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Mutagenesis of essential functional residues in acetylcholinesterase

(esterase mutants/serine hydrolases/site-directed mutagenesis)

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ABSTRACT **The cholinesterases are serine hydrolases that show no global similarities in sequence with either the trypsin or the subtilisin family of serine proteases. The cholinesterase superfamily includes several esterases with distinct functions and other proteins devoid of the catalytic serine and known esterase activity. To identify the residues involved in catalysis and conferring specificity on the enzyme, we have expressed wild-type *Torpedo* acetylcholinesterase (EC 3.1.1.7) and several site-directed mutants in a heterologous system. Mutation of serine-200 to cysteine results in diminished activity, while its mutation to valine abolishes detectable activity. Two conserved histidines can be identified at positions 425 and 440 in the cholinesterase family; glutamine replacement at position 440 eliminates activity whereas the mutation at 425 reduces activity only slightly. The assignment of the catalytic histidine to position 440 defines a rank ordering of catalytic residues in cholinesterases distinct from trypsin and subtilisin and suggests a convergence of a catalytic triad to form a third, distinct family of serine hydrolases. Mutation of glutamate-199 to glutamine yields an enzyme with a higher K_m and without the substrate-inhibition behavior characteristic of acetylcholinesterase. Hence, modification of the acidic amino acid adjacent to the serine influences substrate association and the capacity of a second substrate molecule to affect catalysis.**

Efficiently catalyzed hydrolysis of the neurotransmitter acetylcholine is critical to proper functioning of the cholinergic nervous system, and several pharmacologic and toxicologic agents act by inhibiting acetylcholinesterase (AChE; acetylcholine acetylhydrolase, EC 3.1.1.7). AChE functions as a serine hydrolase (1–3) and enzyme inhibitors used clinically or as insecticides typically serve as alternative substrates by acylating the active-center serine with slower leaving groups. The primary structure of AChE revealed that it lacks global sequence similarities with serine hydrolases of the trypsin and subtilisin families and only possesses residue identity with trypsin immediately around the active-center serine (4). Surprisingly, AChE is homologous to the carboxyl-terminal domain of a large secreted glycoprotein, thyroglobulin, the precursor to thyroid hormone (4). Subsequent primary structures showed that the cholinesterases belong to a distinct, but functionally eclectic, family of serine hydrolases. Included in this family are the butyrylcholinesterases, hepatic microsomal carboxylesterases, the *Drosophila* Est-6, lysophospholipases, cholesterol esterases, and two proteins found in inclusion bodies of *Dictyostelium* (5–11).

The cholinesterases are characterized by their specificity for choline esters but can be subdivided on the basis of acyl group selectivity. The true AChEs (EC 3.1.1.7) show weak catalytic activity for choline esters with acyl groups larger than propionylcholine; the butyrylcholinesterases (EC 3.1.1.8) efficiently hydrolyze esters with larger acyl groups (2, 12). These enzymes can also be distinguished by inhibitor specificity. In addition, the butyrylcholinesterases do not exhibit substrate inhibition characteristic of AChEs (12).

To further define the catalytic residues of the cholinesterases, we mutated several amino acids expected to be critical for function. This has enabled us to delineate some of the residues involved in catalysis and substrate inhibition.

MATERIALS AND METHODS

Mutagenesis and Expression Vectors. All manipulations of nucleic acid sequences were performed using commercially available reagents and standard recombinant DNA techniques (13, 14). The cDNA construction encoding the glycophospholipid-anchored form of *Torpedo californica* AChE (hydrophobic AChE, H-AChE) is described elsewhere (15, 16). Mutant sequences were generated by oligonucleotide-directed substitutions of M13 templates using MutaGene (Bio-Rad). The entire coding sequence of each mutant was confirmed by dideoxy sequencing (Sequenase, United States Biochemical). Wild-type and mutant AChE cDNAs were inserted into the simian virus 40-derived expression vector pcD (17) at *Bam*HI sites flanking the cloning region. Expression plasmids, amplified in *Escherichia coli* DH5, were purified by two sequential bandings in cesium chloride/ethidium bromide gradients.

