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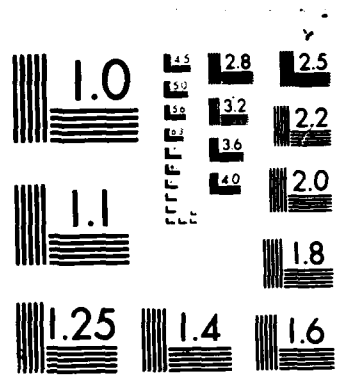
THE PRIMARY STRUCTURE OF ACETYLCHOLINESTERASE AND  
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THE PRIMARY SEQUENCE OF ACETYLCHOLINESTERASE  
AND SELECTIVE ANTIBODIES FOR THE DETECTION  
OF ORGANOPHOSPHATE TOXICITY

ANNUAL REPORT  
PALMER TAYLOR, Ph.D.

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## SUMMARY

The proposed work has been directed to determination of the structure of acetylcholinesterase. During the project period we have determined the primary structure of the Torpedo enzyme through amino acid sequencing and the isolation and sequencing of a c-DNA clone encoding for the 11S form of the enzyme. Peptides corresponding to the active center of the enzyme and a C-terminal region have been synthesized and antibodies are being raised for the purpose of detecting the phosphorylated enzyme and delineating functional regions of the molecule.

## FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and use of Laboratory Animals and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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## Introduction

During this period we have completed the primary structure of acetylcholinesterase through amino acid sequencing and c-DNA cloning and sequencing. This information should prove important to the many groups working on acetylcholinesterase structure.

## Background

The high turnover number of acetylcholinesterase and availability of selective inhibitors have allowed extensive study of its distribution, catalytic mechanism, and functional role in cholinergic neurotransmission. The recent elucidation of the primary structure of the enzyme through protein chemistry and the isolation of its gene have added a molecular dimension to continuing studies on this protein, which controls the residence time of acetylcholine in the synapse. Acetylcholinesterase exhibits an extensive polymorphism of structure and, since the catalytic parameters of the individual enzyme species are largely invariant, the structural diversity appears critical only to the regulation of the cellular disposition of this molecule. Recent structural studies clearly show that acetylcholinesterase behaves as a secreted rather than an integral membrane protein. The post-translational modifications provide an appropriate link to tether the enzyme to specific extracellular locations. Being an extracellular enzyme, modifications of structure critical to its disposition should occur prior to export to its site of residence. Thus, variations in structure responsible for cellular localization must either be encoded in the genome or be differentially affected by posttranslational events of biosynthesis.

### Acetylcholinesterase Polymorphism

Since the initial finding of Massoulié and Reiger (1) that a native form of acetylcholinesterase contains an elongated tail unit linked to defined number of catalytic subunits, the control of individual species of acetylcholinesterase in relation to innervation, developmental processes and activity of excitable cells has received considerable attention (2). Two general classes of acetylcholinesterase species exist. The most unique is the elongated or dimensionally asymmetric species, which contains a filamentous tail unit disulfide-linked to tetrameric sets of catalytic subunits. The tail unit contains a collagen-like sequence distal to the catalytic subunits. Each strand of the triple helix is joined to a tetramer of catalytic subunits. Since each catalytic subunit is approximately 70,000 daltons, elongated species close to a molecular weight of one million are generated. In the case of Torpedo, but not Electrophorus, a second type of structural subunit has been identified as a non-collagenous, 100,000 dalton peptide (3). It will be of interest if this structural entity also prevails in higher species. Treatment of the asymmetric form with collagenase markedly shortens the tail unit and a light tryptic digestion will remove the structural subunits without apparently altering catalytic parameters or the structure of the catalytic subunit (cf. 2). The asymmetric species appear to be fully assembled in the Golgi apparatus prior to export from the cell (4,5).

The second class are the globular forms, which show considerable structural variegation in subunit assembly (monomers to tetramers) and in hydrophobicity. The hydrophobic forms identified to date result from the cotranslational addition of glycopospholipid to the C-terminal carboxyl group of the nascent peptide chain (6,7). This modification resembles that seen in the variable surface glycoprotein of trypanosomes and the Thy-1 antigen (8). It is quite possible that the nature of the glycopospholipid additions are not identical in the various tissues and may, in themselves, provide a basis for microscopic regional localization. Hence, the globular forms range from totally soluble species to species with particular hydrophobic glycopospholipids conjugated to the peptide chain.

### Methods

The methods used for generation of the data described below have been documented in our manuscripts now published in the open literature and will only be described briefly.

A. Determination of the primary structure of acetylcholinesterase Primary structure determinations relied on both tryptic and CNBr fragmentation. The peptides were initially size separated on Sephadex G-50 and then subjected to reverse phase HPLC on C-18 or C-4 columns. Sequencing initially involved dansyl-Edman and the Spinning cup, but after the first 6 months of the contract employed the gas phase method. Details may be found in (9).

B. Antibody Generation and Assessment of Reactivity. Both monoclonal and polyclonal antibodies were made to the 11S and 5.6S species of acetylcholinesterase. Similar methods were used for generation of antibodies to the individual peptides. Antibody reactivity and titers were determined by the enzyme-linked immunoassay (ELISA) method and by radioimmunoassay using <sup>125</sup>I-acetylcholinesterase. Details may be found in references 3 and 10.

C. Cloning and Sequencing of a cDNA-clone Encoding the 11S Species of Acetylcholinesterase. As described in reference 11, nucleotide probes to tandem sequences contained within a CNBr peptide were used to hybridize recombinants in a  $\lambda$ -gt 10 library. Positive clones were isolated, tested by hybridization and sequenced using M-13 sequencing vectors. Details are found in reference 11.

