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PRINCIPAL INVESTIGATOR: Palmer Taylor, Ph.D.

CONTRACTING ORGANIZATION: University of California, San Diego
Department of Pharmacology
La Jolla, California 92093-0636

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Analysis of Structure and Specific Functional Groups Involved in Acetylcholinesterase Catalysis and Inhibition

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Palmer Taylor, Ph.D.

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

U.S. Army Medical Research & Development Command
Fort Detrick
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The acetylcholinesterase genes from Torpedo, mouse and man and the butyrylcholinesterase gene from mouse have been expressed in mammalian and baculovirus based expression systems. The former system has enabled us to rapidly ascertain the kinetic properties and inhibitor specificity of recombinant DNA-derived, site-specific mutants and chimeras of the cholinesterases. The latter expression system has proven useful for large-scale production of cholinesterases for physical-chemical characterization. A large number of mutant enzymes and chimeras have been analyzed to define the residues responsible for substrate and inhibitor specificity. To this end we have assessed the role of charge in catalysis and binding, defined residues responsible for acyl pocket selectivity and for the specificity of acetyl- and butyrylcholinesterase selective inhibitors. In other studies, epitopes for molecular form specific and nonspecific monoclonal antibodies have been defined. Chemical modification studies with azidopropidium analogues have also identified peptides within the AChE structure contributing to the peripheral site.

Acetylcholinesterase; Acetylcholine; Enzymes; Genetic Engineering; Binding Sites; Active Sites; Lab Animals; RA V

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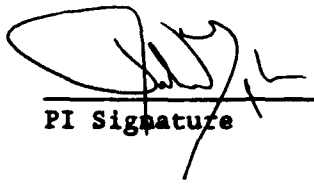

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INTRODUCTION

A. Definition of the Problem and Specific Aims

The proposed work is directed to gaining a detailed knowledge of the catalytic function and inhibitor binding specificity of acetylcholinesterase (AChE) in relation to its structure. The approach relies heavily on recent advances in recombinant DNA technology used to clone, ascertain the structure and express the recombinant *Ache* gene. The work is based on prior advances in cloning Torpedo and mammalian *Ache* genes and recent crystallographic analysis of the Torpedo AChE structure.

B. Background

Enzyme Structure

Acetylcholinesterase (AChE; EC 3.1.1.7) is a membrane-associated enzyme typically localized in cholinergic synapses and in certain differentiated cells of the hematopoietic system (Quinn, 1987; Massoulié & Toutant, 1988; Taylor, 1991). Its only well-described function is the rapid hydrolysis and termination of the action of the neurotransmitter acetylcholine. Analysis of AChE primary and secondary structures reveals that the cholinesterases form part of a large gene family that not only includes a variety of serine hydrolases but also encompasses several other proteins without apparent hydrolase activity. Interestingly, apart from four residues around the active center, this family of enzymes shows no global homology with two other families of serine hydrolases, the pancreatic serine proteases and the subtilisins (Schumacher et al., 1986; Taylor, 1991; Taylor, 1992). Moreover, the ordering of residues within the catalytic triad would suggest that the cholinesterases arose by convergent evolution rather than by divergent evolution from the other hydrolases. Recently the three-dimensional structure of the AChE molecule was determined by crystallographic analysis; the data confirm the catalytic roles of Ser^{200} and His^{440} and establish that Glu^{327} is the third participant in the triad (Sussman et al., 1991). Of particular significance is the hydrophobic gorge, nearly 20 Å deep, at the base of which the catalytic serine resides. The charged residue within the base of the cavity which appears closest to the site of binding of the quaternary ammonium group is Glu^{199}. This charge might be expected to be critical to substrate specificity and catalytic efficiency.

Gene Structure

Analyses of the sequences of Torpedo AChE (Gibney et al., 1988), their encoding cDNAs (Sikorav et al., 1988; Schumacher et al., 1986), and the structure and organization of the *Ache* gene (Maulet et al., 1990) indicate that a single *Ache* gene yields two mRNA species through alternative splicing. Upon translation, the two nascent peptides forming catalytic subunits differ only at their carboxyl termini. They are distinctively processed to add a glycophospholipid and/or to assemble with other catalytic subunits or structural subunits to form homomeric or heteromeric oligomers. This sequence of steps accounts for the wide structural diversity of AChE species that have been identified over the past 25 years (see Massoulié & Toutant (1988) and Taylor (1991) for reviews).

Mammalian and avian AChEs also appear to be encoded by a single gene (Rachinsky et al., 1990; Li et al., 1991; Rotundo et al., 1988). The human

and mouse genes have been localized to regions of synteny, 7q22 in human and the distal region of chromosome 5 in mouse (Getman et al., 1992; Rachinsky et al., 1992). Alternative mRNA processing of the mouse *Ache* gene yields three transcripts encoding peptides which also differ only at their carboxyl termini (Li et al., 1991). Alternative polyadenylation signals with tissue-specific usage are also found in the mouse gene (Li et al., 1993a&b).

The Cholinesterase Gene Family

Recombinant DNA technology was a natural direction for cholinesterase research in the 1980s. The initial cloning of the *Ache* gene indicated that AChE shared global sequence homology only with thyroglobulin (Schumacher et al., 1986), but over the past 6 years, proteins homologous to cholinesterase have enlarged to an extensive but functionally eclectic family, not all members of which function as hydrolases. The family now includes the tactins which are important for heterologous cell contacts. This structural similarity may provide an indication for noncatalytic function of the cholinesterases in developmental processes. The recent report of a high-resolution crystal structure of Torpedo AChE adds the critical third dimension to structure (Sussman et al., 1991). A comparison of three-dimensional structures also indicates that certain carboxypeptidases and dehalogenases share a common folding pattern in their backbone despite the absence of appreciable sequence identity. These proteins share a common structure known as the α/β hydrolase fold.

Our studies and progress to date might be divided into four areas:

1) Development of Expression Systems for Recombinant Acetylcholinesterase and Butyrylcholinesterase
2) Analysis of Structure-Function Relationships Through the Formation of Chimeras and Site-Specific Mutants
3) Probing Enzyme Structure with Antibodies
4) Labeling and Selective Chemical Modifications of Acetylcholinesterase.