Expression in Mammalian Cells. COS-1 cells (American Type Culture Collection CRL 1650) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 10% CO_2, humidified atmosphere.

Cells were plated at 10^6 per 100-mm plate 16–20 hr before transfection. Plasmid DNA (5 μg) was precipitated in ethanol, resuspended at 125 μg/ml in Tris-buffered saline (TBS: 137 mM NaCl/5.13 mM KCl/0.025 mM Na_2HPO_4/1.35 mM $CaCl_2$/1.05 mM $MgCl_2$/24.8 mM Tris·HCl, pH 7.5) and added to 2 volumes of DEAE-dextran solution (10 mg/ml in TBS) that was prewarmed to ≈50°C. The transfection mixture, diluted 33-fold in serum-free DMEM, was applied to cells rinsed in serum-free DMEM. After incubation at 37°C for 4 hr and aspiration of the transfection medium, cells were subjected to a 3-min shock in DMEM containing 10% (vol/vol) glycerol. Following two rinses in phosphate-buffered isotonic saline (PBS), DMEM/10% fetal bovine serum was added. For replicate platings, cells were seeded at 2.5 × 10^6 per 150-mm plate, transfected with 12.5 μg of plasmid DNA, and subcultured 16–24 hr later onto 100-mm dishes.

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Abbreviations: AChE, acetylcholinesterase; H-AChE, hydrophobic AChE.

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Cells were transferred from 37°C to a 28°C incubator 24–36 hr after transfection. All subsequent procedures were carried out within 6–36 hr of incubation at 28°C.

Measurement of AChE Activity of Transfected Cells. AChE purified from *T. californica* electric organ as a 5.6S species (18) was the reference for comparing catalytic parameters. AChE activity was assayed by the method of Ellman *et al.* (19) 48–72 hr after transfection. Cells transfected on 100-mm plates were incubated at 28°C for the final 24 hr. Plates were then placed on ice and rinsed twice with ice-cold PBS. Cells were scraped from the plates, sedimented in an Eppendorf microcentrifuge at 3000 rpm for 1 min at 4°C, and resuspended twice in 0.2 ml of PBS. Final resuspension was in 0.2 ml of PBS with 1% (vol/vol) Triton X-100 for measurement of total cellular activity. Unless specified, activity was measured at pH 8.0 in the presence of 0.5 mM acetylthiocholine.

Metabolic Labeling and Immunoprecipitation. For labeling with [^{35}S]methionine (≈1000 Ci/mmol; Trans^{35}S-label, ICN; 1 Ci = 37 GBq); transfected cells were rinsed with PBS and incubated for 1 hr in methionine-free DMEM supplemented with 1.5% bovine serum albumin, then for 1 hr with [^{35}S]methionine (100 μCi per 100-mm plate) in 2 ml of DMEM with 1.5% albumin.

After labeling, cells were placed on ice, rinsed in PBS, and harvested in 1.0 ml of lysis buffer (100 mM NaCl/0.5% sodium deoxycholate/0.1% SDS/1% Triton X-100/0.01% NaN_3/5 mM EDTA/1 mM phenylmethanesulfonyl fluoride/10 mM sodium phosphate, pH 7.4). Samples were immunoprecipitated twice using a polyclonal antibody to 11S *Torpedo* AChE (15) and protein A-Sepharose CL-4B (Pharmacia). Immunoprecipitates were reduced with 10 mM dithiothreitol, electrophoresed in the presence of SDS in 10% polyacrylamide gels, subjected to fluorography, and quantitated by scanning densitometry.

RESULTS

Expression of the Wild-Type and Mutant AChE in COS Cells. Heterologous expression of the glycophospholipid-linked form of AChE resulted in over 80% of AChE expressed on the outer cell surface (15). Retention of AChE on the cell surface provided a convenient means for monitoring total synthesis. Release of AChE from intact cells by treatment with phosphatidylinositol-specific phospholipase C and incorporation of [^{3}H]ethanolamine into the expressed enzyme were also indicative of processing by addition of glycophospholipid (15).

