenyl butyrate	5.3	5.3	0	present work
α-naphthyl acetate	4.6	4.6	0	Valentino et al., 1981

[a] Values of competitive inhibition constants (K_i) for the mixed inhibitor dibucaine determined in the present work were 0.66 μM for wild type and 19 μM for D70G. Pralidoxime was a competitive inhibitor with a K_i of 0.20 mM for wild type and 3.1 mM for D70G. $\Delta G = -2.3RT \log K$, where $R = 1.98 \times 10^{-3}$ kcal mol^{-1} deg^{-1}, $T = 298$ K, and $K = K_m$, K_i, or I_{50}.

mM failed to inhibit hydrolysis of 5−40 mM butyrylthiocholine. This was taken to mean that the binding affinity for succinyldithiocholine had decreased by orders of magnitude in W82A. These results indicated that Trp 82 contributed much more to the binding stability of positively charged substrates than did Asp 70. Table 2 shows the stabilization energy ($\Delta\Delta G$) from interaction of butyrylthiocholine with Asp 70 to be 1.4 kcal/mol. The stabilization energy ($\Delta\Delta G$) from interaction of butyrylthiocholine with Trp 82 was calculated to be 4.6 kcal/mol by subtracting the ΔG for binding butyrylthiocholine to W82A ($\Delta G = 1.80$ kcal/mol) from the ΔG for binding to wild-type BuChE ($\Delta G = 6.40$ kcal/mol). In addition to its effect on affinity, the W82A mutation radically reduced k_{cat} for positively charged substrates. In contrast, the effect of W82A on the neutral substrate *o*-nitrophenyl butyrate was minimal, amounting only to a 10-fold weakening in affinity.

Glu 197 Gly Mutant. To determine whether Glu 197 or Trp 82 provided the better site for interaction with the choline moiety, we expressed the mutant E197G. With this mutant, the K_m for succinyldithiocholine was increased only 10-fold, to 30 mM. This is clearly a minor contribution when compared to the effect of mutating Trp 82. The absence of a stronger interaction between Glu 197 and the choline head group of succinyldithiocholine was surprising since Glu 197 (*199*) has been found to interact directly with the quaternary ammonium group of a transition state analog in the X-ray structure of AChE (Harel et al., 1996).

Trp 231 Ala Mutant. We mutated Trp 231 to alanine with the expectation that the affinity for the bisquaternary succinyldithiocholine would be weakened. Unfortunately, the k_{cat} values of W231A were reduced by at least 1 order of magnitude for all substrates. For succinyldithiocholine, the apparent rate was no more than 1% of the rate with wild-type BuChE and could not be accurately discriminated from background. We therefore determined the affinity of succinyldithiocholine for W231A by its ability to inhibit benzoylcholine hydrolysis. Unexpectedly, we found the inhibition constant to be 0.25 μM. That is, the affinity of W231A for succinyldithiocholine was 10-fold stronger than the affinity of wild-type BuChE for succinyldithiocholine. This suggests that steric constraints imposed on succinyldithiocholine by Trp 231 outweigh any binding advantage that possible charge−aromatic interactions may offer. In general, the W231A mutation caused a slight, 2−4-fold, weakening of affinity for both monoquaternary and neutral substrates. More significant was the reduction in k_{cat} values for these substrates, which suggests a disorganization of catalytically important residues such as Ser 198 and His 438.

Mechanistic Model for Hydrolysis of Positively Charged Substrates

It is accepted that the mutation of Asp 70 to Gly in the atypical variant of human serum butyrylcholinesterase results in an enzyme that shows lower affinity for positively charged substrates, normal affinity for neutral substrates, and a normal k_{cat} for both neutral and positively charged substrates (Lockridge, 1992). These properties are faithfully reproduced in recombinant BuChE carrying the D70G mutation (Table 1). These features alone would suggest that Asp 70 might be part of the catalytic binding site and that binding is a simple one-step process. At one time, this was thought to be the case, but from the very earliest studies of Kalow (1962), it was difficult to rationalize a one-step binding model with the observation that bisquaternary substrates such as succinyldithiocholine had a 100-fold decrease in binding affinity while monoquaternary ligands had only a 10-fold decrease (Tables 1 and 2).

With the advent of the X-ray crystal structure of the homologous *Torpedo* AChE, it became apparent that Asp 70 was not part of the catalytic site but was located at the entrance of a long gorge leading into the catalytic site. This new information required that cationic substrates bind in at least a two-step process. The initial encounter complex

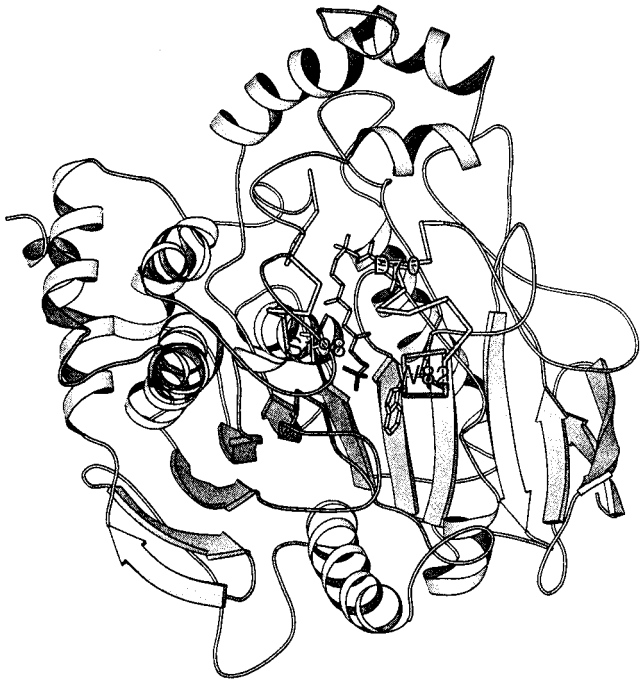


FIGURE 1: Ribbon diagram of succinyldicholine bound to wild-type BuChE. The most energetically stable model of the BuChE−succinyldicholine complex is shown. Asp 70 is near the top of the gorge, and Ser 198 and Trp 82 are near the bottom.

would occur at Asp 70 on the rim of the gorge, followed by re-equilibration of the substrate down into the catalytic site at the bottom of the gorge. Though a two-step model is more consistent with the X-ray structure of AChE, it still does not provide a basis for rationalizing the differential effect of the D70G mutation on mono- and bisquaternary substrates because a two-step process would have both types of substrate interact with position 70 only once during the binding process.

In an effort to resolve this issue and to gain better insight into the nature of substrate interactions with BuChE, we turned to molecular modeling. A computer model for the structure of BuChE based on the structure of the homologous AChE was the starting model. We evaluated the effects on the structure of introducing (1) the substrate succinyldicholine and (2) the D70G mutation. These studies implicated a number of residues, both in the gorge and in the catalytic site, which appear to be important to the binding of positively charged substrates and led us to propose a three-step binding model.