The most exciting developments involve the first and second areas. Accordingly, they will receive the most emphasis. The overall methods and results are distinct for each section and therefore are grouped together.

BODY (Methods and Results)

A. Development of Expression Systems for Recombinant Acetylcholinesterase and Butyrylcholinesterase

Earlier in the development of this study, we had cloned Torpedo AChE (Schumacher et al., 1986) and mouse AChE and butyrylcholinesterase (BuChE) (Rachinsky et al., 1990). We had also developed an expression system for the various molecular species of Torpedo AChE. More recently, we have obtained human genomic clones of AChE (Li et al., 1991). To devise an expression strategy, we employed transient transfection systems, i.e., SV-40 or cytomegalovirus (CMV)-based expression vectors, to rapidly analyze mutants for activity and kinetic parameters. For production-scale levels of recombinant

AChE, we have utilized Spodoptera cells with baculovirus-based vector infections.

1. Cloning and Expression of Torpedo and Mouse Acetylcholinesterases. The Torpedo AChE clone was constructed by ligation of an AChE cDNA containing the 5' coding region with a 3' coding region prepared from genomic sequence by loopout mutagenesis of an intron between the invariant exon and one of the alternative 3' exons (Gibney and Taylor, 1990). The construction was inserted into an SV-40 expression plasmid, and when transfected into Cos-1 cells, expressed the glycophospholipid-linked form of AChE (Gibney and Taylor, 1990). Expression in the baculovirus system required intermediate cloning into a pGEM vector, which enables insertion of the cDNA in front of the polyhedron promoter at a unique NhE-1 site in the linker of the transfer vector (Luckow & Summers, 1988; Summers & Smith, 1987).

A similar construction was used to express a previously isolated cDNA encoding the hydrophilic form of mouse AChE (Rachinsky et al., 1990). The baculovirus vector contained the β-galactosidase gene driven by the P10 promoter, enabling selection of recombinants by β-galactosidase activity (Vialard et al., 1990). Production of site-specific mutants and their verification by sequencing have been previously described (Gibney et al., 1990).

2. Spodoptera frugiperda (Sf9). Cells were typically grown in 250 mL of medium in a 500-mL spinner flask with slow stirring at 27°C in Excell 401 media. Purified viral stock was usually added to cells at a density of 2 x 10^6 cells/mL.

3. Purification of the Recombinant Enzymes. The Torpedo AChEs containing the glycophospholipid linkage were prepared from sedimented cells by initial solubilization in approximately 10 volumes of a 10 mM HEPES buffer, pH 8.0, containing 1% Triton X-100, 100 mM NaCl, 40 mM $MgCl_2$, 0.1 mM EDTA, aprotinin (0.1 mg/mL), bacitracin (1.0 mg/mL), and benzamidine (1.6 mg/mL). After sedimentation at 14000g for 20 min, the supernatant was loaded onto an acridinium affinity column at a flow rate of 2 mL/h (Lee et al., 1982). Following successive washes with 10 column volumes of loading buffer with NaCl increased to 1.0 M, and the loading buffer again, elution was accomplished with 0.1 mM edrophonium in loading buffer. Activity was assayed by the procedure of Ellman et al. (1961) and related to protein concentration. Active-site stoichiometries were determined by titration of the irreversible loss of activity with either 2,2-dimethylbutyl methyl phosphonofluoridate or 7-(methylethoxyphosphinyloxy)-1-methylquinolinium (MEPQ) (Levy & Ashani, 1986).

Kinetic parameters, K_s, K_{ss}, k_{cat}, were determined. We also examined acylation and deacylation of the enzyme using carbamoylating and phosphorylating inhibitors and a detailed kinetic analysis. Binding with reversible inhibitors was also examined, and the mode of inhibition was delineated to ascertain true inhibition constants. We have taken pains to determine primary constants consistent with the inhibition mechanism. This should enable one to correlate data between research groups.

4. Characteristics of the Expressed Wild-Type Cholinesterases.

Expression of AChE in COS cells using a SV-40 based expression system or in human embryonic kidney (HEK) cells with a CMV-based expression system yielded quantities of enzyme sufficient for characterization of the kinetic parameters. The glycophospholipid-linked species was extracted from the cell surface with Triton X-100, while the hydrophilic form of the mouse enzyme was concentrated from the serum-free medium in which the cells were grown for the final 24 hours. The recombinant DNA-derived enzymes appeared stable when on ice for several days to weeks.

Expression in *Sf*9 cells yields far greater quantities of enzyme, although the constructions and cloning of viral stocks with recombinant vector remain as time-consuming endeavors. Expression from *Sf*9 cells in spinner culture yielded quantities of AChE up to 3.7 mg/L which could be purified by affinity chromatography in good yields. Hence, we have available a rapid mammalian cell expression system for screening mutants and reasonably good expression systems in Spodoptera for preparing large quantities of enzyme.

The enzymes prepared from the baculovirus-Spodoptera system were purified to homogeneity by affinity chromatography. Single bands were obtained on polyacrylamide-SDS gel electrophoresis with coincident staining by Western blotting with an anti-AChE antibody and by amido-black staining. Glycosylation could be demonstrated by electrophoretic migration rates of the enzyme prior to and following endoglycosidase F treatment (Radic' et al., 1992).

B. Analysis of Structure-Function Relationships for the Recombinant DNA-derived Enzymes

Figures 1 and 2 summarize all the mutants and chimeras currently under study or used in our previous studies. Positions in the three-dimensional structure are shown for the chimera junctions and site-specific mutants.