### Results

#### 1. Amino acid sequencing of Torpedo acetylcholinesterase

Our sequencing strategy is designed to fulfill several objectives:

- a. To obtain a sequence sufficient for the design of multiple nucleotide probes for c-DNA library sequencing.
- b. To employ the sequence to verify inferred amino acid sequence resulting for c-DNA sequencing and correlate the c-DNA sequences with the multiple acetylcholinesterase gene products.
- c. To elucidate differences in sequence between the molecular forms of acetylcholinesterase.

d. To identify critical regions in the molecule: active center, chemically modified residues, N-terminal sequence, C-terminal sequence, glycosylation sites, cysteine-containing peptides and other sites of post-translational modification.

e. To provide a peptide fractionation scheme by which other cholinesterases of lower abundance can be sequenced and homologous regions identified.

To date, we have sequenced about 80% of the tryptic peptides of the 11S enzyme and 30% of the tryptic peptides of the 5.6S enzyme. All of the CNBr peptides have been isolated from the 11S enzyme and ~30% sequenced. A smaller number have been sequenced in the 5.6S enzyme. The sequences are summarized in Table 1. Several findings should be highlighted:

a. Large tryptic peptides for the active center (24aa) were isolated and sequenced. Sequence was verified by chymotryptic digested and the position of this peptide in the whole enzyme could later be verified. The active center serine is serine 200. These peptides are identical in the 11S and 5.6S species (1) (fig. 1).

b. The N-terminal peptides of the 11S and 5.6S enzymes were identified and sequenced through 42 and 30 residues, respectively. These peptides were also identical in the 11S and 5.6S species. These sequences were later verified by the c-DNA sequence and, more important, a leader peptide was demonstrated for the unprocessed acetylcholinesterase. Cleavage occurred C-terminal to an Ala, giving rise to the N-terminal Asp residue in the processed protein. A candidate C-terminal tryptic peptide ending in leucine was also identified in the 11S species. That this peptide was a C-terminal tryptic peptide was later verified by finding a stop codon at amino acid position 575, which followed the leucine code and thus ended the open reading frame on our cloned cDNA. An analogous C-terminal peptide has not been found for the 5.6S enzyme, and we believe a posttranslational modification occurred here, providing one of the points of structural departure of the two enzyme forms.

c. The cysteine-containing peptides were identified by reduction and subsequent alkylation by [<sup>14</sup>C]iodoacetate. We obtained more cysteine peptides than would be predicted by the c-DNA sequence, but they arose simply from incomplete cleavages. All of these peptides can be placed in the inferred amino acid sequence on the basis of either their total sequence of their N-terminal residue identification and partial sequences. We have initiated fractionations of the unreduced enzyme with the essential aim of establishing the positions of the inter- and intrasubunit disulfid bridges. One of the eight cysteines appears to exist as a free sulfhydryl group and has been labeled with biamine in the 5.6S enzyme. Isolation and sequencing of the peptide reveals that the cysteine is at position 232.

Sites of glycosylation have been identified by lectin blotting of the individual peptides and by broad elution profiles that reflect microscopic heterogeneity within the peaks and their coalescence following endoglycosidase F treatment. An example is shown in fig. 2. Three of the four potential N-linked glycosylation sites have been located by peptide isolation (asparagine positions 56, 457, and 533), while it appears that position

TABLE I: Sequences of Torpedo californica Acetylcholinesterase Peptides\*

11S Acetylcholinesterase

I46 ivgywa2fa-c  
 I77 vpegcvfane-f-nnci  
 III88 fsivpvddgqfw(yst)k  
 II61 kpwsgvw-asnyp (carbohydrate  
 and CM cysteine)  
 II61 kpwigvwfhnypl  
 IV33 dnhsellvntksgkvmgtrvpvlsshisaf1  
 givfaeqgidv (N-terminal)  
 IV67 tvtifgesaggasvghilspgsr  
 (active site)  
 IV14 tgnpneptsqesk  
 IV26 le-ea  
 IV62 fgbgtyly-pdt--yr  
 IV63 ailqsg-vdcepa  
 I46 ivgywaa2fa-c  
 I77 vpegcvfane-f-nnci  
 VI87 vqvcwfnqflp  
 VII17 rpepk  
 V02 fidlntepmnk  
 V04 galqvwhdniqffggdpmk  
 I67 iteahh  
 II49 nlbbglncl-nsaelihicl  
 III65 -(av)dedcly-niwspgca  
 IV69 v-afalig  
 II67 l2vphandlgld(5)v(g)lqytdwmdnngik  
 IV26 hescael (c-term)  
 II61 kpw(i)gvw-as(n)yp1 (carbohydrate,  
 CM cysteine)  
 I46 ivgywa2fa-(c)I77  
 I77 v(p)vegcvfane(f)(lp)nnci  
 III88 f(s)ivpv(d)dgqfw(ystk)  
 II63 dglddivgbhnicplmhf  
 II62 kpwphawdlg-p  
 II64 lsvphandlgldvt  
 III01 dhn1vwpew-gvi(h)gyei--g-l-p  
 II68 lsvphandlgldtvglqytdwmd(ing)  
 4e7 immunoreactive)  
 I73 v(aph)vegcvfane(yf)(lp)(np)nc(f)-  
 (hg)v(ife)  
 II49 nlbbglncl-n sagglihicl (carbo)  
 III59 -(av)(sd)edcly-niw(s)pgca  
 IV57 ailqsgspncpwasvsv(aZg)r  
 VI87 vq(v)cxwfnqflp  
 I61 kpw(l)gvw(f)(h)(n)y(p)l  
 I73 v-vegcvfane--ncf--v(ip)g  
 III64 b-dedcly-niw-pgc  
 V65 ailqsgspncpwasvsv(azg)r  
 V01 tgnpnepshqesk  
 II54 lgvp-a  
 IV64 aieag  
 IV71 tvtifg-s