The introduction of a third step into the binding model was prompted when modeling indicated that succinyldicholine may form a stable complex in the gorge leading to the catalytic site (Figure 1). One choline group interacted with Asp 70 at the rim, and the other choline interacted with Trp 82 at the bottom of the gorge (Figure 2). This model is supported by the crystal structure of decamethonium in AChE, where the bisquaternary ligand decamethonium is oriented along the narrow gorge leading to the active site with one quaternary group apposed to the indole of Trp (*84*) and the other to that of Trp (*279*) near the top of the gorge (Harel et al., 1993). The complex for a monoquaternary substrate would place the choline head at the bottom of the gorge to interact with Trp 82 and not with Asp 70. This complex in the gorge provides us with a species in which

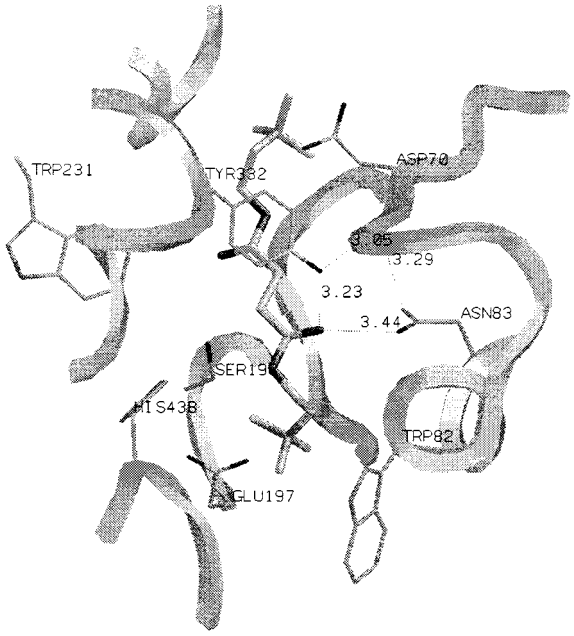


FIGURE 2: Closeup view of the most stable model of succinyldicholine in wild-type BuChE. Succinyldicholine is oriented along the length of the active site gorge with one choline head stabilized by interaction with Asp 70 and the other choline head by interaction with Trp 82. Hydrogen bonds are shown between Tyr 332, the main chain of Asp 70, Asn 83, and succinyldicholine. There is no H bond between the side chain of Asp 70 and Tyr 332. A portion of the omega loop including Asp 70, Asn 83, and Trp 82 is on the right. This structure is represented by ES2 in Figure 3.

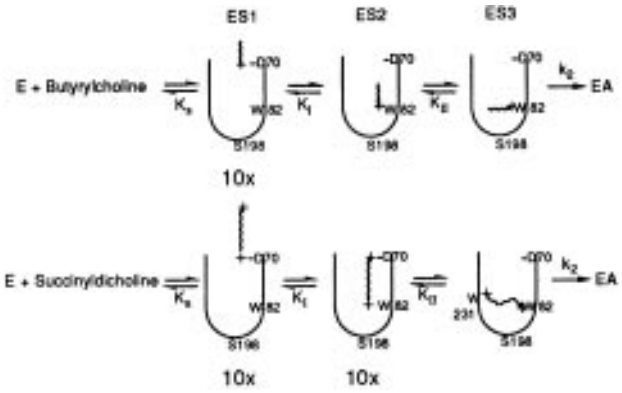


FIGURE 3: Three enzyme−substrate complexes. A mechanism with three enzyme−substrate complexes is proposed to explain the observation that the K_m for butyrylthiocholine differs by 10-fold between wild type and D70G, whereas the K_m for succinyldithiocholine differs by 100-fold between wild type and D70G. The first enzyme−substrate complex, ES1, forms when substrate binds to Asp 70. The second complex, ES2, forms when the substrate slides down the gorge to bind to Trp 82. The third complex, ES3, forms when the substrate rotates to a position where it can be hydrolyzed by S198. This last step is unfavorable for succinylcholine because the fit is poor. Only one binding step is affected when the substrate is butyrylthiocholine and the enzyme contains the D70G mutation, thus accounting for a 10-fold difference in the K_m between wild type and D70G. Two binding steps are affected when the substrate is succinyldicholine and the enzyme contains the D70G mutation, thus accounting for a 100-fold difference in K_m.

bisquaternary substrates interact with Asp 70 differently from monoquaternary substrates.

The three-step model is schematically shown in Figure 3. The first complex (ES1) involves a charge−charge interaction between the choline group and Asp 70. This interaction is the same for both mono- and bisquaternary substrates. The

second intermediate (ES2) finds the substrate positioned vertically in the gorge between Asp 70 and Trp 82. In the third complex (ES3), the substrate is positioned horizontally at the bottom of the gorge, in the catalytic site.

Scheme 2 depicts a mechanism with three enzyme−substrate complexes.

Scheme 2

$$E + S \underset{K_s}{\rightleftharpoons} ES1 \underset{K_I}{\rightleftharpoons} ES2 \underset{K_{II}}{\rightleftharpoons} ES3 \xrightarrow{k_2} EA + P_1 \xrightarrow{k_3} E + P_2$$

E is enzyme. S is substrate. ES1, ES2, and ES3 are enzyme−substrate complexes. EA is the acylated enzyme intermediate. P_1 and P_2 are products. The dissociation constant of the ES1 complex is K_s. The isomerization constant of the ES2 complex is K_I and for ES3 is K_{II}. By making steady-state and rapid equilibrium assumptions, one can derive expressions for k_{cat} and K_m:

$$k_{cat} = \frac{k_2}{K_I K_{II} + K_{II} + \frac{k_2}{k_3} + 1} \quad (3)$$

$$K_m = \frac{K_S K_I K_{II}}{K_I K_{II} + K_{II} + \frac{k_2}{k_3} + 1} \quad (4)$$

$$\frac{k_{cat}}{K_m} = \frac{k_2}{K_S K_I K_{II}} \quad (5)$$

The 10-fold difference in binding affinity for butyrylthiocholine between wild type and D70G can be explained with Figure 3 and eq 5. Butyrylthiocholine interacts with Asp 70 in the ES1 complex but not in the ES2 and ES3 complexes. For binding of butyrylthiocholine, the equilibrium constants K_s and K_I are the only constants which differ between wild type and D70G. A 10-fold increase in K_m is therefore explained by a 10-fold increase in the value of K_sK_I. The 100-fold increase in K_m for succinyldicholine binding to D70G compared to that of wild type can be explained by a similar analysis. Succinyldicholine interacts with Asp 70 in complexes ES1 and ES2. This means three equilibrium constants, K_s, K_I, and K_{II}, are increased in the D70G mutant. Since k_{cat} and k_2 are identical for D70G and wild type, eq 5 can be rearranged to give $K_m = K_sK_IK_{II}$ (constant). Therefore, a 10-fold increase in K_sK_I combined with a 10-fold increase in K_{II} yields a 100-fold increase in K_m.

