Expression of Recombinant Acetylcholinesterase in a Baculovirus System: Kinetic Properties of Glutamate 199 Mutants†

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ABSTRACT: The glycophospholipid-linked, amphiphilic form of acetylcholinesterase (AChE) from *Torpedo californica* and the hydrophilic form from mouse were overexpressed in Sf9 insect cells using the baculovirus expression system. Recombinant baculovirus, constructed by inserting AChE cDNA's into the genome of *Autographa californica* nuclear polyhedrosis virus adjacent to the strong polyhedron promoter, yielded recombinant enzyme varying between 0.5 and 3.8 mg/L. The recombinant enzyme was glycosylated although it migrated slightly more rapidly in SDS gel electrophoresis than enzyme purified from the electric organ of *Torpedo*. Kinetic properties of the recombinant DNA- and tissue-derived enzymes are identical. The detailed catalytic properties and susceptibility to inhibitors were examined for two enzyme mutations of the glutamate residue N-terminal to the active site serine. The Glu^{199} to Gln mutation shifted both the K_m and K_{ss} to higher substrate concentrations and resulted in a k_{cat} of 28% of the wild type. Mutation of Glu^{199} to Asp also yielded a reduction in k_{cat} but with no change in K_m. Substrate inhibition normally apparent in wild-type AChE was eliminated with the Asp mutation, suggesting that substrate catalysis and substrate inhibition are not directly linked. Both mutations decreased the affinity of reversible inhibitors and reduced the rates of phosphorylation and carbamoylation; these changes were more striking with the Gln^{199} mutation. Decarbamoylation rates were unaffected by these mutations. Glu^{199} is the charged residue found deep within the active center gorge close to the site of acetylcholine binding, and our findings indicate it influences, but is not essential for, efficient catalysis.

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 (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). Moreover, the ordering of residues within the catalytic triad would suggest that the cholinesterases arose by convergent rather than 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. Our initial study showed that substitution of aspartate or glutamine for this glutamate in the enzyme retained activity, while the histidine substitution produced an inactive enzyme (Gibney et al., 1990).

Achieving high levels of recombinant AChE expression in the baculovirus–*Spodoptera* system enabled us to purify the recombinant DNA-derived enzymes to homogeneity, titrate their active sites, and determine detailed catalytic parameters. With this system we show that conservative substitutions at the 199 position affect not only k_{cat} but also inhibitor and substrate specificity. Substrate inhibition has also been analyzed for these mutants.

EXPERIMENTAL PROCEDURES

Materials. The baculovirus transfer vector pJVP10Z was obtained from InVitrogen, San Diego, CA. Sf9 cells and AcNPV were obtained from Dr. Max Summers, Texas A & M University. Edrophonium and 3-hydroxy-*N*-methylpyridinium were gifts of Dr. W. E. Scott, Hofmann La Roche, Nutley, NJ. The other inhibitors were purchased from Sigma and Aldrich Chemical Co. Grace's insect media was purchased from Hazelton; Excell 401 media was from J. R. Scientific.

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 et al., 1990a). The construction in an SV-40 expression plasmid, when transfected into Cos-1 cells, was shown to express the glycophospholipid-linked form of AChE (Gibney et al., 1990a). 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;

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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., 1990b).

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 2×10^6 cells/mL.

Purification of the Recombinant Enzymes. The *Torpedo* AChE's 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 14000*g* 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, the 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. Initial studies showed that elution with 100 mM decamethonium resulted in AChE coeluting with another protein. Activity was assayed by the procedure of Ellman et al. (1961) and related to protein concentration. Active sites 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).

Endoglycosidase Treatment. The purified enzyme, denatured with 0.1% SDS, 2% β-mercaptoethanol, and 20 mM EDTA, was incubated with 20 units/mL endoglycosidase F at 37 °C for 14 h in the presence of 1% β-octylglucoside.

Estimation of Enzyme Purity. Purity and electrophoretic migration were ascertained under reducing conditions on 8% polyacrylamide gels in the presence of SDS. Total protein was detected by amido black staining and AChE migration by Western blots. Polyclonal antibodies directed against *Torpedo* (Lee et al., 1982) and bovine AChE (Doctor et al., 1990) were employed.