11S Acetylcholinesterase (includes CNBr  
 peptides)

bpd1 mwnpdre(p)  
 bpd2 mnfvsnypfgpgvlyflsieapd  
 bt1 mddnngiknrdglddivgdhnicplm  
 bt2 m-wfg-p-pepgkpwnv-was--y-n  
 bt3 mlntgnfkk(s)qillgvn(yk)(s)fgif(f)  
 lyga(v)(g3)f  
 bt4 mhvwatfaktgnpnepeg-(t)kwplifik-  
 (fq)-(e)  
 VI87 vq-cwfnqflp  
 VII17 rpepk  
 V01 tgnpnepshqesk  
 V02 fidlntepmnk  
 V03 ailqsgspncpwasvsv  
 V04 galqvwhdniqffggdpmk  
 III65 --dedcly-niw-pgc  
 II61 pw-gv--a--vpl  
 IV26 hescael (C-terminal peptide)  
 I77 v-vegcvfane--nnci  
 II01 d-n1vwpew-gvi-gy  
 II49 dlbbglncl-nsaeli--cl  
 II59 lgvpda  
 II67 l-vphandlgldtvglqytdwmd  
 III88 f-ivpv-dgqfw  
 IV14 tgnpnepshqesk  
 IV62 fgdgtyly  
 IV64 aieag  
 IV71 tvtifg-s  
 I46 ivgywa-fa  
 I67 iteah  
 IV69 v-afali

5.6 Acetylcholinesterase

IV18 tgnpnep  
 IX02 gpha-a  
 VI64 ail-e--pncpwatv-va  
 dnhsqllvntksgkvmgt (N-terminal)  
 tvtifgesaggasvghilspgsr (active  
 site)



416, despite the presence of an Asn, X, Ser/Thr, is not glycosylated. Overall carbohydrate compositions suggest that we may have an O-linked site, but this remains to be established.

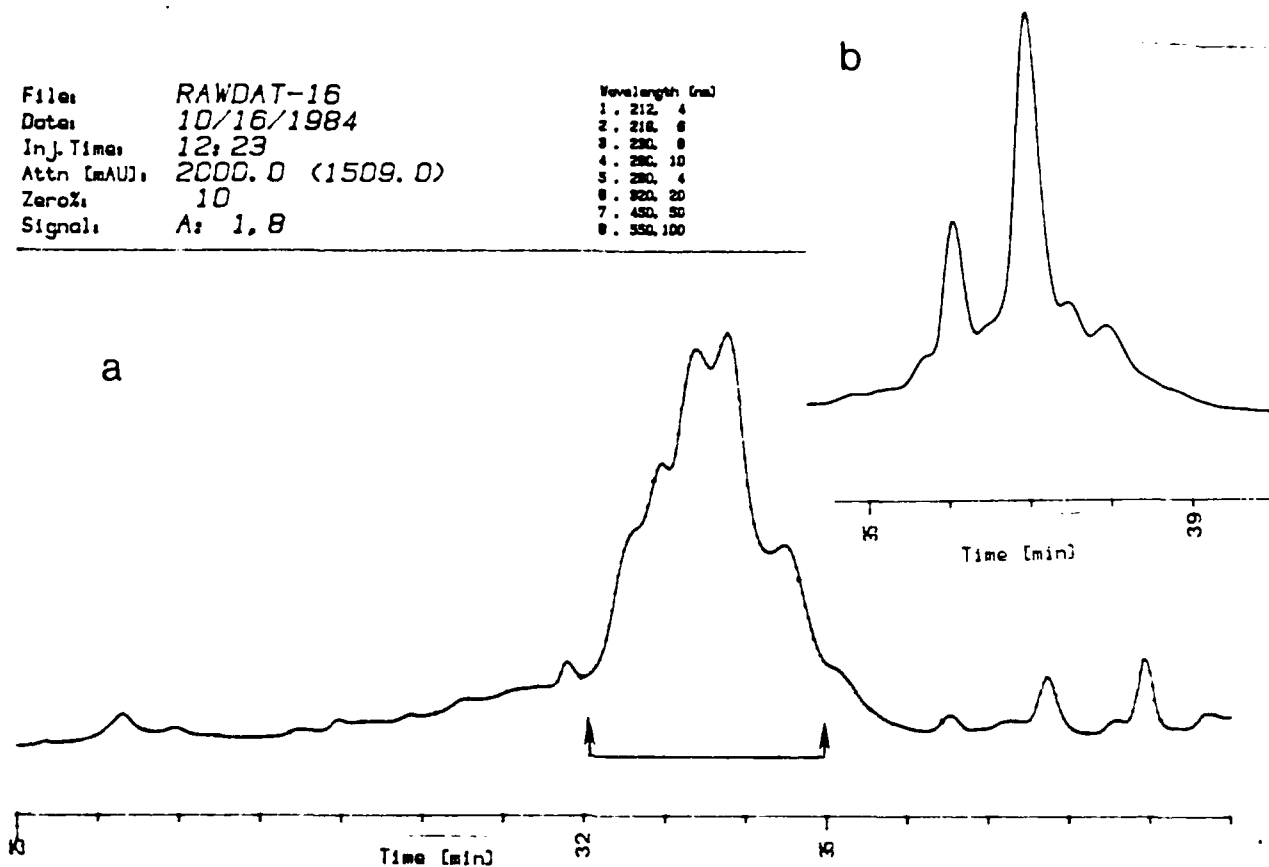


Figure 2: HPLC Profiles of 11S acetylcholinesterase peptides prior to (a) and following (b) endoglycosidase F treatment. Fractions 32-35 were isolated, lyophilized and treated with endoglycosidase F. The fractions were run on the same column (C-18 reverse phase), using an identical trifluoroacetic acid-H<sub>2</sub>O-acetonitrile gradient. The elution profile with its altered elution positions and decreased complexity is shown in the inset.

d. Potential sites that serve as epitopes for the monoclonal antibodies raised by B.P. Doctor have also been identified. The two of particular interest are 4E-7 and AE-2. 4E-7 reacts selectively with the 5.6S enzyme (2) and has been found to react only with the glycosylated form

of the enzyme. Treatment with endoglycosidase F but not endoglycosidase H eliminates the antigenicity. 4E-7 reacts equally well with the native and denatured enzyme. A peptide extending between residues 358 and 386 shows the greatest reactivity with 4E-7 as determined by antibody blotting and competitive immunoprecipitation. We expect this peptide to be one of those unique to the 5.6S enzyme.