There is one important constraint on this model which must be pointed out. Namely, k_{cat} remains the same for both D70G and wild-type BuChE (Table 1). If we assume that the D70G mutation does not affect the rate-limiting step, then for k_{cat} to remain constant it is necessary that the final equilibrium concentration of ES3 be essentially unaffected by the mutation. This requirement can be accommodated in the following way. If the equilibrium between ES1 and ES2 is highly favorable (for formation of ES2) while that between ES2 and ES3 is highly unfavorable (for formation of ES3), then nearly all the enzyme will be present as ES2 and a 10-fold change in K_s will not significantly alter the ES3 concentration. As can be seen in Figure 4, succinyldicholine does not fit well into the catalytic site, making it probable that the ES3 complex is not very stable. Thus, the three-step binding model is consistent with the observation that k_{cat} appears to be unchanged in the D70G mutant.

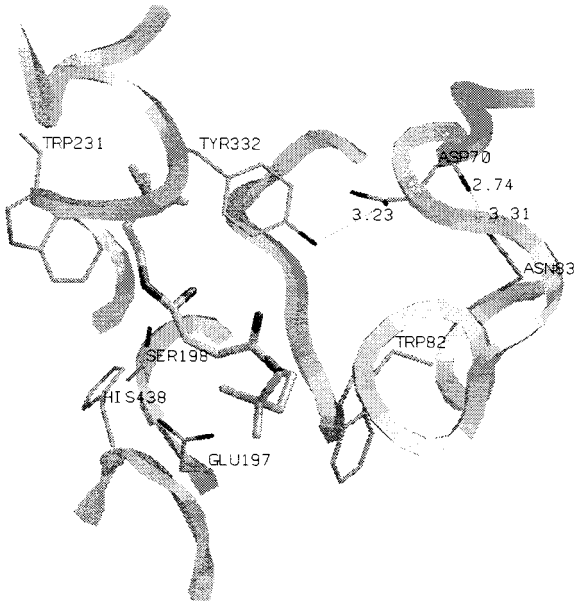


FIGURE 4: Closeup view of succinyldicholine after it has rotated to the hydrolysis position. Succinyldicholine is positioned between Trp 82 and Trp 231 in a twisted conformation made necessary by the small space available. The distance between Oγ of Ser 198 and the nearest carbonyl of succinyldicholine is 1.6 Å. The omega loop has a different conformation in this model than in Figure 2, especially in the region near Asp 70. This structure is represented by ES3 in Figure 3.

Description of the Three Substrate Binding Complexes

Complex ES1. A model for this complex was not attempted. However, Asp 70 appears to play a significant role in this complex. Asp 70 is located at the entrance to the active site gorge (Figure 5) and is likely to be part of the encounter complex for positively charged substrates. We have considered charge−charge interactions between Asp 70 and positively charged substrates to be a major stabilizing influence in the formation of ES1. In addition, the side chain of Asp 70 is directly above the ring of Trp 82 (Figure 5). Tryptophan 82 is the binding site for quaternary ammonium ions in ES2 and ES3 complexes. The location of Asp 70 relative to Trp 82 suggests that another function of Asp 70 in ES1 is to orient the positively charged portion of a substrate such that it can slide down the gorge onto Trp 82. Its strategic location at the mouth of the gorge gives Asp 70 this gatekeeper function. It should also be pointed out that, in the absence of substrate, Asp 70 is hydrogen bonded to Tyr 332 (Figure 5). Formation of ES1 will release Tyr 332 and could thereby trigger protein conformational change.

Complex ES2. Figure 2 shows succinyldicholine in the ES2 complex with wild-type BuChE. Succinyldicholine fits along the length of the gorge to bridge the peripheral anionic site (Asp 70) and the catalytic "anionic" site (Trp 82). The dominant, stabilizing interactions appear to be a charge−charge interaction between one choline head and Asp 70 and a cation−π interaction (Dougherty, 1996) between the other choline head and Trp 82. Kinetic studies on both Trp 82 and Glu 197 mutants clearly demonstrated that Trp 82 was the site of the major interaction. Dougherty (1996) has noted that cation−π interactions cannot be correctly estimated by

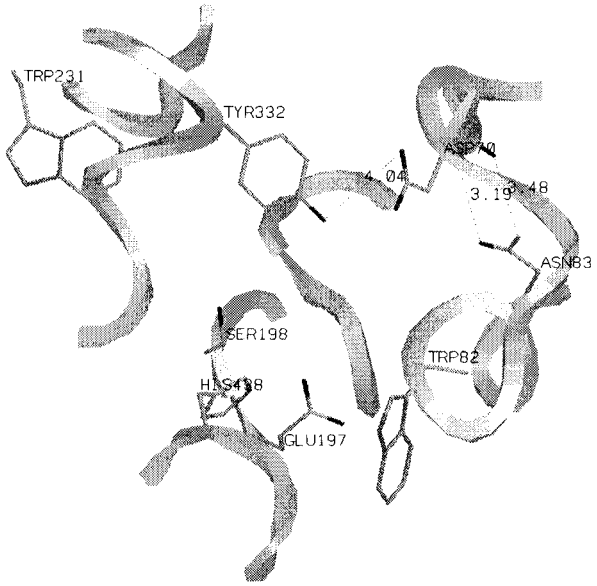


FIGURE 5: Empty wild-type BuChE showing the H bond between Tyr 332 and Asp 70. Asp 70 is near the top of the gorge, and Ser 198 and His 438 are at the bottom. A hydrogen bond between Tyr 332 and Asp 70 is shown. The omega loop is formed by amino acids Cys 65−Cys 92 and includes Asp 70 and Trp 82. Asp 70 is located directly above the side chain of Trp 82, suggesting that it functions to attract the positively charged choline end of butyrylcholine. From this position, butyrylcholine is correctly oriented to transfer down into the choline binding site at Trp 82.

molecular mechanics calculations. Weaker stabilizing interactions are with Asn 83, Gly 116, Gly 117, Gln 119, Pro 285, Leu 286, Ser 287, and His 438. In addition, there is substantial rearrangement of the omega loop (residues Cys 65−Cys 92), especially in the region including Asp 70 (Figure 6A).