Determination of Kinetic and Inhibition Parameters. Kinetic constants were determined from the Ellman assay in a 0.1 M phosphate buffer, pH 7.0, at 22 °C. To account for substrate inhibition, the Haldane equation was used (Haldane, 1930):

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

where K_m is the Michaelis constant and K_{ss} is a substrate inhibition constant representing dissociation of a second substrate molecule.

Reversible inhibitors were added prior to substrate addition. K_I's for reversible inhibitors were determined from reciprocal plots of reaction velocity and substrate concentration in the absence and presence of inhibitors. Replots of the apparent K_m and V_{max} values versus inhibitor concentration yielded K_I's for the respective competitive and noncompetitive components.

Irreversible inhibition entailed prior reaction with the acylating agent for varying periods, addition of substrate, and immediate measurement of residual activity with the Ellman reaction. The time course of loss of enzyme activity yielded k, a first-order inhibition constant characteristic for each inhibitor concentration. Reactions of irreversible acylating inhibitors were analyzed by the following equation:

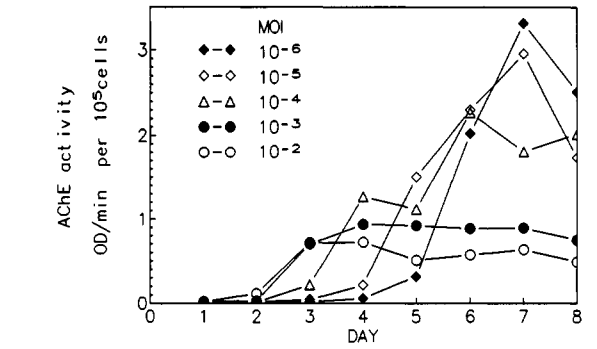
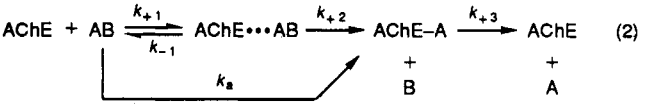


FIGURE 1: Expression of recombinant acetylcholinesterase in *Spodoptera* Sf9 cells at various days after infection. Cells at a density of 3.0×10^5 cells/mL in 24-well plates were infected at day zero with baculovirus containing the amphiphilic form of *Torpedo* AChE cDNA in front of the polyhedron promoter. The MOI is the viral multiplicity of infection. AChE activity was measured by the Ellman assay. MOIs: ◆, 10^{-6}; ◇, 10^{-5}; △, 10^{-4}; ●, 10^{-3}; ○, 10^{-2}.



(2)

where k_{+1} and k_{-1} are association and dissociation rate constants for the initial reversible complex of enzyme and inhibitor AB, while k_{+2} and k_{+3} are rate constants for the enzyme acylation and deacylation. k_a is a second-order rate constant for inhibition or acylation. Measurement of k at different substrate (AB) concentrations enabled determination of k_a, Michaelis-type constant K_a, and maximal acylation rate constant k_{+2}, which are defined by

$$k = k_{+2}/(K_a/[AB] + 1) \quad (3)$$

$$K_a = (k_{-1} + k_{+2})/k_{+1} \quad (4)$$

$$k_{+2}/K_a = k_a \quad (5)$$

RESULTS

Production of Recombinant DNA-Derived AChE. Integration of the cDNA's encoding AChE from *Torpedo* or mouse into the genome of AcNPV by homologous recombination produced quantities of AChE ranging between 0.5 and 3.8 mg/L of suspension culture. Yields of enzyme from infected Sf9 cells were greater if low multiplicities of infection (MOIs) and long intervals between infection and harvest were used (Figure 1). Once viral stocks free of polyhedron production were obtained, cells were infected at MOI of $(1–2) \times 10^{-5}$ and grown in multiple 250- or 500-mL spinner bottles for 6–8 days to produce enzyme for purification.

The purified *Torpedo* enzyme expressed in baculovirus migrated as a doublet corresponding to an apparent molecular mass of 58–60 kDa. This compares with a value of 67 kDa for AChE purified from *Torpedo* electric organ. The mouse enzyme expressed in baculovirus also migrated as a diffuse band with an apparent molecular mass of ~60 kDa (Figure 2). In some electrophoretic runs, the diffuse band is a closely spaced doublet.