The other antibody of interest is AE-2, an antibody isolated by Fambrough and colleagues (3) which shows considerable species cross-reactivity. AE2 was found to react with a peptide found by B.P. Doctor in fetal calf serum acetylcholinesterase. This peptide has been found between positions 12 and 18 and considerable homology between species exists in a large portion of this peptide (cf: Table III). Several other antibodies are less well characterized. However, some, such as 4G-7 and 2C-9, show high titers and good immunoprecipitation capacity.

The rather brief description given here describes the bulk of the studies performed during the past 2 years. Extensive fractionation and sequencing were required to achieve this state of progress for an enzyme subunit size of 575 amino acids which exists in multiple enzyme forms. These endeavors have been very much facilitated by the instrumentation provided in the contract. Our basic sequencing strategy was to reduce and alkylate the protein with [<sup>14</sup>C]iodoacetate and size-fractions were collected which were then subjected to reverse phase HPLC on C<sub>4</sub> columns, using an acetonitrile-1% aqueous trifluoroacetic acid gradient. Peptides that fractionated poorly on C<sub>18</sub> columns usually resolved well on C<sub>4</sub> columns. Compositions and N-terminals were ascertained before subjecting the peptides to gas phase sequencing. Profiles of some of the many fractionations can be found in MacPhee-Quigley et al. (9).

## 2. Preparation of antibodies directed to the active venter for acetylcholinesterase

Having obtained the active center peptide sequence, we then synthesized a 25 mer peptide to generate antibodies to the active center of acetylcholinesterase. The peptide was synthesized by the Merrifield solid phase methods, using Dr. Russell Doolittle's facility, Department of Chemistry, University of California, San Diego. An N-terminal lysine was added to promote solubility, giving the sequence:

Lys-Thr-Val-Thr-Ile-Phe-Gly-Glu-Ser-Ala-Gly-Ala-Ser-Val-Gly-Met-Ile-Leu-Ser-Pro-Gly-Ser-Arg.

Antibodies are being generated in three ways: Monoclonals are being raised by B.P. Doctor and M.K. Gentry at Walter Reed. The fusions are now complete, positives colonies have been selected, and we should be screening for precise titers and selectivity next month. Polyclonal antibodies are also being raised in rabbits at San Diego, using two forms of immunogens: the peptide dispersed in liposomes and the peptide conjugated to hemocyanin. The monoclonal antibodies will have the potential of obtaining isolated antibodies directed to small peptidic domains, some of which show little species cross-reactivity. Other, owing to extensive homology, will exhibit considerable species cross-reactivity. The polyclonal antibodies can be expected to have the higher titers and will prove most useful for screening in vitro translation products and the development of highly sensitive assays

for the active center of acetylcholinesterase. Antibodies to synthetic peptides have the advantage of not showing cross-reactivity to contaminant proteins in biological preparations.

### 3. Comparative sequencing of Torpedo and other cholinesterases

Our initial findings showing extensive homology of the active center peptides of Torpedo acetylcholinesterase and human butyrylcholinesterase (Table II) and the substantial homology in the N-terminal region of the two proteins prompted a further homology search in conjunction with Drs. Oksana Lockridge and Bert LaDu at the University of Michigan, and we see extensive similarity throughout the two molecules. Several peptides showing corresponding sequences can readily be found if our peptides (Table I) and their peptides are compared. The Michigan group also has about 80% of the peptides sequenced and with a total inferred sequence available in Torpedo, it should be possible for them to place the remaining peptides within the linear sequence.