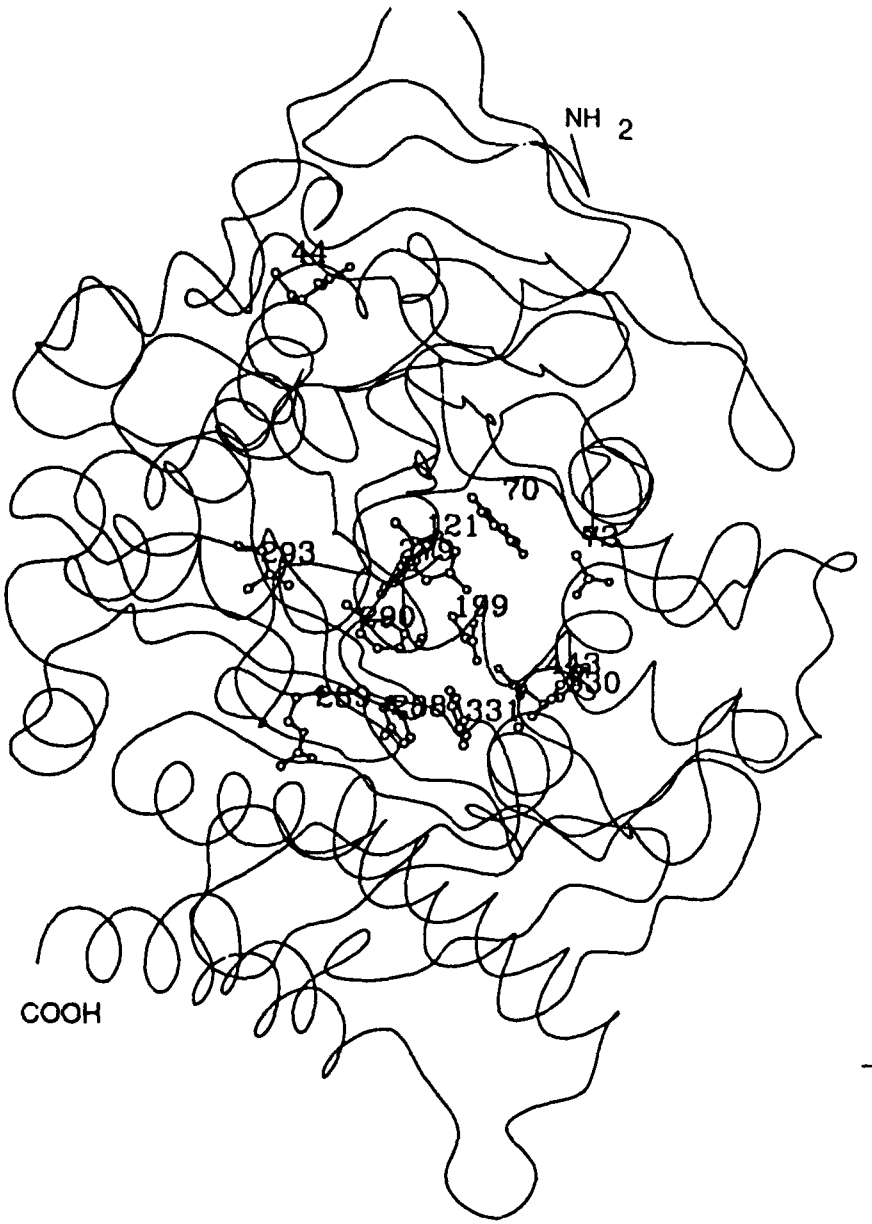
Our initial study was directed toward a detailed analysis of the influence of charge on the binding of substrate. Glutamate 199 is the proximal anionic residue to the choline head group of acetylcholine when bound (~5 Å between the centers of the most proximal oxygen and the hydrogen in the trimethylammonio moiety). An analysis reveals several interesting features of this mutation, which are detailed in Tables I through V. First, the removal of charge by changing Glu to Gln (E to Q) reduces k_{cat} and increases K_m. Hence, k_{cat}/K_m is reduced by a factor of 50 (Table I). The Glu to Asp (E to D) mutation reduces only k_{cat} but curiously eliminates substrate inhibition. Carbamoylation rates can be similarly affected for E → Q (reduction in rate by a factor of 20 for those inhibitors with a dispersed charge) (Table III). Decarbamoylation is only marginally affected by the E → D and E → Q mutation (Table IV). Phosphorylation is also influenced to a greater extent by the E → Q mutation (Table V). Quaternary reversible inhibitors of high affinity are markedly affected by the E → Q mutation (Table II). Consistent with its influence on substrate inhibition, the E → D mutation influences propidium's affinity, while the E → D mutation has little effect.

Our second round of mutants included $E_{443} \rightarrow D$, I or Q, $S_{226} \rightarrow A$. In this set we found that $E_{443} \rightarrow Q$, $E_{443} \rightarrow I$ and $S_{226} \rightarrow A$ inhibit activity to less than 1% of wild-type, while $E_{443} \rightarrow D$ retains partial activity. This suggests, but does not prove, that these residues stabilize a critical H_2O molecule which may be essential for deacylation. A more detailed analysis of these mutants is still under way. Included in this set were $E_{199} \rightarrow I$, $R_{44} \rightarrow K$ and $R_{44} \rightarrow Q$. The first is a control for the above studies, while the other two are being studied collaboratively with Dr. Goran Bucht in Umea, Sweden.

A third set of mutants was designed to address two issues: (a) what residues outline the acyl pocket, and (b) what residues confer the selectivity seen in AChE versus BuChE inhibitors.

Our approach is multifaceted and involves the use of chimeras and site-specific mutants (Table VI). Since we had cloned AChE and BuChE in mouse, we have used the mouse template for this and subsequent studies. The numbering reflects residue numbers in the mammalian enzyme. The frame of reference can be found in Table VI. Numbers in parentheses cited below denote the position in the Torpedo sequence. Our findings show that chimeras of AChE in which the amino-terminal 174 and the carboxyl-terminal 88 amino acids have been converted to the BuChE sequences ($B_{4-175}A_{176-487}B_{488-575}$ and $B_{4-175}A_{176-575}$) still exhibit acetyl-like substrate specificity (Table VII). Four nonconserved amino acids which are within the central sequence and appear to surround the acyl pocket, F_{295}, R_{296}, F_{297}, and V_{300}, have been mutated alone and in combination with the corresponding residues found in BuChE, L_{286}, S_{287}, I_{288}, and G_{291}. The V_{300} and R_{296} mutants slightly enhance butyrylthiocholine hydrolysis while the F_{295} and F_{297} mutants alone and in combination confer BuChE character by enhancing activity to butyrylthiocholine, and diminishing activity to acetylthiocholine. The double F_{295}, F_{297} mutant was examined by Chanal et al. (1992), but they overlooked the interesting distinctions in the single mutations. The F_{297} mutation eliminates substrate inhibition. F_{295} and F_{297} may form a clamp around the acetoxy methyl group. They have distinctive roles in affecting catalysis of the two acylcholines and precisely control acyl ester specificity. The F_{295} mutant actually shows a five-fold improved catalytic efficiency for butyrylcholine hydrolysis over BuChE (Table VIII). Comparisons of the susceptibilities of the chimeras and site-specific mutants to cholinesterase-specific inhibitors isoOMPA, ethopropazine, and BW284c51 suggest that inhibitor selectivity for isoOMPA is attributable to residues limiting the size of the acyl pocket, while residues in the amino-terminal domain presumably near the lip of the gorge affect BW284c51 selectivity (Table VIII). Ethopropazine specificity is governed by residues in the central portion of the sequence but not those in the acyl pocket.