Treatment of the *Torpedo* (not shown) and mouse enzymes (Figure 2) with endoglycosidase F increases their migration rates upon SDS gel electrophoresis. The migration position

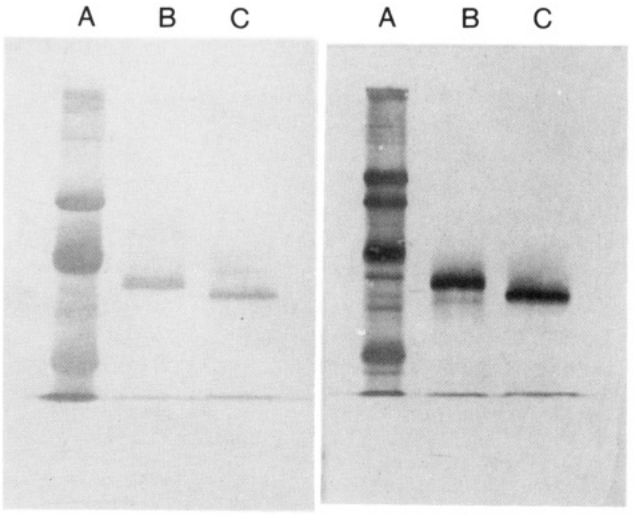


FIGURE 2: Polyacrylamide gel electrophoresis of recombinant DNA-derived mouse acetylcholinesterase. AChE produced from the baculovirus–mouse AChE gene was expressed in Sf9 cells and purified by affinity chromatography from the cell supernatants. The edrophonium eluants were dialyzed and subjected to polyacrylamide gel electrophoresis in the presence of SDS and reducing agent. Left panel: Amido black staining of (A) molecular weight standards, (B) purified enzyme, and (C) purified enzyme treated with endoglycosidase F. Right panel: Antibody staining (A) biotin-conjugated molecular weight standards, (B) purified enzyme, and (C) purified enzyme treated with endoglycosidase F.

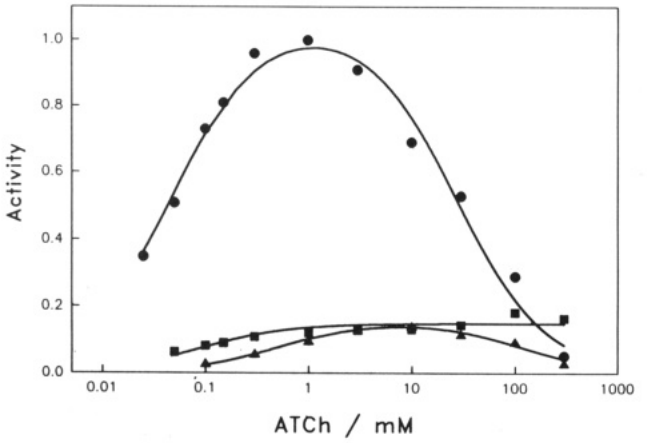


FIGURE 3: Dependence of catalytic activity on acetylthiocholine for the wild-type and mutant recombinant DNA-derived acetylcholinesterase. The enzymes were purified to homogeneity, and activity was measured by the Ellman assay in 0.1 M sodium phosphate at pH 7.0. The relative activity values have been normalized to equivalent active site concentrations determined by titration with the high-affinity alkyl phosphate, MEPQ. ●, wild-type recombinant; ▲, $Glu^{199} \rightarrow$ Gln; ■, $Glu^{199} \rightarrow$ Asp.

of the recombinant *Torpedo* enzyme corresponded closely with the electric organ purified enzyme deglycosylated under the same conditions (not shown), suggesting that the apparent size difference is due to processing differences (Vialard et al., 1990) at the four glycosylation sites on the *Torpedo* enzyme (Schumacher et al., 1986).

Analysis of Kinetic Constants for the Recombinant-Produced Enzyme. Our early measurements of recombinant AChE involved transient expression in Cos-1 cells. Low expression limited measurements at high substrate concentration where general-base catalysis was of the same magnitude as enzyme catalysis. Moreover, the quantities of enzyme produced were insufficient for purification and precise determination of k_{cat}. Therefore, relative k_{cat}'s could only be inferred from comparing V_{max} with labeled enzyme precipitated with antibody.