TABLE II Sequences Of Active Site Regions

		5	P	10	15	20															
TORPEDO ACETYLCHOLINESTERASE	NH <sub>2</sub> -	<u>THR</u>	<u>VAL</u>	<u>THR</u>	<u>ILE</u>	<u>PHE</u>	<u>GLY</u>	<u>GLU</u>	<u>SER</u>	<u>ALA</u>	<u>GLY</u>	<u>GLY</u>	<u>ALA</u>	<u>SER</u>	<u>VAL</u>	<u>GLY</u>	<u>MET</u>	<u>HIS</u>	<u>ILE</u>	<u>LEU</u>	<u>SER</u>
EEL ACETYLCHOLINESTERASE							<u>GLY</u>	<u>GLU</u>	<u>SER</u>	<u>SER</u>	<u>GLU</u>	<u>GLY</u>	<u>ALA</u>	<u>ALA</u>	<u>GLY</u>						
HUMAN PSEUDOCOLINESTERASE	NH <sub>2</sub> -	<u>SER</u>	<u>VAL</u>	<u>THR</u>	<u>LEU</u>	<u>PHE</u>	<u>GLY</u>	<u>GLU</u>	<u>SER</u>	<u>ALA</u>	<u>GLY</u>	<u>ALA</u>	<u>ALA</u>	<u>SER</u>	<u>VAL</u>	<u>SER</u>	<u>LEU</u>	<u>HIS</u>	<u>LEU</u>	<u>LEU</u>	<u>SER</u>
EQUINE PSEUDOCOLINESTERASE						<u>PHE</u>	<u>GLY</u>	<u>GLU</u>	<u>SER</u>	<u>ALA</u>	<u>GLY</u>	<u>SER</u>	<u>ALA</u>	<u>ALA</u>							
EQUINE ALIESTERASE						<u>PHE</u>	<u>GLY</u>	<u>GLU</u>	<u>SER</u>	<u>ALA</u>	<u>GLY</u>	<u>ALA</u>	<u>ALA</u>	<u>SER</u>							
BOVINE TRYPSINOGEN		<u>LYS</u>	<u>ASP</u>	<u>SER</u>	<u>CYS</u>	<u>GLN</u>	<u>GLY</u>	<u>ASP</u>	<u>SER</u>	<u>GLY</u>	<u>GLY</u>	<u>PRO</u>	<u>VAL</u>	<u>VAL</u>	<u>CYS</u>	<u>SER</u>	<u>GLY</u>	<u>LYS</u>			
PORCINE TRYPSIN		<u>LYS</u>	<u>ASP</u>	<u>SER</u>	<u>CYS</u>	<u>GLN</u>	<u>GLY</u>	<u>ASP</u>	<u>SER</u>	<u>GLY</u>	<u>GLY</u>	<u>PRO</u>	<u>VAL</u>	<u>VAL</u>	<u>CYS</u>	<u>ASN</u>	<u>GLY</u>	<u>GLN</u>			
S. GRICEUS TRYPSIN		<u>VAL</u>	<u>ASP</u>	<u>THR</u>	<u>CYS</u>	<u>GLN</u>	<u>GLY</u>	<u>ASP</u>	<u>SER</u>	<u>GLY</u>	<u>GLY</u>	<u>PRO</u>	<u>MET</u>	<u>PHE</u>	<u>ARG</u>	<u>LYS</u>	<u>ASP</u>	<u>ASN</u>			
E. COLI ALKALINE PHOSPHATASE		<u>LYS</u>	<u>PRO</u>	<u>ASP</u>	<u>TYR</u>	<u>VAL</u>	<u>THR</u>	<u>ASP</u>	<u>SER</u>	<u>ALA</u>	<u>ALA</u>	<u>SER</u>	<u>ALA</u>	<u>THR</u>	<u>ALA</u>	<u>TRP</u>	<u>SER</u>	<u>THR</u>			

Human butyrylcholinesterase and Torpedo acetylcholinesterase can be expected to diverge on a phylogenetic basis and the basis of distinct enzymatic properties (i.e., the butyrylcholinesterase will accommodate substrates with large acyl groups, it does not show substrate inhibition and it is preferentially inhibited by different alkylphosphates). Therefore, one might expect that other mammalian acetylcholinesterases will possess structures showing structural divergence between these two limiting cases. In this regard, the fetal bovine serum acetylcholinesterase has proven useful. The trend in sequence divergence that we might expect can be seen in examining the N-terminal region of four cholinesterases (Table III). A more complete analysis of this mature should prove very useful in identifying various functional and antigenically cross-reactive regions.

Table III N-Terminal Sequences Of The Cholinesterases

	5	10	15	20	25	30	35	40	45	50	55	60	65																					
DNA SEQUENCE (42-8)	DDMS	ELLVNTKSG	AVMGT	RVPVLSSM	S	AF LG	P	A	P	P	G	N	R	FRRPEPEKRPB	SGYLA	A	S	T	Y	F	A													
TORPEDO (5.65)	D	E	L	L	V	N	T	K	S	G	A	V	M	G	T																			
TORPEDO (115)	DDMS	ELLVNTKSG	AVMGT	RVPVLSSM	S	AF LG	P	A	P	P	G	N	R	FRRPEPEKRPB	SGYLA	A	S	T	Y	F	A													
BOVINE FETAL SERUM AChE	EGPEDP	ELLV	VSG	EL	GL	L	H	A	P	R	G	P	V	S	A	F	L	G	I	V	F	A	P	D	Y	R	F	E	Y	Y	F			
HUMAN BuChE	EDDII	IATR	N	G	G	V	R	G	H	L	V	F	G	G	N	V	T	A	F	L	G	I	P	T	L	Q	V	L	G	A	V	L	A	D

4. Isolation of c-DNA clones encoding for acetylcholinesterase

Although this portion of the work was initiated and sustained with the support of the National Institutes of Health, the protein chemistry and molecular biological approaches are integrally linked, and it would have been impossible to proceed as rapidly without having both approaches in the same laboratory. Our library screening employed strategies that relies on hybridization with tandem but not overlapping probes, since we initially found that screening with a single probe yielded a very high incidence of false positives. When sequenced, the false positives were found to be repeating sequences of ~500 bp with rather good base matches (14 of the 17 bases in the mixed probe). The tandem probes eliminated this artifact and were preferable to using probes coding for separate peptides. The latter approach will miss short length sequences. The tandem probe approach usually requires that more amino acid sequence be known, since rather long peptidic stretches are usually required to minimize code redundancy in the probes. Positives to both tandem probes were then screened to a probe encoding for the N-terminal region. This reduced the number of positives and enhanced the likelihood of obtaining full-length inserts. By this approach we have now obtained 7 inserts which clearly encode for acetylcholinesterase and 13 more candidates. Their lengths and locations of Eco RI sites are detailed in fig. 3. Only lambda 2-4 (AChE-1) has been fully sequenced. The sequencing strategy (Fig. 4) and sequence (Fig. 5) are shown.