The fourth series of mutants is directed to assigning the residues responsible for inhibitor specificity; this series is designed to follow up on the interesting findings from the chimeras (Vellom et al., 1993). The basic concepts are that isoOMPA selectivity is influenced by the acyl pocket outline with the side chains of F_{295} and F_{297} being the most critical. BW284c51 will be influenced by $Y_{72(70)}$, $Y_{124(121)}$ and $W_{286(279)}$ as based on the chimeras and by neighboring residues.



Summary of mutants*

Glu199Gln Glu199Asp	Expressed in Baculovirus. Characterized for inhibition by reversible and acylating inhibitors. Also for decarbamylation.
Arg44Lys Arg44Glu Glu199Ile Glu443Gln Glu443Asp Glu443Ile Ser226Ala	Expressed in HEK293 cells. Characterization in progress. All except Glu443Asp very low activity. Glu443Asp characterized: - pS curve, - phosphorylation, -carbamoylation
Phe288Leu Arg289Ser Phe290Ile Val293Gly	*Single site, double (288,290), and triple (288,289,290) mutants.* Expressed in HEK293. *Characterized for reaction with substrates acetylthiocholine (ATCh) and butyrylthiocholine (BTCh), and inhibitors BW284C51, isoOMPA and ethopropazine.*
Tyr70Asn Asp72Asn Tyr121Gln Trp279Arg Trp279Ala Phe288Tyr Phe290Tyr Tyr330Ala Tyr330Phe Phe331Gly	Single site, double (70,121; 70,279; 121,279), triple (70,121,279) and quadruple (70,72,121,279) mutants. Most of them already expressed in HEK293 cells. Initial characterization for reaction with ATCh and reversible inhibitors done.

* All numbers refer to Torpedo AChE. The first two groups were done in Torpedo AChE, the last two in mouse AChE.

Figure 1: Compilation of progress on site-specific mutants.