As previously found for the acetylcholine receptor from *Torpedo* (20), expression of active AChE from this poikilotherm was at least 10-fold greater at 28°C even though the expression of enzyme protein as detected by anti-AChE antibody was slightly greater at 37°C (15). Thus, expression was maximized by transfection at 37°C and, after 24–36 hr, transfer of cells to an incubator at 28°C.

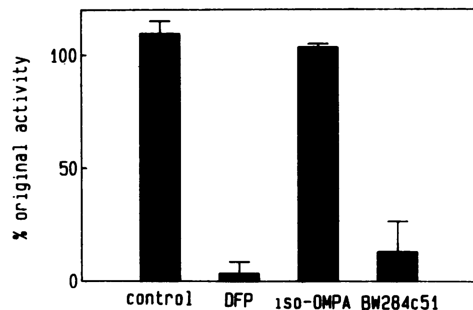

FIG. 1. Sensitivity of expressed H-AChE to cholinesterase inhibitors. Aliquots of cells transfected with SV-H were incubated in PBS or in PBS with 0.1 mM diisopropyl fluorophosphate (DFP), 0.1 mM tetraisopropylphosphoramide (iso-OMPA), or 0.1 mM BW284c51 for 20 min at room temperature. Cell-surface AChE activity retained after incubation is related to the activity of untreated cell aliquots held on ice. Data represent the mean ± SEM of triplicate measurements in two independent experiments.

Table 1. Oligonucleotides used in site-directed mutagenesis of H-AChE

Mutation	Wild-type codon	Mutagenic oligonucleotide
Ser^{200} → Val	AGT	CTTCGGAGAG<u>GTT</u>GCCGGCG
Ser^{200} → Cys	AGT	CTTCGGAGAG<u>TGT</u>GCCGGCG
His^{425} → Gln	CAC	TTCTTCAAC<u>CAA</u>CGAGCC
His^{440} → Gln	CAC	GCGTCATC<u>CAA</u>GGCTATG
Glu^{199} → Gln	GAG	ATCTTCGGA<u>CAG</u>AGTGCCGGC
Glu^{199} → Asp	GAG	ATCTTCGGA<u>GAT</u>AGTGCCGGC
Glu^{199} → His	GAG	CCATCTTCGGA<u>CAT</u>AGTGCCGGC

Mutant codons are underlined.

Activity in COS cells transfected with wild-type cDNA (SV-H) exhibited the expected susceptibility to cholinesterase inhibitors (Fig. 1). Activity was abolished by 0.1 mM diisopropyl fluorophosphate, greatly diminished by the AChE-specific reversible inhibitor BW284c51 at 0.1 mM, but relatively insensitive to 0.1 mM tetraisopropylphosphoramide, a specific inhibitor of butyrylcholinesterase.

Table 1 shows the mutations studied and the mutagenic oligonucleotides used to create them. Sequencing the mutants in their entirety showed that mutagenesis generated two point mutations in addition to the primer-directed changes. The H440Q mutant also replaces a guanine with adenine at base 1338. This mutation changes the third base of the Gly^{335} codon but does not alter the translated sequence. E199H is altered at base 169, which results in substitution of threonine for Ala^{36}. All other sequences contain only the designed mutations.

Typically, transfected cells were subcultured onto four replicate plates; one plate was incubated with [^{35}S]methionine within 6–18 hr after transfer to 28°C. A representative autoradiogram of ^{35}S-labeled immunoprecipitated protein is shown in Fig. 2. The remaining plates were maintained at 28°C for 24 hr, after which activity was measured. Table 2 summarizes the results of activity measurements and quantitation of immunoprecipitated AChE.