Complex ES3. Figure 4 shows succinyldicholine at the bottom of the gorge in a position suitable for hydrolysis. One choline head is near Trp 82, and the second is near Trp 231. The space between Trp 82 and Trp 231 provides a poor fit for succinyldicholine. Stabilization of this complex comes from interactions with Trp 82, Gly 115, Gly 116, Gly 117, Glu 197, Ser 198, Ala 199, Trp 231, Ser 287, Phe 329, Phe 398, His 438, Gly 439, and Tyr 440. Many of the residues of the omega loop which had moved in ES2 have returned to their original positions (Figure 6B). This is particularly evident in the position of Asp 70.

Succinyldicholine in the D70G Mutant

Molecular modeling suggested that the structure of the most energetically stable complex of succinyldicholine with the D70G mutant (Figure 7) was similar to that of the complex with wild-type BuChE (Figure 2). That is, succinyldicholine spanned the gorge from the rim to the active site despite the absence of the stabilizing interaction with Asp 70. Hydrolysis cannot occur until succinyldicholine has shifted to a location between Trp 82 and Trp 231 as in Figure 4. The identical k_{cat} value of 350 min^{-1} for wild type and D70G (Table 1) suggests that the binding pocket between Trp 82 and Trp 231 has the same shape in the wild type and the D70G mutant during the rate-limiting step for hydrolysis which is probably deacylation (Masson et al., 1996).

Molecular dynamics analysis was undertaken to gain insight into the effect of mutation D70G on the motion of the omega loop. This was achieved over 30 ps at 300 K

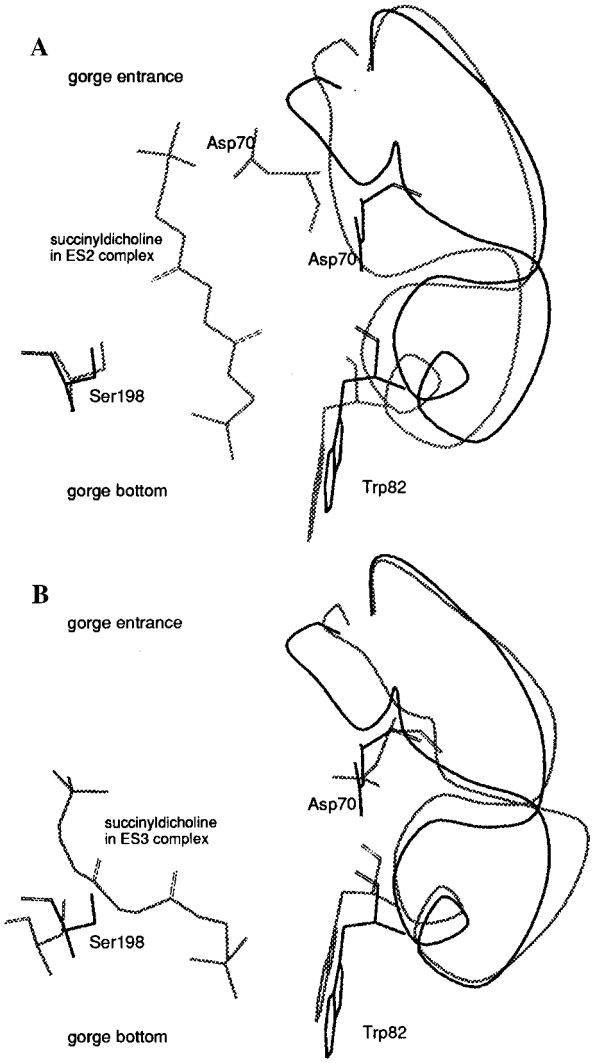


FIGURE 6: Omega loop movement. (A) The omega loop (Cys 65−Cys 92) of empty wild-type BuChE (solid line) is superimposed on the omega loop of wild-type BuChE, to which succinyldicholine is bound in the ES2 complex (light line). (B) Comparison of omega loop structures of empty wild-type BuChE (solid line) and succinylcholine bound to wild-type BuChE in the ES3 complex (light line).

using the empty enzyme structures (wild type and D70G) and the respective ES2 complexes. H bond and nonbond interaction pair lists were updated each 25 steps, and coordinates were stored every 0.1 ps. Molecular dynamics calculations suggested that the omega loop structure was highly flexible in the D70G mutant when it was complexed to succinyldicholine (Figure 8). Over the 30 ps simulations, the greatest mean fluctuation of 3 Å was at P74 (Figure 8). By contrast, mean fluctuations were similar (1−1.5 Å) for empty wild type, empty D70G, and for wild type complexed to succinyldicholine. A tentative explanation for the flexibility of the omega loop in the ES2 complex with the D70G mutant was the presence of glycine in position 70 in the mutant enzyme leading to a reduced number of hydrogen bonds and electrostatic interactions. Residues 71−74 had zero H bonds in D70G complexed to succinyldicholine, one H bond in empty D70G, one H bond in wild type complexed to succinyldicholine, and five H bonds in empty wild type. Flexibility in the omega loop structure may be a second factor, in addition to loss of the negative charge, that accounts for the loss of approximately 3 kcal/mol in binding succi-

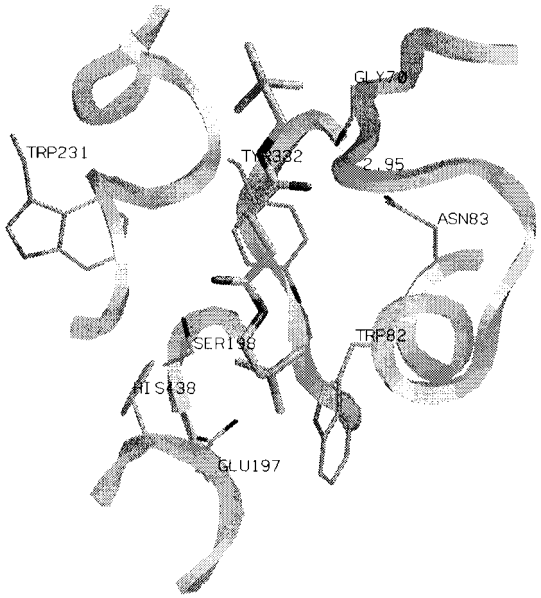


FIGURE 7: Succinyldicholine bound to the D70G mutant. The conformation of the omega loop is similar in the D70G mutant bound to succinyldicholine and in wild-type BuChE bound to succinyldicholine (Figure 2).