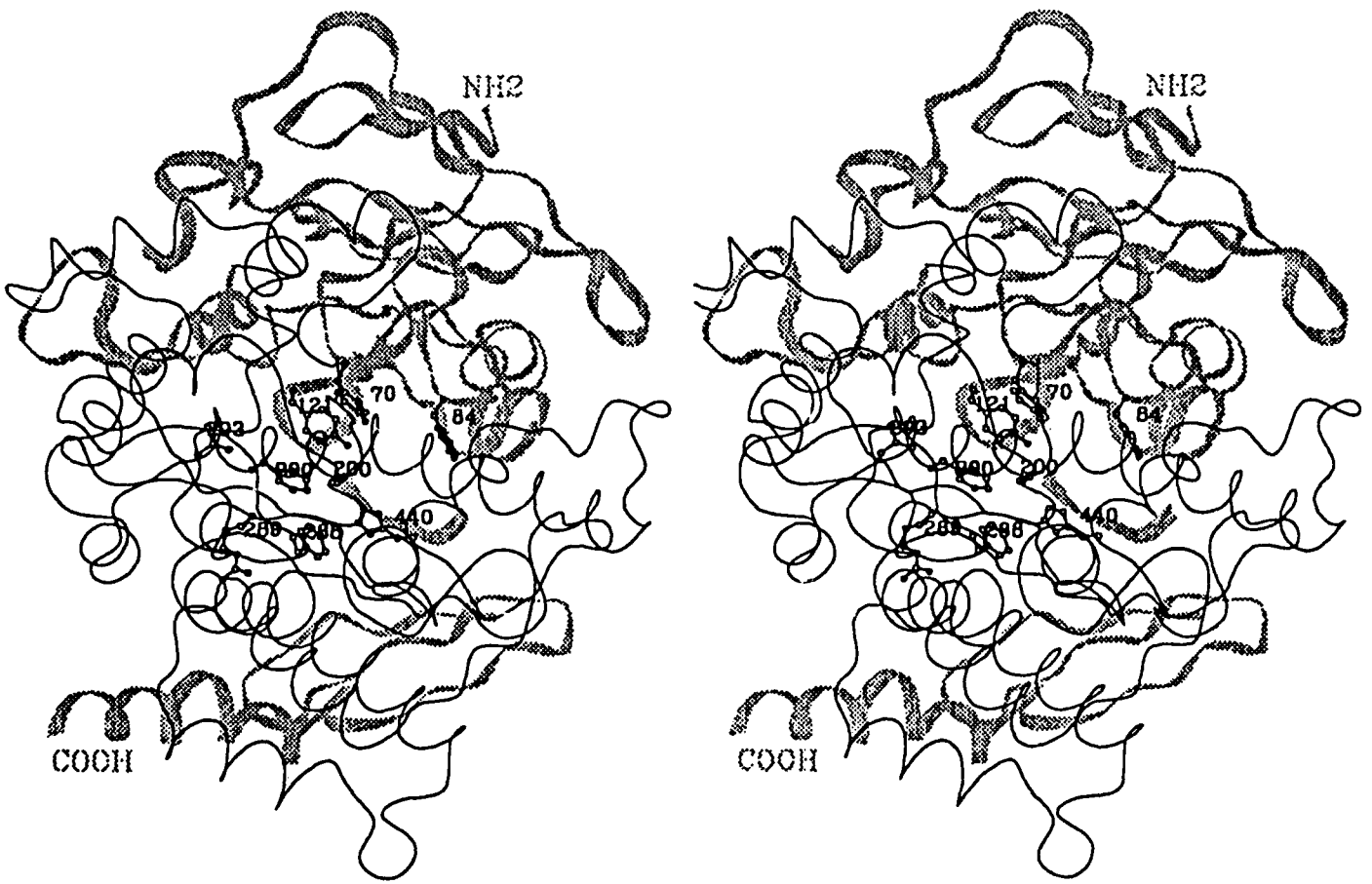


Figure 2: Structure of Torpedo acetylcholinesterase. The crystal structure is from Sussman et al.(1991). The amino- and carboxyl-terminal domains are displayed as wide ribbons to represent chimeras made by substitution of BuChE sequence. The short, unconnected segment links to the end of the carboxyl-terminal sequence, but the intervening residues are not visible in the crystal structure. Shown are side chain positions of residues mutated in the acyl pocket (F_{288}, R_{289}, F_{290}, V_{293}), catalytic residues (S_{200}, H_{440}), a residue (W_{84}) which stabilizes the choline moiety, and the two aromatic residues within the gorge (Y_{70}, Y_{121}) not conserved in BuChE.

TABLES I, II & III

Table I: Kinetic Parameters (±Standard Errors) for Wild-Type and Mutant Acetylcholinesterases Expressed in a Baculovirus–*Spodoptera* System[a]

enzyme	$10^3 K_m$ (M)	$K_{m\ wt}/K_{m\ mut}$	$10^3 K_{ss}$ (M)	$K_{ss\ wt}/K_{ss\ mut}$	$10^5 k_{cat}$ (min^{-1})	$10^9 k_{cat}/K_m$
Torpedo wild type (tissue derived)	0.076		25		2.5 ± 0.5	3.3
Torpedo wild type (recombinant)	0.048 ± 0.005	1	32 ± 3	1	1.5 ± 0.3	3.1
Torpedo Glu^{199} → Gln	0.66 ± 0.16	0.07	151 ± 18	0.2	0.42 ± 0.14	0.064
Torpedo Glu^{199} → Asp	0.043 ± 0.014	0.9	–	–	0.30 ± 0.13	0.7
mouse wild type	0.076 ± 0.012		25 ± 2		1.3 ± 0.1	1.7

[a] Kinetic constants were determined by measuring activity versus substrate concentration and fitting the curves according to eq 1. The dash indicates that no substrate inhibition was detected up to concentrations of 300 mM substrate. Mean values and standard errors for K_m and K_{ss} were determined by curve fitting to eq 1. Data come from three to eight experiments and three preparations of enzyme. k_{cat} was determined from measurements of V_{max} and titration of active site concentrations on three preparations of enzyme. Standard errors are shown only for primary constants where three or more determinations have been made on separate preparations of enzyme. Dashes indicate the lack of substrate inhibition. Details of the kinetic analyses are found in Radić et al., 1992.

Table II: Reversible Inhibition of Torpedo Acetylcholinesterase [a]

recombinant enzyme	K_i[a] ± standard error (μM)									
	propidium		edrophonium		phenyltrimethyl-ammonium		3-hydroxypyridine		3-hydroxy-*N*-methylpyridinium	
	comp	noncomp	comp	noncomp	comp	noncomp	comp	noncomp	comp	noncomp
wild type	0.35 ± 0.08	0.67 ± 0.08	0.23 ± 0.07	–	80	500	2.2×10^4	3.5×10^4	$6.3 \pm 0.9 \times 10^3$	$1.9 \pm 0.6 \times 10^4$
Glu^{199} → Gln	0.75 ± 0.35	–	5.7 ± 0.9	–	440	–	$4.8 \pm 0.7 \times 10^4$	$1.8 \pm 1.2 \times 10^5$	$1.2 \pm 0.8 \times 10^4$	1.6×10^4
Glu^{199} → Asp	4.5 ± 2.5	8.0	2.5 ± 1.5	–	50	–	3.3×10^4	–	nd	nd

[a] The dashes indicate that the constant for the other mode of inhibition was indeterminate or at least 10-fold greater than the paired constant determined for the specified mode of inhibition. nd, not determined. Inhibition was determined from reciprocal plots of velocity and substrate concentrations. Replots of the apparent K_m and V_{max} versus inhibitor concentration yielded the competitive and noncompetitive components. Standard errors are shown when separate preparations of enzyme were analyzed.

Table III: Progressive Inhibition of Recombinant Acetylcholinesterases[a]

inhibitor	AChE	inhibition constants			$k_{a\ wt}/k_{a\ mut}$
		K_a (M)	k_{+2} (min^{-1})	k_a (M^{-1} min^{-1})	
M7C	*Torpedo* wt	$(8.5 \pm 3.7) \times 10^{-6}$	5.8 ± 4.4	$(8.5 \pm 2.4) \times 10^5$	
	Torpedo Gln^{199}	$(2.4 \pm 0.15) \times 10^{-5}$	1.1 ± 0.1	$(4.5 \pm 0.5) \times 10^4$	19
	Torpedo Asp^{199}	$(8.7 \pm 2.4) \times 10^{-7}$	2.0 ± 0.4	$(2.4 \pm 0.2) \times 10^6$	0.4
	mouse	$(1.3 \pm 0.63) \times 10^{-6}$	5.3 ± 2.5	$(6.0 \pm 1.1) \times 10^6$	
eserine	*Torpedo* wt	–	–	$(1.1 \pm 0.24) \times 10^6$	
	Torpedo Gln^{199}	9.0×10^{-6}	3.9	$(4.3 \pm 0.7) \times 10^5$	3
	Torpedo Asp^{199}	5.6×10^{-6}	2.1	$(3.7 \pm 0.1) \times 10^5$	3
	mouse	–	–	$(8.5 \pm 0.8) \times 10^5$	
neostigmine	*Torpedo* wt	–	–	6.4×10^5	
	Torpedo Gln^{199}	6.0×10^{-6}	0.57	9.5×10^4	7
	Torpedo Asp^{199}	–	–	$(1.3 \pm 0.1) \times 10^5$	5
	mouse	–	–	5.7×10^6	
pyridostigmine	*Torpedo* wt	$(1.0 \pm 0.2) \times 10^{-4}$	0.17 ± 0.003	$(2.0 \pm 0.44) \times 10^3$	
	Torpedo Gln^{199}	$(1.9 \pm 0.19) \times 10^{-3}$	0.18 ± 0.006	$(9.4 \pm 0.06) \times 10^1$	21
	Torpedo Asp^{199}	$(6.3 \pm 1.8) \times 10^{-4}$	0.59 ± 0.03	$(1.0 \pm 0.3) \times 10^3$	2
	mouse	9.5×10^{-6}	4.6	4.9×10^5	