**Acetylcholinesterase c-DNA Clones**  
 (Eco R1 and Hind III Indicate Restriction Sites)

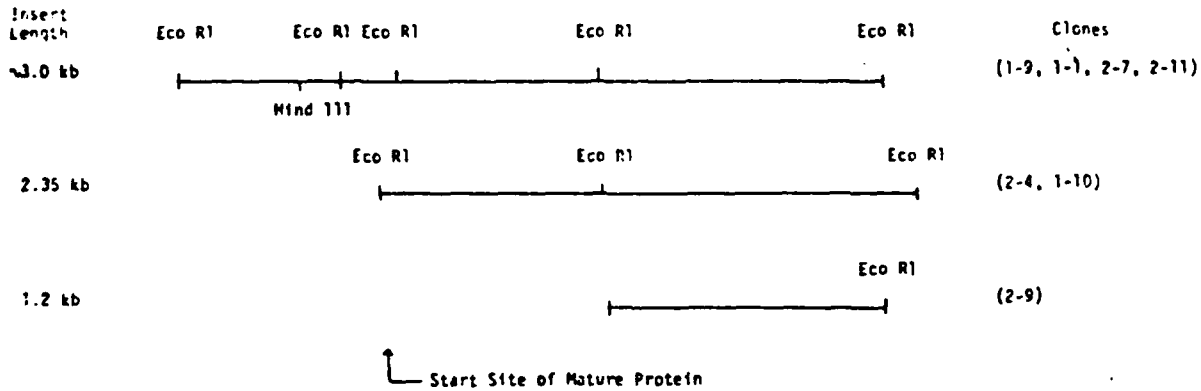


Figure 3: Characterization of several clones encoding for acetylcholinesterase. The length of the clones, Eco R1 and Hind III sites are shown.

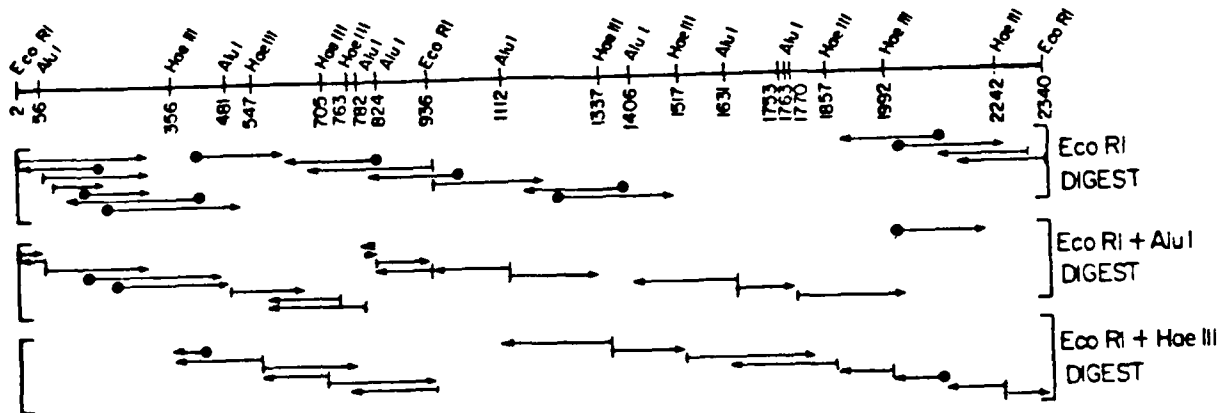


Figure 4: Sequencing strategy for acetylcholinesterase clone AchE-1 ( $\gamma$ 2-4). Critical restriction sites (Eco R1, Hae III, Alu I) and sequencing primer sites are shown.