Activity was abolished as expected when the active-center serine [as identified both by labeling with diisopropyl fluorophosphate and by similarity of surrounding residues (ref. 4; see also Table 3)] was replaced with valine. Substitution of

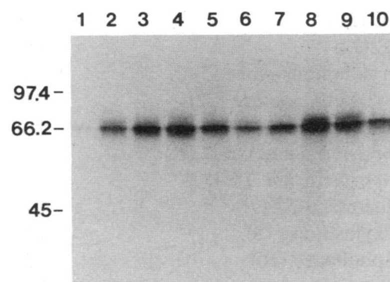

FIG. 2. Antibody precipitation of wild-type and mutants of H-AChE protein. COS cells were subcultured onto replicate plates 22 hr after transfection and transferred 8 hr later to an incubator at 28°C. Forty-six hours after transfection, one plate of cells from each transfection was labeled with [^{35}S]methionine for 1 hr and immunoprecipitated. The remaining replicate plates were held at 28°C for measurements of activity. The results of SDS/PAGE of the reduced immunoprecipitates are shown. The indicated lanes on the autoradiogram correspond to cells transfected with SV (vector alone) (lane 1), SV-H (lane 2), SV-H S200V (lane 3), SV-H S200C (lane 4), SV-H H425Q (lane 5), SV-H H440Q (lane 6), SV-H H425Q, H440Q (lane 7), SV-H E199Q (lane 8), SV-H E199D (lane 9), and SV-H E199H (lane 10). Molecular size markers (kDa) are at left.

Table 2. Activities associated with transfection of wild-type and mutant H-AChE sequences

Mutant	Activity	[35S]AChE	Activity/35S
Wild type	100	1.0	100
S200V	<0.1	2.4 ± 0.2	—
S200C	1.2 ± 0.6	3.7 ± 0.8	0.3 ± 0.2
H425Q	59 ± 6	1.3 ± 0.2	52 ± 11
H440Q	<0.1	0.8 ± 0.1	—
H425Q, H440Q	<0.1	1.1 ± 0.3	—
E199Q	50 ± 15	2.9 ± 0.3	17 ± 5
E199D	23 ± 9	2.8 ± 0.7	9 ± 5
E199H	<0.1	2.9 ± 1.9	—

Mutant names are based on one-letter amino acid symbols (e.g., S200V represents $Ser^{200} \rightarrow Val$). "Activity" is the mean ± SEM, expressed as percent of wild type, from two or three replicate plates from three transfections. Activity was measured with 0.5 mM acetylthiocholine. "[35S]AChE" is the relative mean ± SEM of AChE radioactivity immunoprecipitated from a replicate plate from each transfection. "Activity/35S" is each activity normalized to its ^{35}S-signal value, reported as the mean ± SEM of the three transfections of separate plates.

the sulfhydryl amino acid cysteine resulted in a major reduction, but not complete abolition, of activity (Table 2).

Two histidines at positions 425 and 440 are conserved throughout the family of cholinesterases and many homologous serine hydrolases with esterase activity. In some of the shorter esterases, only one histidine is found in this region (6–11), but it is difficult to ascertain its positional reference to AChE (Table 3). The essential histidine appears at position 440 in AChE, since its mutation to glutamine resulted in abolition of activity (≤0.1% of wild-type expression) whereas 50–60% of the activity was retained with a His → Gln substitution at position 425. The apparent K_m of the H425Q mutant also did not differ appreciably from that of the native enzyme (Table 4). Mutation of both histidines resulted in undetectable activity.

Mutations at position 199, the glutamic residue that is amino-terminal to the active-site serine, yielded enzymes of diminished activity. No activity was detected with a Glu → His

Table 3. Related sequences in the vicinity of the active-center serine

Enzyme (ref.)	Sequence
Serine esterases	
Torpedo AChE (4)	TIFGESAGGASV
Bovine AChE (21)	TLFGESAGAASV
Mouse AChE (22)	TLFGESAGAASV
Human butyrylcholinesterase (5)	TLFGESAGAASV
Mouse butyrylcholinesterase (22)	TIFGESAGAASV
Drosophila cholinesterase (23)	TLFGESAGSSSV
Dictyostelium esterosome CP (11)	TIYGESAGAFSV
Dictyostelium esterosome D2 (11)	TIWGESAGAFSV
Rat carboxylesterase E1 (7)	TIFGESAGGVSV
Rabbit carboxylesterase (8)	TIFGESAGGQSV
Rat lysophospholipase (10)	TIFGESAGGAIV
Drosophila Est-6 (6)	LLVGHSAGGASV
Serine proteases, trypsin family	
Bovine trypsin (24)	SCQGDSGGPVVC
Drosophila trypsin-like (24)	ACQGDSGGPLVS
Streptomyces trypsin-like (24)	TCQGDSGGPMFR
Hornet chymotrypsin (24)	ACHGDSGGPLVA
Crab collagenase (24)	TCDGDSGGPLNY
Pig elastase (24)	GCQGDSGGPLHC
Serine proteases, subtilisin family	
Subtilisin BPN (24)	AYNGTSMASPHV

The serine hydrolases are divided into three families and sequences around the active-center serine (boxed; Ser^{200} in *Torpedo*) are shown.