[a] Enzyme inhibition was measured in 0.1 M $NaPO_4$, pH 7.0, as a function of time and inhibitor concentration. At least three inhibitor concentrations were used. Kinetics were analyzed according to the eq 3–5. Where the dashes are shown, the rates were too rapid to ascertain K_a and k_2 by conventional mixing techniques. Standard errors are shown in the cases where determinations were made on two or more preparations. Ka is the reversible dissociation constant of inhibitor and enzyme determined from the concentration dependence of inhibition, while k_2 is the first order rate constant for irreversible inhibition determined at saturating inhibitor concentration. Details of the kinetic analysis may be found in Radić et al., 1992.

Table IV: Spontaneous Reactivation of Recombinant Acetylcholinesterase

$$EA \xrightarrow[H_2O]{k_{+3}} E + A$$

inhibitor[a]	AChE	$10^3 k_{+3}$[b] (min^{-1})	$k_{+3\ wt}/k_{+3\ mut}$
M7C	*Torpedo* wt	1.6 ± 0.12	
	Torpedo Gln^{199}	0.58 ± 0.27	3
	Torpedo Asp^{199}	1.2 ± 0.35	1
	mouse	1.9	
eserine	*Torpedo* wt	5.4 ± 1.2	
	Torpedo Gln^{199}	1.5 ± 0.1	4
	Torpedo Asp^{199}	1.4 ± 0.22	4
	mouse	8.1	
pyridostigmine	*Torpedo* wt	1.5 ± 0.30	
	Torpedo Gln^{199}	0.86 ± 0.47	2
	Torpedo Asp^{199}	nd[c]	
	mouse	2.3	

[a] AChE was initially inhibited ~80–90% with listed inhibitors. Return of enzyme activity was monitored as a function of time upon 1000-fold dilution of inhibition mixture. No reactivation was detected for methanesulfonyl fluoride or haloxon inhibition. [b] Standard errors are shown when measurements were made on multiple enzyme preparations. [c] Not determined.

Table V: Inhibition of Recombinant Native and Mutant Acetylcholinesterases by Organophosphates and Sulfonates [a]

		inhibition constants[a]			
inhibitor	AChE	K_a (M)	k_{+2} (min^{-1})	k_a ($M^{-1}\ min^{-1}$)	$k_{a\ wt}/k_{a\ mut}$
haloxon	wt	$(1.4 \pm 0.45) \times 10^{-6}$	0.60 ± 0.4	$(4.7 \pm 1.8) \times 10^5$	
	Gln^{199}	$(2.2 \pm 0.67) \times 10^{-5}$	0.18 ± 0.012	$(9.4 \pm 0.24) \times 10^3$	50
	Asp^{199}	1.7×10^{-5}	1.6	$(8.0 \pm 1.2) \times 10^4$	6
	mouse	1.0×10^{-5}	2.5	2.5×10^5	
paraoxon	wt	$(5.8 \pm 1.1) \times 10^{-5}$	3.8 ± 1.2	$(7.7 \pm 1.6) \times 10^6$	
	Gln^{199}	$(7.3 \pm 4.7) \times 10^{-4}$	0.54 ± 0.23	$(9.3 \pm 2.8) \times 10^2$	83
	Asp^{199}	–	–	$(3.1 \pm 0.12) \times 10^4$	3
	mouse	2.7	9.0	1.5×10^6	
MEPQ	wt	–	–	$(2.9 \pm 0.45) \times 10^8$	
	Gln^{199}	–	–	9.6×10^6	30
	Asp^{199}	–	–	7.5×10^7	4
	mouse	–	–	1.3×10^8	
DFP	wt	–	–	$(1.0 \pm 0.19) \times 10^4$	
	Gln^{199}	$(8.0 \pm 1.5) \times 10^{-3}$	0.30 ± 0.05	$(3.8 \pm 0.8) \times 10^1$	263
	Asp^{199}	7.4×10^{-4}	1.6	$(1.8 \pm 0.35) \times 10^3$	6
	mouse	–	–	1.1×10^4	
methanesulfonyl fluoride	wt	–	–	2.2×10^2	
	Gln^{199}	–	–	4.7×10^1	5
	Asp^{199}	–	–	$(9.1 \pm 4) \times 10^1$	2
	mouse	–	–	2.9×10^2	

[a] Rates of inhibition were determined as described in Table III. Standard errors are shown when measurements were made on multiple enzyme preparations.

TABLES VI, VII & VIII

Table VI Amino Acid Residues Surrounding the Putative Acyl Pocket in Cholinesterase and Site-Directed Mutants[a]

mouse AChE	F^{295} R F S F V^{300}
human AChE	F^{295} R F S F V^{300}
fetal bovine AChE	F^{295} R F S F V^{300}
Torpedo AChE	F^{288} R F S F V^{293}
Drosophila cholinesterase	F^{329} P – S – A^{332}
mouse BuChE	L^{286} S I N F G^{291}
human BuChE	L^{286} S V N F G^{291}
rabbit BuChE	L^{286} S V N F G^{291}

Site-Directed Mutants Residue(s)			
mouse AChE	*Torpedo* AChE	mouse BuChE	mutation
295	(288)	286	F → L
296	(289)	287	R → S
297	(290)	288	F → I
300	(293)	291	V → G
295, 297	(288, 290)	286, 288	F, F → L, I
295, 296, 297	(288, 289, 290)	286, 287, 288	F, R, F → L, S, I

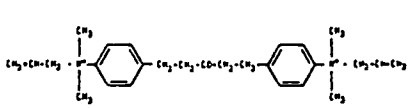
[a] Numbers refer to residues in the mature protein.