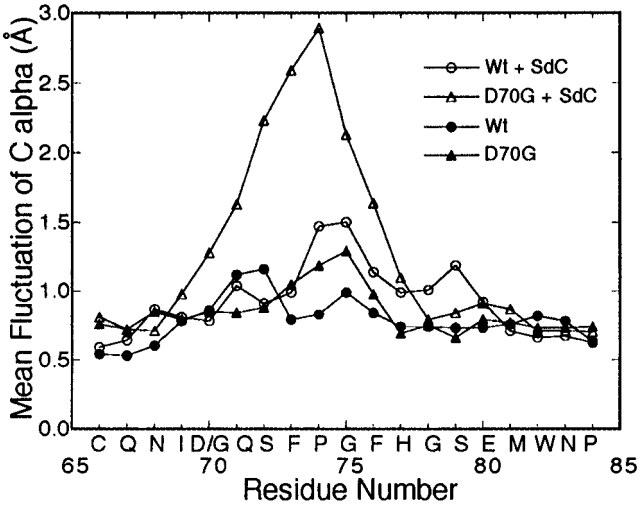


FIGURE 8: Fluctuation of the omega loop. The mean fluctuation of the α carbon in amino acids 66−84 of the omega loop is shown in the absence and presence of succinyldicholine (SdCh) for wild-type BuChE and the D70G mutant. The greatest fluctuation is at Pro 74 for the D70G mutant binding succinyldicholine.

nyldicholine to D70G compared to wild-type BuChE (Table 2). Flexibility in the omega loop structure may depend on the specific amino acid in position 70 and may explain why the D70N mutant behaves in a manner different from that of D70G (Table 1). In addition, in the ES2 complex with the D70G mutant, the omega loop is expected to be more mobile because succinyldicholine has a single tight anchor point (on Trp 82) instead of the two anchors (Asp 70 and Trp 82) in the ES2 complex of wild-type enzyme.

Induced Fit Step in Binding of Succinyldicholine

In the absence of ligand, Asp 70 is hydrogen bonded to Tyr 332 (Figure 5). In the presence of succinyldicholine, this H bond breaks and a new interaction forms between the choline group and Asp 70 (Figure 2). The side chain of Asp 70 is directed out of the gorge, whereas in empty BuChE, it had pointed across the gorge. The new bond to the choline group induced a conformational change in the omega loop of wild-type BuChE (Figure 2). A similar global conformation is found in the omega loop of the D70G mutant complexed to succinyldicholine (Figure 7), even though the omega loop of D70G is more flexible in the Pro 74 region (Figure 8). The change in the shape of BuChE that results from binding of succinyldicholine is interpreted in terms of induced fit in BuChE. The induced fit complex is depicted as the ES2 enzyme−substrate intermediate in Figure 3. Movement of the omega loop changed the orientation of Trp 82, leading to a more favorable conformation for interaction with the quaternary ammonium group of succinyldicholine.

Substrate Activation and Inhibition

The substrate activation phenomenon takes place when a second molecule of butyrylthiocholine binds to BuChE, causing an increase in the apparent maximum velocity (Masson et al., 1996). Since the rate-limiting step in the hydrolysis of butyrylthiocholine could be either formation or breakdown of the acyl−enzyme intermediate, it is not clear which form of the enzyme binds the second butyrylthiocholine: the productive Michaelis complex or the acyl intermediate EA. We propose that the second butyrylthiocholine binds in the gorge between Asp 70 and Trp 82 to cause activation. The second substrate molecule in this position induces a conformational change in the protein which involves the omega loop and at least one catalytically important group, His 438. We propose that substrate activation is a consequence of this induced conformational change. Such a mechanism could also account for substrate inhibition, as seen with benzoylcholine, since the different substrate could induce a different conformation which could cause inhibition of the rate-limiting step instead of activation. In an effort to identify specific amino acids which may be critical for substrate activation/inhibition, we tested the mutants in Table 1 at high substrate concentrations. Mutants D70G and D70K showed complete loss of both substrate activation with butyrylthiocholine and no substrate inhibition with benzoylcholine. This suggested that Asp 70 was critical for these phenomena.

The simple picture of a critical role for Asp 70 in substrate activation/inhibition was confounded when the D70N mutant was examined. This mutant showed greater substrate activation with butyrylthiocholine ($b = 10$) than wild-type BuChE ($b = 3.2$), but no substrate inhibition with benzoylcholine ($b = 1$). Once again, we are faced with a situation in which Asp 70 appears to play a significant role, but the nature of that role is unclear. The Y332F mutant, like D70N, showed a mixed effect, having substantial substrate activation and no substrate inhibition. The W82A mutant showed no substrate activation, suggesting that Trp 82 is important for substrate activation. The varied response of activation and inhibition to these amino acid replacements suggests that the structural changes which lead to substrate inhibition/activation are intricately related to the protein structure in the gorge region. A more precise description of the events leading to substrate inhibition/activation is unwarranted at this time. However, since the omega loop contains both Asp 70 and Trp 82, activation/inhibition may result from a conformational change involving the omega loop.

DISCUSSION

Site-Directed Mutagenesis and Molecular Modeling Support the Three-Step Binding Model. The existence of three complexes prior to the covalent acyl−enzyme intermediate is postulated to explain the experimental fact that bisquaternary compounds have a 100-fold decrease in binding affinity while monoquaternary compounds have a 10-fold decrease in binding affinity to the D70G mutant. Kinetic analysis of mutant enzymes and molecular modeling support the proposed three-step binding model. (1) Support for the existence of an ES1 complex comes from analysis of the D70G mutant which shows that Asp 70 is important for binding positively charged compounds but has no effect on binding of neutral compounds. Asp 70, being 12 Å from the active site Ser 198, can have only a minor effect on the final complex when the positively charged substrate is at the bottom of the gorge. Therefore, a transient charge−charge interaction with substrate as it enters the gorge is a likely role of Asp 70. Stopped-flow kinetic analysis of BuChE interacting with the bisquaternary ligand D-tubocurarine (Stojan & Pavlic, 1991) shows the existence of a rapidly formed initial complex at the peripheral site which may correspond to the ES1 complex. Transient complexes with the peripheral site of AChE are also thought to occur (Haas et al., 1992; Rosenberry et al., 1996). (2) Support for the existence of an ES2 complex comes from our molecular modeling result which showed that the most stable orientation of succinyldicholine in BuChE was along the length of the gorge, spanning the distance from Asp 70 at the rim of the gorge to Trp 82 at the bottom of the gorge. This model is similar to the X-ray structure of decamethonium in acetylcholinesterase (Harel et al., 1993) which shows decamethonium spanning the length of the gorge from the peripheral site to Trp (84). Since succinyldicholine and decamethonium have similar structures, both molecules having the same chain length and both molecules being bisquaternary amines, it is reasonable that succinyldicholine should mimic decamethonium in its fit in the active site gorge. Additional support for the ES2 complex is the finding that the W82A mutant does not bind succinyldicholine, indicating that Trp 82 has an important role in binding succinyldicholine. (3) Support for the ES3 complex is the fact that succinyldicholine is hydrolyzed by BuChE. This means one of the carbonyl groups of succinyldicholine must be close enough to Ser 198 for hydrolysis to occur. When this constraint was imposed on molecular modeling, the result was the ES3 complex. In ES3, the nearest carbonyl is 1.6 Å from the Ser 198 hydroxyl, a distance almost the same as that of a typical C−O covalent bond. Hydrolysis cannot occur when succinyldicholine is in the ES2 position because the nearest carbonyl of succinyldicholine is 6.0 Å from Ser 198, a distance too far to allow hydrolysis. The mutant W82A supports a role for Trp 82 in binding succinyldicholine, because succinyldicholine was not bound or hydrolyzed by the W82A mutant. The mutant W231A bound succinyldicholine more tightly than wild-type BuChE, suggesting that removal of Trp 231 gave more room for succinyldicholine to fit in the ES3 position.