14 31 44  
 8 GAT TCC GTG CTT CAG TTT GTC CTC TGC TGC CAG GCG GAC GAT CAC TCT  
 Aun Ser Val Leu Leu His Leu Val Val Leu Cys Gln Ala Asp Asp His Ser  
 41 76 91 106  
 GAG CTC CTC ACC ACC AAG TCG GGA AAA GTC ATG GCA ACA GCA GTC CCA GTC  
 Gln Leu Leu Val Aun Thr Lys Ser Gly Lys Val Met Gly Thr Arg Val Pro Val  
 121 136 151  
 CTC TCC ACC CAC ATC ACC GCT TTC CTG GGG ATT CCC TTT GCC GAG CTT CCA GTT  
 Leu Ser Ser His Ile Ser Ala Phe Leu Gly Ile Pro Phe Ala Gly Pro Pro Val  
 144 181 211  
 GGG AAC ATG AAG TTT AAG ACG CCT GAG CCC AAG AAA CCG TGG TCG GGA GTC TGG  
 Gly Aun Met Arg Phe Arg Arg Pro Gly Pro Lys Lys Pro Lys Ser Gly Val Trp  
 41 226 241 256  
 AAC GCT TCC ACC TAT CCC AAC AAC TGC CAG CAG TAC TTT GAC GAG CAG TTC CCT  
 Aun Ala Ser Thr Tyr Pro Aun Aun Cys Gln Gln Tyr Val Asp Gly Gln Phe Pro  
 271 286 301 316  
 GGA TTT TCA GAG ATG TGG AAT CCG AAC AGA GAG ATG AGT GAG GAC TGT  
 Gly Phe Ser Gly Ser Gly Met Trp Aun Pro Aun Arg Gly Met Ser Gly Asp Cys  
 331 346 361 376  
 TGG TAC CTC AAC ATT TGG GTG CCT TCT CCG AGG CCG AAG AGT ACA ACC GTC ATG  
 Leu Tyr Leu Aun Ile Trp Val Trp Val Leu Gly Val Val Leu Val Ser Thr Val Met  
 391 406 421  
 GTG TGG ATC TAC GGA GGC GGT TTC TAC AAC GCG TCC TCG ACG TTG GAC GTC TAC  
 Val Trp Ile Tyr Gly Gly Phe Tyr Ser Gly Ser Thr Leu Asp Val Tyr  
 436 451 466 481  
 AAT GGG AAA TAC CTT GCT TAC ACC GAG GAG GTG GTG CTG GTC TCT CTG AAC TAC  
 Aun Gly Lys Tyr Leu Ala Tyr Thr Gly Gly Val Val Leu Val Ser Leu Ser Tyr  
 496 511 526 541  
 CCG GTG GGC GCT TTT GGT TTT CTC GCC CTC CAC GGC AAC GAG GAG GCA CCA GGA  
 Arg Val Gly Ala Phe Gly Phe Leu Ala Leu His Gly Ser Gln Gly Ala Pro Gly  
 541 556 571 586  
 AAT GTG GGC CTC CTG GAC CAG AAG ATG GCA CTG GAG TGG GTG CAC GAC AAC ATC  
 Aun Tyr Gly Leu Asp Gln Arg Met Ala Leu Gln Trp Val His Asp Aun Ile  
 601 616 631 646  
 CAG TTC TTC GGC GGC ACC AAG ACG GTG ACC ATC TTC GGA GAG AGT GCC GGC  
 Gln Phe Phe Gly Gly Asp Pro Lys Thr Val Thr Ile Phe Gly Gly Ser Ala Gly  
 185 661 676 691  
 GGC GCC TCT GTC GGC ATG CAC ATT CTC TCC CCG GGG AAC CCA GAC CTC TTC GCG  
 Gly Ala Ser Val Gly Met His Ile Leu Ser Pro Gly Ser Arg Asp Leu Phe Arg  
 706 721 736 751  
 CCG GCC ATC CTT CAG AGC GTC TCG CCC AAT TGC CCG TGG GCG TCT GTC TCT GTT  
 Arg Ala Ile Leu Gln Ser Gly Ser Pro Aun Cys Pro Trp Ala Ser Val Ser Val  
 766 781 796 811  
 GCT GAA GGC CCG AAG AAG GCG GTC GAG CTG GGA AGA AAC CTC AAC TGT AAC CTC  
 Ala Gly Gly Arg Arg Ala Val Gly Leu Gly Arg Aun Leu Aun Cys Aun Leu  
 811 826 841 856  
 AAC GGC GAA GAG CTC ATC CAC TGT CTG AGG GAA AAG AAG CCT CAG GAG TTB  
 Aun Ser Asp Gly Gln Leu Ile His Cys Leu Arg Gly Lys Pro Gln Gly Leu  
 871 886 901 916  
 ATT GAC GTG GAG TGG AAT GTC CTT CCC TTT GAC AGT ATC TTC ARG TTC TCC TTC  
 Ile Asp Val Gly Trp Aun Val Leu Pro Phe Asp Ser Ile Phe Arg Phe Ser Phe  
 931 946 961  
 GTT CCC GTC ATC GAT GGG GAA TTC TTC CCA ACC TCC CTG GAA TCT ATG TTB AAC  
 Val Pro Val Ile Asp Gly Gly Phe Phe Pro Thr Ser Leu Gly Ser Met Leu Aun  
 976 991 1006 1021  
 TCT GTC AAC TTC AAG AAG CAG ATC TTA CTG GGA GTC AAC AAG GAC GAG GGC  
 Ser Gly Aun Phe Lys Thr Gln Ile Leu Leu Gly Val Aun Lys Asp Gly Gly  
 1034 1051 1066  
 TCG TTT TTC CTC TTT TAC GGA GCG CCG GGT TTC ACG AAG GAC TCT GAA ACG AAG  
 Ser Phe Phe Leu Leu Tyr Gly Ala Pro Gly Phe Ser Lys Asp Ser Gly Ser Lys  
 329