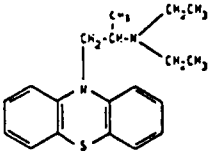
Table VII Kinetic Constants Calculated for Catalysis of Acetylcholine (ATCh) and Butyrylcholine (BTCh) by Recombinant DNA-Derived Cholinesterases ± Standard Error

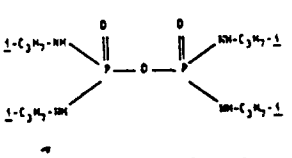
wild-type chimera and mutant cholinesterases	K_m (μM) (ATCh)	K_{ss} (mM) (ATCh)	k_{cat} (min^{-1})[a] (ATCh)	k_{cat}/K_m (M^{-1} min^{-1}) (ATCh)	K_m (μM)[b] (BTCh)	K_{ss} (mM)[b] (BTCh)	k_{cat} (BTCh)/ k_{cat} (ATCh)	k_{cat}/K_m (M^{-1} min^{-1}) (BTCh)
AChE	39 ± 2	38 ± 4	17 ± 1.3 × 10^4	4.4 × 10^9	49 ± 3	76 ± 15	0.005 ± 0.001	0.17 × 10^8
BuChE	451 ± 6	–	11 ± 0.2 × 10^4	0.24 × 10^9	280 ± 84	–	0.67 ± 0.03	2.6 × 10^8
B_{5-174}, $A_{175-575}$	140 ± 10	76 ± 0.5	22 ± 0.3 × 10^4	1.6 × 10^9	330 ± 16	28 ± 2	0.016 ± 0.005	0.11 × 10^8
B_{5-174}, $A_{175-487}$, $B_{484-575}$	130 ± 20	96 ± 18	16 ± 5.0 × 10^4	1.2 × 10^9	234 ± 20	65 ± 17	0.032 ± 0.004	0.22 × 10^8
F_{295} → L	49 ± 4	62 ± 8	4.4 ± 0.7 × 10^4	0.90 × 10^9	9.0 ± 3	90 ± 5	0.27 ± 0.03	13 × 10^8
R_{296} → S	42 ± 5	43 ± 14	11 ± 1.0 × 10^4	2.6 × 10^9	57 ± 15	360 ± 26	0.053 ± 0.003	1.0 × 10^8
F_{297} → I	357 ± 66	–	2.7 ± 0.4 × 10^4	0.08 × 10^9	215 ± 12	–	0.34 ± 0.01	0.43 × 10^8
V_{300} → G	50 ± 4	46 ± 6	16 ± 2 × 10^4	3.2 × 10^9	100 ± 3	64 ± 3	0.18 ± 0.05	2.9 × 10^8
F_{295}, F_{297} → L, I	150 ± 30	355 ± 75	2.0 ± 0.2 × 10^4	0.13 × 10^9	125 ± 5	–	0.58 ± 0.03	0.93 × 10^8
F_{295}, R_{296}, F_{297} → L, S, I	138 ± 62	–	1.0 ± 0.5 × 10^4	0.07 × 10^9	ND	ND	0.44 ± 0.07	ND

[a] k_{cat} was determined from at least three titrations of the inhibition of enzyme activity with MEPQ; measurements represent means and standard errors of 3–4 separate transfections. [b] K_m and K_{ss} were calculated from a computer-generated best fit analysis of data similar to Figures 2 and 3 according to the formula: $v = v_{max}/[1 + (K_m/[S]) + ([S]/K_{ss})]$. The fit employed Sigma Plot and was based on a Marquardt least-squares analysis. Values represent means and standard errors from 2–4 separate transfections. [c] A dash indicates substrate inhibition was not detected. Concentrations of substrate up to 300 mM were used. ND, not determined.

Table VIII Inhibition Constants for the Cholinesterase Chimeras and Site-Directed Mutant Enzymes[a]







	K_I (BW284c51) (nM)		K_I (ethopropazine) (μM)		k_i (isoOMPA) (M^{-1} min^{-1})
chimera/mutant	K_I competitive	αK_I	K_I competitive	αK_I	
AChE		6.5 ± 0.9	≥100		15.9 ± 1.3
BuChE	11300 ± 430	42300 ± 5000	0.062 ± 0.011	0.17 ± 0.03	217000 ± 33000
B_{5-174}, $A_{175-575}$	290 ± 80	1600 ± 200	25	50	51 ± 3
B_{5-174}, $A_{175-487}$, $B_{488-575}$	707 ± 12	1960 ± 50	43	89	108 ± 15
F_{295} → L		3.1 ± 1.2	≥100		1440 ± 60
R_{296} → S		1.1 ± 1.0	≥100		142 ± 6
F_{297} → I		20 ± 17	44	174	3300 ± 400
V_{300} → G		2.2 ± 0.5	≥100		180 ± 11
F_{295}, F_{297} → L, I		19 ± 2	30	111	8500 ± 1250

[a] Inhibition by the reversible inhibitors BW284c51 and ethopropazine was analyzed by construction of reciprocal plots of activity versus substrate concentration. Replots of the slopes and intercepts versus inhibitor concentration yield mixed inhibition from which K_I (competitive) and αK_I are calculated. For ethopropazine, the competitive component is dominant. Standard deviations are derived from the computer-generated replots. No attempt was made to analyze mode of inhibition when K_I exceeded 100 μM. For BW284c51, replots of the slopes for the mutants inhibited at low concentrations do not show linearity, so we did not distinguish the mode of inhibition. k_i for the irreversible inhibitor, isoOMPA, was determined by measuring the rates of inhibition at two isoOMPA concentrations. k_i is directly proportional to inhibitor potency. When inhibition by ethopropazine required high concentrations of inhibitors, standard errors were not determined. Blank values for K_I indicate non-competitive inhibition.

Ethopropazine will be influenced by residues around $W_{86(84)}$ and $F_{337(331)}$. Some of the propidium site should overlap with that of BW284c51, while the edrophonium site may in part be defined by W_{86}, F_{337} and $E_{202(199)}$. The analysis is buttressed by a more extensive structure-activity study as we have done for $E_{202(199)}$ in which we are analyzing binding of congeners of the acetyl versus butyrylcholinesterase selective agents. Fig. 1 outlines the progress to date. While all of the data are not in, the predictions outlined above appear to be borne out.