Table 4. Estimated K_m values for separate transfections of wild-type and mutant AChE

Enzyme	$K_m \times 10^5$, M
Electric organ	13
Wild type	9.8, 9.2, 10.5
H425Q	5.0, 5.4, 8.2, 10.3
E199Q	64, 63, 77, 50

K_m values were estimated from Eadie–Hofstee plots and represent separate cell transfections. The electric organ enzyme was the 5.6S species purified from *T. californica*. Activity from the wild-type and mutant enzymes was measured in Triton X-100 extracts from transfected COS cells.

substitution despite a histidine being found at this position in a homologous esterase, Est-6 (ref. 6 and Table 3). However, a nearly isosteric substitution of glutamine or substitution of a dicarboxylic amino acid, aspartic acid, showed retention of activity (Table 2). The E199Q enzyme exhibited the greater of the two activities and its catalytic parameters were studied in more detail. The pH dependence of activity in the presence of saturating substrate for the wild-type and H199Q mutant did not differ, showing a maximum near pH 8.0 (data not shown).

Substitution of glutamine for Glu^{199} produced a marked effect on the substrate concentration dependence for catalysis. This was reflected in an increase of the apparent K_m of the enzyme (Table 4) and a loss of substrate inhibition typical of AChE (Fig. 3). The substrate concentration dependence for SV-H is indistinguishable from that of the hydrophobic form of the enzyme purified from *Torpedo* (data not shown).

DISCUSSION

Analysis of conserved residues in the cholinesterase family in conjunction with the assignment of a functional role for specific residues in AChE reveals several interesting features for these proteins. Substitution of cysteine for the active-center serine at position 200 results in retention of activity, albeit substantially reduced. Hence, the AChE structure can function as a cysteine hydrolase. Similar substitutions in the trypsin and subtilisin families of proteases, initially by chemical modification (25, 26) and subsequently by site-specific mutagenesis (27), yielded enzymes with substantially reduced esterase and protease activities. Protease activity was reduced by nearly 6 orders of magnitude, while esterase activity using an activated ester, N^{ϵ}-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester, as a substrate was 3% that of the native enzyme. The reduction of cholinesterase activity for choline esters appears to be of similar magnitude, although low expression with a eukaryotic expression vector precludes purification of sufficient enzyme for a detailed kinetic analysis. Owing to the high turnover of native AChE, $k_{cat} = 3 \times 10^5\ min^{-1}$ (2), the thiol-substituted AChE still possesses a reasonably high catalytic efficiency for ester hydrolysis.

Retention of activity with cysteine at the active center is not unexpected, since papain, a naturally occurring cysteine hydrolase, maintains a catalytic triad of Cys^{25}, His^{159}, and Asn^{175}. The $\epsilon 2$ and $\delta 1$ imidazole nitrogens are within hydrogen-bonding distances of the Asn^{175} and Cys^{25} side chains, respectively (27, 28). Moreover, a series of viral cysteine proteases appear to be homologous with the trypsin family of eukaryotic serine proteases (29). Positions of functional residues in the catalytic triad are identical between the viral 2a and 3c families and the small and large serine proteases, respectively (29). The viral proteases contain an aspartic acid instead of the asparagine found in papain for the active-center triad. The role of the aspartic residue in stabilizing the tautomer of the imidazole necessary for the proton transfer in the enzyme has been strengthened by x-ray studies showing the D102N mutant of trypsin to be inactive, yet the asparagine residue exhibits no change in position in the crystal