1081 1096 1111 1126  
 ATC TCT CCG GAA GAC TTC ATG TCA TCG GGG GTC AAG CTA AGC GTT CCC CAC GGC AAT  
 Ile Ser Arg Gly Asp Phe Met Ser Gly Val Lys Leu Ser Val Pro His Ala Aun  
 347  
 1141 1156 1171 1186  
 GAC TTA GCG TTT GAC GCT GTC ACB CTA CAG TAC ACA GAC TGG ATG GAT GAC AAC  
 Asp Leu Gly Leu Asp Ala Val Thr Leu Gln Tyr Thr Asp Trp Met Arg Asp Ser  
 365  
 1201 1216 1231  
 AAT GGT ATA AAG AAC AGA GAT GGA TTB GAC ATC GTA GGG GAC CAC AAC GTC  
 Aun Gly Ile Lys Aun Arg Asp Gly Leu Asp Asp Ile Val Gly Asp His Aun Val  
 383  
 1246 1261 1276 1291  
 ATA TCC CCC TTT ATG CAC TTT GTT AAC AAG TAC ACC AAG TTT GGC AAT GGC ACC  
 Ile Cys Pro Leu Met His Phe Val Aun Lys Tyr Thr Lys Phe Gly Aun Gly Thr  
 401  
 1306 1321 1336  
 TAC CTG TAC TTC AAC CAC CCA GCG TCA AAC CTG GTG TGG CCG GAG TGG ATG  
 Tyr Leu Tyr Phe Phe Aun His Arg Ala Ser Aun Leu Val Trp Pro Gly Trp Met  
 419  
 1346 1361 1376 1391  
 GTC GTC ATC CAC GGC TAT GAG ATT GAG TTC GTC GGG CTG CCT CTG GTG AAG  
 Gly Val Ile His Gly Tyr Gly Ile Gly Phe Val Phe Gly Leu Pro Leu Val Lys  
 437  
 1411 1426 1441 1456  
 GAG CTG AAC TAC ACA GCG GAG GAA GCG CTG ACG CCG AOB ATA ATG CAT TAC  
 Gly Leu Aun Tyr Thr Ala Gly Gln Gly Ala Leu Ser Arg Arg Ile Met His Tyr  
 455  
 1471 1486 1501  
 TGG GCG ACA TTC GCA AAG ACT GGA AAC CCA AAC GAA CCC CAC TCA CAG GAG ACG  
 Trp Ala Thr Phe Ala Lys Thr Gly Aun Pro Aun Gly Pro His Ser Gln Gly Ser  
 473  
 1516 1531 1546 1561  
 AAA TGG CCT CTC TTC ACT ACC AAG GAG CAG AAA TTT ATT GAC CTC AAC ACA GAA  
 Lys Trp Pro Leu Phe Thr Lys Gly Gln Lys Phe Ile Asp Leu Aun Thr Gly  
 491  
 1576 1591 1606  
 CCC ATG AAA GTC CAC CAG CGA CTC CGA GTT CAG ATG TGC GTG TTC TGG AAC CAG  
 Pro Met Lys Val His Gln Arg Leu Arg Val Gln Met Cys Val Phe Trp Aun Gln  
 509  
 1621 1636 1651 1666  
 TTC CTC CCC AAG CTC CTC AAC GCC ACA GAG ACC ATT GAT GAG GCA GAA CCG CAG  
 Phe Leu Pro Lys Leu Leu Aun Ala Thr Gly Thr Ile Asp Gly Ala Gly Arg Gln  
 527  
 1681 1696 1711 1726  
 TGG AAG ACG GAG TTT CAT CCG TGG AGT TCC TAC ATG ATG CAC TGG AAG AAC CAA  
 Trp Lys Thr Gly Phe His Arg Trp Ser Tyr Met Met His Trp Lys Aun Gln  
 545  
 1741 1756 1771 1786  
 TTT GAC CAC TAC ACC ADA CAC GAG ACG TGT GCT GAG CTG TGA GCTCTGCTCT GCGAGTCCG  
 Phe Asp His Tyr Ser Arg His Gly Ser Cys Ala Gly Leu  
 563  
 1808 1818 1838 1848 1858  
 TGGTGAAGCA GAGAGCAGAG TCGGATATTG ACCAGACAC CAGTCTAGTT CCTGGAGACC CTGCTTGGCC  
 1868 1878 1898 1908 1918 1928  
 CTCGTGAGCCC CCGCCCGCCCA CCGCCGCAAA CTCGCTTTC GAGACTGCTT CCTTCCGATCA  
 1938 1948 1958 1968 1978 1988 1998  
 CCGCAGGCTG CCGAGCTGCT CTCGCGACC GTCAGCTGTA AATCTGCGCC ACCAATCTTT CCGCCACCCA  
 2008 2018 2028 2038 2048 2058 2068  
 CTCCTGACAC AGCCCAACT TCCTCTCTCT CTCGCCACC ACTACCCCCC TATTACCCAC TCCTCCACTT  
 2078 2088 2098 2108 2118 2128 2138  
 CCGCAGCTTC TCCTCCGATA CCGTCAGCA CCGATTCGAA CCGCCGATTC TCCTCTCTCT CAGCTATTGA  
 2148 2158 2168 2178 2188 2198 2208  
 CCGTCTACCC CCGCCCGCTC ACTCTGTCTC CCGAAGACT CCGTCCCTCT TCATCCGAGA GTTCCCAACT  
 2218 2228 2238 2248 2258 2268 2278  
 CCGCAGGAGA CTTCTGCTCT CTTCTCTAGT TCGCCCTTCA CCGTGTACAAA TCCTGGGTGAG ACCCAATATG  
 2288 2298 2308 2318 2328 2338 2348  
 ATCTGCTGT ATTCTGCTCT TTATCCAGTT TCCTTGGCCA ATCATTTATAT CCGCCACTC GAAATC

Figure 5: c-DNA and inferred amino acid sequence of clone 2-4.

The insert begins with a 16 amino acid leader peptide, extends through the 575 amino acids of the processed protein, and contains another 570 bases in a 3' noncoding region. It does not contain a poly A termination or a canonical poly A initiation signal, which suggests that this 3' region is not complete (4). Clone 2-4 was sequenced in M-13 by the dideoxy method. Protein sequence, again, provided confirmation that the selected open reading frame was correct and did not diverge due to a skipped base. In addition, the protein sequence enabled us to establish that the clone likely encoded for the 11S species. We are sequencing the other clones based on initial findings, are optimistic that we have found a clone for another acetylcholinesterase species. There is one caveat: The fact that all clones end in EcoR-1 sites suggests incomplete methylation in the library preparation. Clones 1-1, 1-9 and 1-10 are probably identical but reflect another gene of acetylcholinesterase. They are being sequenced. Clone 1-9, which is nearly 2.9 kb in length, is our candidate for obtaining complete 5' and 3' noncoding regions. Clone 2-9 is probably a shortened version of 1-9, terminating at the EcoR-1 site. In short, valuable information will continue to accrue as we compare c-DNA inferred sequences with actual protein sequences. Accordingly, the combination of molecular biology and protein chemistry should enable us to identify all of the structural polymorphisms in Torpedo acetylcholinesterase.

5. General aspects of acetylcholinesterase structure deduced from amino acid and nucleotide sequencing

All of the above data enable us to arrive at the following conclusions:

a. Acetylcholinesterase contains a hydrophobic leader sequence (residues -13-0) but contains no other hydrophobic domains which are candidates for membrane-spanning regions. Thus it is likely to be an exported protein and its membrane attachment site(s) arises as a consequence of posttranslational modifications.

b. The active center serine is at residue 200. The N-terminal location contracts with the serine proteases of similar size that function in the clotting cascade (i.e., factor IX and prothrombin).

c. No significant global or local homology is found with the acetylcholine receptor.

d. Although acetylcholinesterase is closely homologous to human butyrylcholinesterase, no significant global homology and very limited local homology are found with other serine proteases: the largest local homology is seen with liver aliesterase and the carboxylesterases.

e. Substantial homology is found between acetylcholinesterase and thyroglobulin in their C-terminal regions (acetylcholinesterase residues 1-575; thyroglobulin residues 2168-2750). Six of the eight cysteines are conserved, suggesting a similar folding pattern for the two macromolecules. The region between 160 and 190 shows greater than 60% identity.

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