These mutants should enable us to assign regions conferring acetyl versus BuChE specificity. Our analyses of structure employ the Biosym programs for energy minimization and docking of ligands and the Iris Indigo-Elan computer.

An interesting future development comes from the examination of the peptide fasciculin with the chimeras. Fasciculin is highly specific for AChE and extremely potent. Again, the chimeras point to a region around the two tyrosines 72 and 124 as critical for fasciculin binding. The site-specific mutants should enable us to further define the site of fasciculin interaction. The crystal structure of fasciculin was recently published (Le Du, et al., 1992).

Studies on the Identification of Epitopes on Acetylcholinesterase

This section describes ongoing work directed toward identifying epitopes and antigenic domains on the AChE molecule. The approach involves formation of CNBr peptides of AChE (5.6S form), fractionation of the peptides by size exclusion and reverse-phase HPLC. Synthesis of peptides was used to confirm the reactivity of the epitope. Epitope identification relies on specific competition assays for the two monoclonal antibodies 4E7 and 2C9. These studies are a collaborative endeavor with B.P. Doctor and M.K. Gentry at Walter Reed Army Institute of Research.

We have mapped the epitopes to which two monoclonal antibodies against AChE from Torpedo californica are directed. One antibody, 2C9, has equivalent affinity for both the 5.6S, amphiphilic, and 11S, hydrophilic, enzyme forms; the other, 4E7, recognizes only the amphiphilic form and has been shown previously to require an N-linked oligosaccharide residue on the protein. Isolation of cyanogen bromide peptides from the 5.6S form and assay by a competition ELISA for 2C9 and by a direct-bind ELISA for 4E7 identified the same CNBr peptide, residues 44-82, to contain epitopes against both antibodies. The epitope for 4E7 includes the oligosaccharide conjugated to Asp 59, an N-linked glycosylation site not present in mouse AChE. A 20-amino-acid synthetic peptide, RFRRPEPKKPWSGVWNASTY, representing residues 44-63, was synthesized and found to completely inhibit 2C9 binding to 5.6S enzyme at molar concentrations comparable to the cyanogen bromide peptide. It was unreactive with 4E7. Fractionation of the synthetic peptide further localized the 2C9 epitope. Peptides RFRRPEPKKPW and KPWSGVWNASTY both reacted, but less than the entire synthetic peptide, at equivalent molar concentrations, while the peptide RPEPKKPWSGVWNASTY was as effective as the larger synthetic peptide. The crystal structure of AChE shows the peptide to be on the surface of the molecule as part of a convex hairpin loop starting before the first alpha helix.

Identification of Sites of Inhibitor Binding by Protein Modification

Our chemical modification studies have emphasized the peripheral anionic site, since this region of AChE is less well defined than other domains of the molecule. To this end, we have synthesized two azidopropidium analogues, 3'-azidopropidium and 8'-azidopropidium, and have examined their interactions with AChE. We purified 35 mg of Torpedo AChE, the 11S hydrophilic form. A two-step purification by affinity chromatography with an intervening treatment with Staphalococcal nuclease was used to remove any nucleic acid carryover. The photoactivation reaction was conducted at 2.4×10^{-5} M AChE with a 1.5-molar excess of 3'-azidopropidium. The enzyme was exposed to 340 nm light at 4°C for 30 min (75-watt xenon). An equivalent control contained a 20-fold molar excess of propidium. Tryptic peptides were prepared as we have done in the past (Gibney et al., 1988) and fractionated by size exclusion. Peptides were monitored at 219 and 280 nm while the azido conjugate was monitored at 480 nm. Full characterization of the peptides is not yet complete, but the initial analysis of the high molecular weight excluded fractions looks most promising. In collaboration with Dr. Gabriel Amitai at the Israel Institute for Biological Research, individual fractions have been analyzed by mass spectroscopy. In the excluded fraction, we find major peaks at m/z's of 1999.6 and 1905; the latter is a peptide encompassing residues 251 through 267. We also found a peak with an m/z of 2352, which is interpreted as the 1905 + 447 (the latter being the molecular weight of the conjugated monoazidopropidium). Further fractionation is currently under way. This work should prove complementary to and extend the work of Hucho and colleagues (Weise et al., 1990) who, by indirect protection experiments, identified a peptide contributing to the peripheral site.

DISCUSSION AND CONCLUSIONS

A. We have developed mammalian expression systems for screening recombinant DNA-derived mutants of Torpedo, mouse and human AChE, and mouse BuChE. Those mutants appropriate for a detailed structural analysis have been expressed in a baculovirus-Spodoptera system yielding up to 3.7 mg/liter in a suspension culture. The value of this system lies in producing sufficient enzymes to analyze substrate inhibition and inhibitor interactions with the cholinesterases in mechanistic detail. This is currently being pursued.

B. An exhaustive analysis of the structural features of the AChE important to catalysis and conferring specificity for inhibition has been undertaken. Both site-specific mutants (single and multiple) and chimeras have been employed. The analysis is based on a detailed examination of inhibitor specificity in which competitive and noncompetitive components are ascertained for reversible inhibitors and rates of inhibition are determined for the irreversible inhibitors. The analysis has relied heavily on the crystal structure. We should be able to define distinct domains responsible for AChE and BuChE substrate and inhibitor specificity and the role of various physiochemical and structural parameters in governing substrate specificity.

1) In particular, we hope to assess the role of charge in substrate and inhibitor association.
2) delineate the role of H_2O and other nucleophiles in deacylation.

3) establish the loci of binding and bonding forces for the AChE- and BuChE-specific inhibitors: BW284c51, isoOMPA and ethopropazine as well as for active center (edophonium, N-methylacridinium) and peripheral site (propidium) selective inhibitors.
4) establish the molecular basis for fasciculin specificity for AChE (cf Le Du et al., 1992).
5) ascertain the role of the peripheral anionic site in substrate inhibition (Radić et al., 1991).

C. The amino acid and oligosaccharide epitopes for the two monoclonal antibodies, 2C9 and 4E7, on AChE have been mapped. The analysis required isolation of peptides and production of synthetic peptides.

D. The peripheral anionic site on AChE is being mapped by chemical modification of the structure using monoazidopropidium. The light-activated conjugate is being characterized by mass spectroscopy of the tryptic peptides.

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