Functions of Asp 70. Asp 70 is the point of interaction between positively charged substrates and BuChE in the ES1 complex, providing the binding energy to stabilize that complex. A second function of Asp 70 is to guide positively charged substrates to the bottom of the active site gorge in an orientation that leads to rapid hydrolysis (Massoulié et al., 1993). A third function of Asp 70 is to anchor one end of bisquaternary compounds such as succinyldicholine while the other end is stabilized by Trp 82. A fourth function is in substrate activation, where Asp 70 transiently binds a molecule of butyrylthiocholine and guides it to Trp 82 while the first molecule of butyrylthiocholine is at the bottom of the gorge. Substrate inhibition by benzoylcholine occurs via a similar process. Asp 70 and Trp 82 cooperate in all these functions.

The interaction energy to form the ES1 complex and the guiding function of Asp 70 explain why the D70G mutant, called the atypical variant, has a decreased binding affinity for all positively charged substrates and positively charged inhibitors. Without these functions of Asp 70, a positively charged molecule has the same statistical probability as a neutral molecule to find itself correctly positioned for interaction with Trp 82. Asp 70 contributes 1−2 kcal/mol to the binding of compounds with one positive charge and 3 kcal/mol to binding of compounds with two positive charges (Table 2). Its partner Trp 82 has a much more important role as it contributes 5 kcal/mol to the binding of the monoquaternary butyrylthiocholine.

Function of Trp 82. Trp 82 is the dominant factor in the stabilization of positively charged substrates in the ES2 and ES3 complexes. Our results fully support conclusions made with AChE that Trp 82 has the role attributed to the anionic site (Sussman et al., 1991; Ordentlich et al., 1993). In view of the absence of an anionic charge on Trp 82, it would be more useful to rename this site the cation-π site (Dougherty, 1996).

Two-Point Attachment of Succinyldicholine. An unanswered question of long standing in the literature, first posed by Kalow (1962), is the question of why succinyldicholine and other bisquaternary compounds have a 100-fold difference in binding affinity for wild-type BuChE compared to the atypical variant (D70G), whereas monoquaternary compounds have only a 10-fold difference in binding affinity. To explain these differences, we propose the existence of three enzyme−substrate complexes preceding formation of the acyl−enzyme. The substrate enters the gorge to form ES1, a complex with transient binding to Asp 70. Then the substrate moves down onto Trp 82 to form ES2. Finally, the substrate rotates into a position where it can be attacked by S198 in the ES3 intermediate.

The differential effect of the D70G mutation on bisquaternary and monoquaternary compounds can now be rationalized, as follows. Neutralization of the negative charge at position 70 will destabilize the ES1 complex for all positively charged substrates. In addition, it will destabilize the ES2 complex for bisquaternary substrates but not for monoquaternary substrates. The weakening of two complexes in the binding process of bisquaternary substrates and only one complex in the binding process of monoquaternary substrates predicts a larger effect of the D70G mutation on binding of bisquaternary substrates than on binding of monoquaternary substrates, as is observed. For example, there is a 10-fold decrease in the binding of butyrylthiocholine to the D70G mutant which can be attributed to a 10-fold decrease in affinity at ES1. There is a 100-fold decrease in affinity for succinyldicholine, which can be attributed to

a 10-fold decrease in affinity at ES1 and an additional 10-fold decrease in affinity at ES2.

AChE Does Not Hydrolyze Succinyldicholine. Our model of succinyldicholine binding and hydrolysis explains why human AChE is inhibited by succinyldicholine but does not hydrolyze it (Evans et al., 1952; Ikarashi et al., 1990). Inhibition is the result of binding of succinyldicholine along the length of the gorge, in an orientation similar to that of decamethonium (Harel et al., 1993). The inability to hydrolyze succinyldicholine is explained by the inability of succinyldicholine to rotate within the constricted active site pocket of AChE. Succinyldicholine cannot fit between residues equivalent to Trp 231 and Trp 82 because access to Trp 231 is blocked by aromatic residues in the AChE active site. Thus, in AChE, the ester groups never come close enough to Ser 198 to undergo nucleophilic attack.

Substrate Activation. Substrate activation in wild-type BuChE is observed in the range of 0.4−40 mM butyrylthiocholine when the buffer is 0.1 M potassium phosphate at pH 7.0. The extrapolated V_{max} determined from this range of butyrylthiocholine concentrations is 3-fold higher than the V_{max} calculated with low butyrylthiocholine concentrations from 0.01 to 0.1 mM (Masson et al., 1996; Millard et al., 1995). The 3-fold increase in V_{max} at high butyrylthiocholine concentrations is called substrate activation and is expressed as the *b* value (Radic et al., 1993; Masson et al., 1996). Substrate activation in BuChE is seen with acetylthiocholine (Wetherell & French, 1986; Masson et al., 1993), propionylthiocholine, and butyrylthiocholine (Masson et al., 1993, 1996; Millard et al., 1995; Radic et al., 1993; Cauet et al., 1987; Eriksson & Augustinsson, 1979) but not with benzoylcholine, succinyldithiocholine, or *o*-nitrophenyl butyrate. Other positively charged compounds have been reported to accelerate activity. Choline, tetramethylammonium ion, and tryptamine increased the hydrolysis rate of benzoylcholine (Erdös et al., 1958; Clark et al., 1968); dibucaine (>25 μM) increased the rate of hydrolysis of *o*-nitrophenyl acetate (Masson et al., 1993), and amiloride accelerated the rate of hydrolysis of *p*-nitrophenyl esters (Clery et al., 1995).

Which enzyme−substrate intermediate binds a second substrate molecule? Binding accelerates V_{max}; therefore, binding must speed the rate-limiting step. If the rate-limiting step is deacylation, then the enzyme form that binds the second substrate molecule is the acyl−enzyme intermediate. Substrate activation would accelerate the rate of deacylation in a way similar to acceleration of deacylation by organic and inorganic cations (Kitz et al., 1969). A mechanism to explain how positively charged compounds could accelerate the deacylation step has been proposed by Wlodek et al. (1995) to involve the protonation state of His 438. His 438 must be deprotonated before His 438 can activate a water molecule for nucleophilic attack on the acyl−enzyme intermediate. Conformational changes induced by binding of a positively charged compound could increase either the speed or extent of deprotonation of His 438 and thus increase the rate of deacylation.