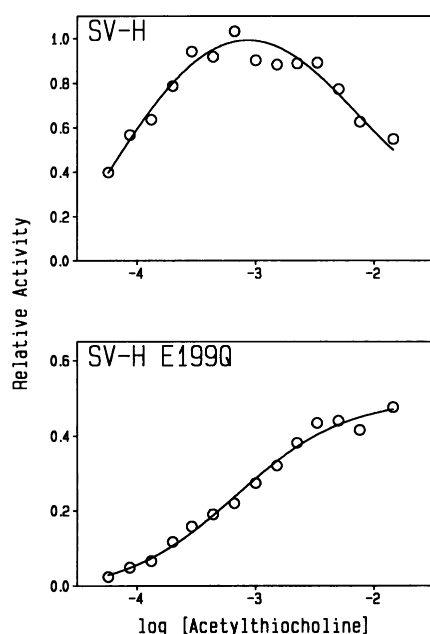

FIG. 3. Substrate concentration dependencies for catalysis of acetylthiocholine by wild-type (*Upper*) and E199Q (*Lower*) AChE. Relative activities shown on the ordinate have been normalized to the specific activities found in comparable experiments when immunoreactive protein was measured (cf. Table 2).

structure (30). However, it has been suggested on the basis of proton inventories that an involvement of the aspartic residue in a charge-relay or proton-shuttle mechanism within the catalytic triad may differ between the cholinesterase family and the trypsin or subtilisin families of proteases (31, 32). The location of the putative aspartic residue in the sequence potentially fulfilling this role is unknown.

Mutagenesis of the conserved histidines implicates His^{440} in the catalytic mechanism. Indirect evidence, including a pH dependence for catalysis of neutral and charged substrates (33), inhibition by ethoxyformic anhydride and diethyl pyrocarbonate (34, 35), residual reaction with diisopropyl fluorophosphate (8), and retention of a proton donor–acceptor function for the nucleophile in a variety of serine and cysteine hydrolases, indicates a role for a proximal histidine in catalysis. Sequence analysis of cholinesterases from *Drosophila* to humans delineates only two histidines conserved at positions corresponding to residues 425 and 440 in *Torpedo* (refs. 4, 23, and 36; Table 3). Both appear in the third intrasubunit disulfide loop, between residues 400 and 521 (37). Histidines in analogous positions are found in rabbit and *Drosophila* esterases (6, 7) but only the histidine corresponding to 440 is found in a homologous rat microsomal carboxylesterase (8). The latter three esterases lack the cysteines to enclose the homologous histidine within a intrasubunit disulfide loop. The amino acids around His^{440} are better conserved than those around His^{425}. With one exception, His^{440} is flanked by a glycine and at least one acidic residue within the next three amino acids to its carboxyl-terminal side (Table 5).

The rank order of amino acids for the catalytic triad in the trypsin family (Asp^{102}, His^{57}, Ser^{195}) (38) differs from the order in the subtilisin family (Asp^{32}, His^{64}, Ser^{221}) (39). Divergent sequence orders of functional residues provide a strong argument for convergent evolution of the serine hydrolases. The positions of Ser^{200} and His^{440} extend this argument to cholinesterases and provide additional evidence for a third distinct family of serine hydrolases. Some of its members—i.e., the cholinesterases—contain three disulfide loops while other esterases contain only two. However, the position of His^{440} as identified by a proximal glycine on the carboxyl-terminal side is retained in both the three- and two-disulfide loop structural variants of this family (Table 5).

The carboxyl-terminal region of thyroglobulin is homologous to the entire cholinesterase sequence (4, 40) and thyroglobulin also contains the three disulfide loops structured by the six conserved cysteines. As anticipated for a protein devoid of esterase activity, thyroglobulin lacks a serine near position 200 and a histidine near 440. Similarly, AChE lacks tyrosine at the requisite positions in thyroglobulin that become iodinated, cleaved, and conjugated to form the thyroid hormones T_3 and T_4.