We have identified two residues important for substrate activation, Asp 70 and Trp 82. It is expected that other residues in the active site, especially those near His 438, also affect substrate activation and inhibition in BuChE.

Induced Fit. The omega loop, which starts and ends at disulfide bond C65−C92, is a classical omega loop (Fentrow, 1995) having definite structure through its network of hydrogen bonds and characterized by its important function of orienting Asp 70 and Trp 82. Molecular dynamics suggested that the omega loop is flexible in BuChE, changing conformation upon binding succinyldicholine. This change in conformation is reminiscent of Rosenberry's proposal that the catalytic pathway for AChE includes an induced fit conformational change of the initial enzyme−substrate complex (Rosenberry, 1975; Rosenberry et al., 1996). A conformational change is also supported by stopped-flow analysis of the reaction of horse serum BuChE with D-tubocurarine (Stojan & Pavlic, 1991). These authors showed that there is instantaneous binding of D-tubocurarine to a peripheral site, followed by a relatively slow conformational transition in the enzyme.

Loop movement changed the orientation of Trp 82, though not as dramatically as in the computer simulations of AChE (Barak et al., 1995; Faerman et al., 1996) probably because Trp 82 in BuChE is hydrogen bonded to Tyr 440 whereas Trp (*84*) in *Torpedo* AChE has no hydrogen bonds. Conformational changes in the omega loop of AChE are thought to be induced by ligand binding to the peripheral site (Ordentlich et al., 1993; Radic et al., 1995; Barak et al., 1995), leading to a change in orientation of the side chain of Trp (*84*) and a decrease in the rate of acylation when the ligand is an inhibitor (Eastman et al., 1995; Radic et al., 1995).

Clinical Relevance. The consequence for a person who has the D70G mutation is an abnormal response to the muscle relaxants succinyldicholine and mivacurium (Kalow & Grant, 1995; Ostergaard et al., 1993). Another drug that may cause an abnormal response is pyridostigmine. During the Gulf War, 41 650 soldiers who were instructed to take pyridostigmine under the threat of nerve agent attack were observed for adverse effects (Keeler et al., 1991). One Israeli soldier with atypical BuChE developed nausea, insomnia, weight loss, general fatigue, and depression upon taking the standard prophylactic dose of 30 mg orally every 8 h for 1−7 days (Loewenstein-Lichtenstein et al., 1995). In a group of that size, one can expect 14−20 people to have the homozygous atypical genotype and to experience severe side reactions. To date, none of the 28 Americans whose pyridostigmine therapy was discontinued because of intolerance to the drug (Keeler et al., 1991) have been genotyped for BuChE, though it can be anticipated that many of these 28 have a genetic variant of BuChE. It is likely that cocaine is a third drug which is poorly metabolized by people with atypical BuChE since BuChE is the major detoxifying enzyme for cocaine and it is known that the D70G mutant has a reduced affinity for cocaine (Jatlow et al., 1979; Kalow & Grant, 1995; Schwartz & Johnson, 1996). It is expected that people with atypical BuChE are at greater risk for myocardial infarction and fatalities following the use of cocaine (Kalow & Grant, 1995).

To explain how a mutation located far from the active site can have such dramatic effects, we propose a role for Asp 70 in binding positively charged substrates. To explain the exaggerated inability of the atypical variant to bind bisquaternary compounds such as succinyldicholine, we propose the formation of three enzyme−substrate complexes preceding the acyl−enzyme intermediate.

ACKNOWLEDGMENT

We thank Professor Palmer Taylor and Dr. Zoran Radic (La Jolla, CA) for helpful discussions and Dr. Yacov Ashani (Ness Ziona, Israel) for the gift of MEPQ.

REFERENCES

Barak, D., Kronman, C., Ordentlich, A., Ariel, N., Bromberg, A., Marcus, D., Lazar, A., Velan, B., & Shafferman, A. (1994) *J. Biol. Chem. 264*, 6296−6305.

Barak, D., Ordentlich, A., Bromberg, A., Kronman, C., Marcus, D., Lazar, A., Ariel, N., Velan, B., & Shafferman, A. (1995) *Biochemistry 34*, 15444−15452.

Brooks, B. R., Bruccoleri, R. E., Olafson, B. D., States, D. J., Swaminathan, S., & Karplus, M. (1983) *J. Comput. Chem. 4*, 187−217.

Cauet, G., Friboulet, A., & Thomas, D. (1987) *Biochem. Cell. Biol. 65*, 529−535.

Clark, S. W., Glaubiger, G. A., & La Du, B. N. (1968) *Ann. N. Y. Acad. Sci. 151*, 710−722.

Clery, C., Heiber-Langer, I., Channac, L., David, L., Balny, C., & Masson, P. (1995) *Biochim. Biophys. Acta 1250*, 19−28.

Cornish-Bowden, A. (1974) *Biochem. J. 137*, 143−144.

Davies, R. O., Marton, A. V., & Kalow, W. (1960) *Can. J. Biochem. Physiol. 38*, 545−551.

Dewar, S., Zoebisch, E. G., Healy, E. F., & Stewart, J. J. P. (1985) *J. Am. Chem. Soc. 107*, 3902−3909.

Dougherty, D. A. (1996) *Science 271*, 163−168.

Eastman, J., Wilson, E. J., Cerveñansky, C., & Rosenberry, T. L. (1995) *J. Biol. Chem. 270*, 19694−19701.

Ellman, G. L., Courtney, K. D., Andres, V., & Featherstone, R. M. (1961) *Biochem. Pharmacol. 7*, 88−95.

Erdös, E. G., Foldes, F. F., Zsigmond, E. K., Baart, N., & Zwartz, J. A. (1958) *Science 128*, 92−93.

Ericksson, H., & Augustinsson, K. B. (1979) *Biochim. Biophys. Acta 567*, 161−173.

Evans, F. T., Gray, P. W. S., Lehmann, H., & Silk, E. (1952) *Lancet 1*, 1229−1230.

Faerman, C., Ripoll, D., Bon, S., Le Feuvre, Y., Morel, N., Massoulié, J., Sussman, J. L., & Silman, I. (1996) *FEBS Lett. 386*, 65−71.

Fentrow, J. S. (1995) *FASEB J. 9*, 708−717.

Gilson, M. K., Straatsma, T. P., McCammon, J. A., Ripoll, D. R., Faerman, C. H., Axelsen, P. H., Silman, I., & Sussman, J. L. (1994) *Science 263*, 1276−1278.

Goochee, C. F., Gramer, M. J., Andersen, D. C., Bahr, J. B., & Rasmussen, J. R. (1991) *Bio/Technology 9*, 1347−1355.

Haas, R., Adams, E. W., Rosenberry, M. A., & Rosenberry, T. L. (1992) in *Multidisciplinary Approaches to Cholinesterase Functions* (Shafferman, A., & Velan, B., Eds.) pp 131−139, Plenum Press, New York.

Harel, M., Sussman, J. L., Krejci, E., Bon, S., Chanal, P., Massoulié, J., & Silman, I. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10827−10831.

Harel, M., Schalk, I., Ehret-Sabatier, L., Bouet, F., Goeldner, M., Hirth, C., Axelsen, P. H., Silman, I., & Sussman, J. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9031−9035.

Harel, M., Quinn, D. M., Nair, H. K., Silman, I., & Sussman, J. L. (1996) *J. Am. Chem. Soc. 118*, 2340−2346.

Ikarashi, Y., Hada, T., Way, E. L., & Maruyama, Y. (1990) *J. Chromatogr. 533*, 23−33.

Jatlow, P., Barash, P. G., Van Dyke, C., Radding, J., & Byck, R. (1979) *Anesth. Analg. 58*, 235−238.

Jenkins, N., & Curling, E. M. A. (1994) *Enzyme Microb. Technol. 16*, 354−364.

Kalow, W. (1962) *Pharmacogenetics: heredity and the response to drugs*, pp 69−136, W. B. Saunders Co., Philadelphia.

Kalow, W., & Davies, R. O. (1958) *Biochem. Pharmacol. 1*, 183−192.

Kalow, W., & Grant, D. M. (1995) in *The Metabolic and Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., & Valle, D., Eds.) Vol. 1, pp 303−326, McGraw-Hill Inc., New York.

Keeler, J. R., Hurst, C. G., & Dunn, M. A. (1991) *JAMA 266*, 693−695.

Kitz, R. J., Braswell, L. M., & Ginsberg, S. (1969) *Mol. Pharmacol. 6*, 108−121.

Kozak, M. (1991) *J. Biol. Chem. 266*, 19867−19870.

Kronman, C., Velan, B., Gozes, Y., Leitner, M., Flashner, Y., Lazar, A., Marcus, D., Sery, T., Papier, Y., Grosfeld, H., Cohen, S., & Shafferman, A. (1992) *Gene 121*, 295−304.

Levy, D., & Ashani, Y. (1986) *Biochem. Pharmacol. 35*, 1079−1085.

Lockridge, O. (1992) in *Pharmacogenetics of Drug Metabolism* (Kalow, W., Ed.) pp 15−50, Pergamon Press, New York.

Lockridge, O., Mottershaw-Jackson, N., Eckerson, H. W., & La Du, B. N. (1980) *J. Pharmacol. Exp. Ther. 215*, 1−8.

Loewenstein-Lichtenstein, Y., Schwarz, M., Glick, D., Norgaard-Pedersen, B., Zakut, H., & Soreq, H. (1995) *Nat. Med. (N.Y.) 1*, 1082−1085.

Main, A. R., Miles, K. E., & Braid, P. E. (1961) *Biochem. J. 78*, 769−776.

Masson, P., Adkins, S., Gouet, P., & Lockridge, O. (1993) *J. Biol. Chem. 268*, 14329−14341.

Masson, P., Froment, M.-T., Bartels, C. F., & Lockridge, O. (1996) *Eur. J. Biochem. 235*, 36−48.

Massoulié, J., Pezzementi, L., Bon, S., Krejci, E., & Vallette, F.-M. (1993) *Prog. Neurobiol. 41*, 31−91.

McGuire, M. C., Nogueira, C. P., Bartels, C. F., Lightstone, H., Hajra, A., van der Spek, A. F. L., Lockridge, O., & La Du, B. N. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 953−957.

McTiernan, C., Adkins, S., Chatonnet, A., Vaughan, T. A., Bartels, C. F., Kott, M., Rosenberry, T. L., La Du, B. N., & Lockridge, O. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6682−6686.

Millard, C. B., & Broomfield, C. A. (1992) *Biochem. Biophys. Res. Commun. 189*, 1280−1286.

Millard, C. B., Lockridge, O., & Broomfield, C. A. (1995) *Biochemistry 34*, 15925−15933.

Neville, L. F., Gnatt, A., Padan, R., Seidman, S., & Soreq, H. (1990) *J. Biol. Chem. 265*, 20735−20738.

Ordentlich, A., Barak, D., Kronman, C., Flashner, Y., Leitner, M., Segall, Y., Ariel, N., Cohen, S., Velan, B., & Shafferman, A. (1993) *J. Biol. Chem. 268*, 17083−17095.

Ostergaard, D., Jensen, F. S., Jensen, E., Skovgaard, L. T., & Viby-Mogensen, J. (1993) *Acta Anaesthesiol. Scand. 37*, 314−318.

Radic, Z., Pickering, N. A., Vellom, D. C., Camp, S., & Taylor, P. (1993) *Biochemistry 32*, 12074−12084.

Radic, Z., Quinn, D. M., Vellom, D. C., Camp, S., & Taylor, P. (1995) *J. Biol. Chem. 270*, 20391−20399.

Rosenberry, T. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3834−3838.

Rosenberry, T. L., Rabl, C.-R., & Neumann, E. (1996) *Biochemistry 35*, 685−690.

Schwartz, H. J., & Johnson, D. (1996) *Clin. Toxicol. 34*, 77−81.

Shafferman, A., Velan, B., Ordentlich, A., Kronman, C., Grosfeld, H., Leitner, M., Flashner, Y., Cohen, S., Barak, D., & Ariel, N. (1992) *EMBO J. 11*, 3561−3568.

Stojan, J., & Pavlic, M. R. (1991) *Biochim. Biophys. Acta 1079*, 96−102.

Sussman, J. L., & Silman, I. (1992) *Curr. Opin. Struct. Biol. 2*, 721−729.

Sussman, J. L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L., & Silman, I. (1991) *Science 253*, 872−879.

Valentino, R. J., Lockridge, O., Eckerson, H. W., & La Du, B. N. (1981) *Biochem. Pharmacol. 30*, 1643−1649.

Wetherell, J. R., & French, M. C. (1986) *Biochem. Pharmacol. 25*, 939−945.

Whittaker, M. (1986) *Cholinesterase. Monographs in Human Genetics* (Beckman, L., Ed.) Vol. 11, pp 1−132, Karger, Basel.

Whittaker, M., & Britten, J. J. (1980) *Clin. Chim. Acta 108*, 89−94.

Wlodek, S. T., Antosiewicz, J., Gilson, M. K., McCammon, J. A., Clark, T. W., & Scott, L. R. (1995) in *Enzymes of the Cholinesterase Family* (Quinn, D. M., et al., Eds.) pp 97−104, Plenum Press, New York.

BI962484A