The series of mutations involving Glu^{199} suggests that this residue is important to the presumed conformational change in substrate inhibition. Efficient catalysis requires maintaining a precise orientation of the serine within the catalytic triad, and substrate inhibition may result from an alteration of alignment of these residues. A comparison of the sequences around the active center reveals that all of the serine esterases contain glutamate at position 199 except *Drosophila* Est-6, which has a histidine at 199. The serine proteases,

Table 5. Related sequences in the vicinity of the conserved histidines in the serine hydrolases

Enzyme	Sequence
	425 430 435 440 445
Torpedo AChE	YFFNHRASNLVWPEWMGVIHGYEIEFVF
Bovine AChE	YIFEHRASTLSWPLWMGVPHGYEIEFIF
Murine AChE	YIFEHRASTLTWPLWMGVPHGYEIEFIF
Drosophila cholinesterase	YYFTHRTSTSLWGEWMGVLHGDEIEYFF
Human butyrylcholinesterase	YYFEHRSSKLPWPEWMGVMHGYEIEFVF
Murine butyrylcholinesterase	YFFEHRSSKLPWPEWMGVMHGYEIEFVF
Drosophila Est-6	LDLHR.LANRTDYDFGTVHGDDYFLIF
Rabbit carboxylesterase	VARHHR.SSDMRPKTVIGDHGDEIFSVL
Rat carboxylesterase E1	.......VSDQRPQTVQGDHGDEIFSVF
Dictyostelium CP	........HSFEACEGLVCHGTELPMVF
Dictyostelium D2	........HSLDACDDKVCHGTELSLFF
Rat lysophospholipase	YLFSHPSRMPIYPKWMGADHADDLQYVF
Subtilisin BPN	........SETNPFQDNNSHGTHVAGTV
Subtilisin Carlsberg	........SETNPFQDNNGHGTHVAGTV

Residues have been aligned around residue 440 using *Torpedo* AChE as reference. In *Drosophila* Est-6 and rabbit carboxylesterase the histidines aligned with 425 appear 37 and 31 residues amino-terminal to His^{440}; hence, a gap is specified. The rat carboxylesterase E1 does not contain a second histidine in the vicinity of the His-Gly sequence. The *Dictyostelium* esterosome proteins CP and D2 contain three histidines within the 36 amino acids amino-terminal to the His-Gly histidine. No obvious identity with the amino acids surrounding His^{57} in the trypsin family is found. Alignments with the subtilisin family around His^{221} show only the His-Gly similarity.

by contrast, have aspartate amino-terminal to the serine (Table 3). Although we have found the E199H mutant to be devoid of activity, it may have activity towards other substrates.

That the E199Q substitution eliminates substrate inhibition suggests that substrate-induced changes in the orientation of the functional residues may be important to catalytic efficiency. Entrapment of a labeled acetyl-enzyme by denaturation at high substrate concentration indicates that the rates of AChE-catalyzed acylation and deacylation are of similar magnitude at high substrate concentrations (41). Binding of a second substrate molecule at a peripheral site could affect active-center conformation to reduce acylation rates. Alternatively, binding of a second substrate molecule to the acyl-enzyme may selectively influence deacylation (42).

Elimination of substrate inhibition by mutation of a residue proximal to the active-site serine is surprising on two accounts. First, butyrylcholinesterase does not show substrate inhibition, yet Glu[199] and the Phe-Gly-Glu-Ser-Ala-Gly sequence are retained in all cholinesterases (Table 3). Second, AChE has a peripheral anionic site, which at a distance removed from the active center regulates catalysis in an allosteric fashion (2, 43).

It is possible that peripheral-site ligands alter active-site conformation by affecting the position of the loop of amino acids surrounding the active-site serine. Such a proposal is consistent with the kinetic characteristics of inhibition by these ligands and their capacity to affect the fluorescence quantum yield of alkylphosphonates conjugated to the active-site serine (43). The tolerance for substitutions at position 199 appears small; substitutions that retain either the molecular dimensions (Glu $\rightarrow$ Gln) of the side chain or the charge (Glu $\rightarrow$ Asp) allow retention of significant activity, whereas activity is diminished to <0.1% with Glu $\rightarrow$ His substitution. Examination of the specificity of the position 199 mutants for amides, carbamoyl esters, and phosphoryl esters should be of interest.

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