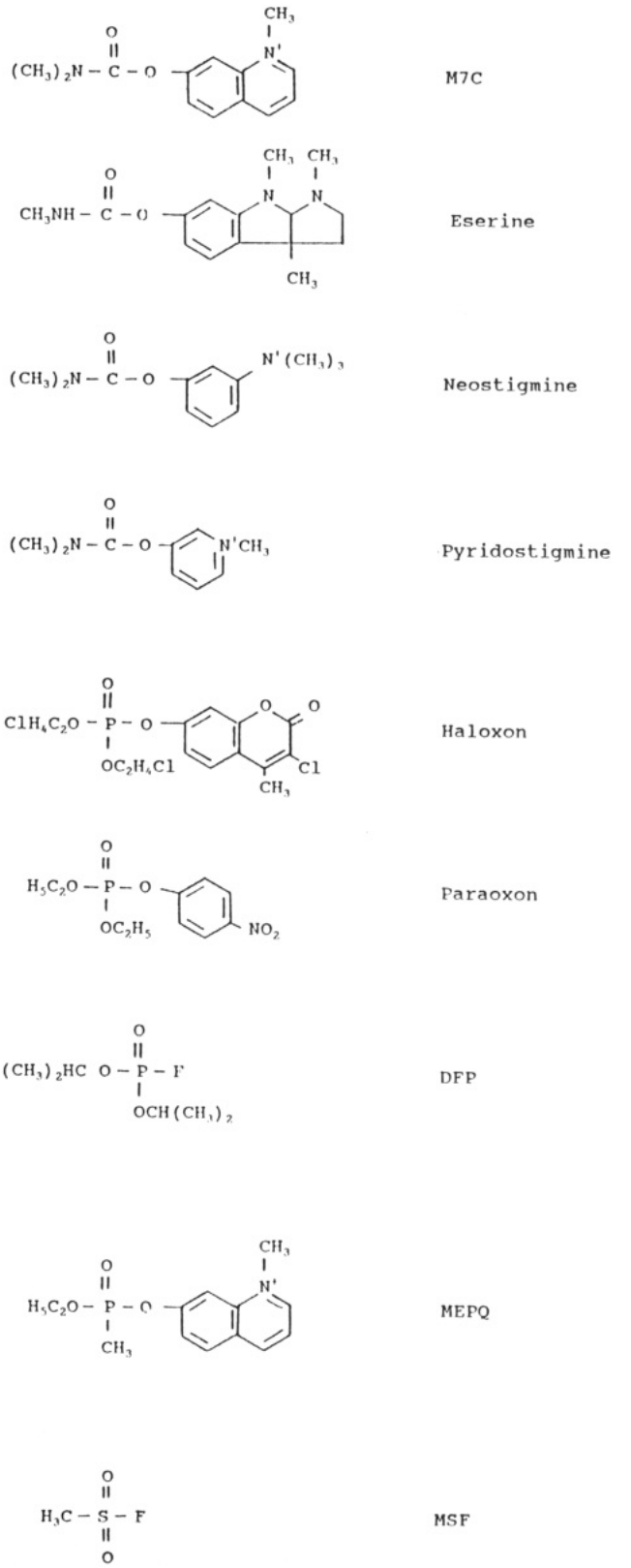


FIGURE 4: Structures of alkyl phosphates and carbamoylating agents used in this study.

The dependence of rate of catalysis on substrate concentration for the recombinant-derived *Torpedo* enzyme and two mutants is shown in Figure 3. Fitting of the data to eq 1 yields the kinetic constants and standard errors shown in Table I. k_{cat} was ascertained from the ratio of activity to active site concentration. Our analysis showed that the kinetic constants for the recombinant-derived *Torpedo* enzyme expressed in baculovirus were virtually identical to those from the enzyme

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

enzyme	10^3K_m (M)	$K_{m\ wt}/K_{m\ mut}$	10^3K_{ss} (M)	$K_{ss\ wt}/K_{ss\ mut}$	10^5k_{cat} (min^{-1})	$10^9k_{cat}/K_m$
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.

Table II: Reversible Inhibition of Torpedo Acetylcholinesterase

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.

purified to homogeneity from the *Torpedo* electric organ (Table I) (Lee et al., 1982). Thus, the different oligosaccharide processing found in *Spodoptera* cells does not appear to alter catalytic activity.

The recombinant enzymes from *Torpedo* and mouse show the classic substrate inhibition behavior typically seen for AChE but not butyrylcholinesterase (Augustinsson, 1948) (Table I). Substitution of Gln for Glu^{199} shifts both K_m and K_{ss} to considerably higher substrate concentrations. In addition, V_{max} or k_{cat} is reduced to ~28% of the value in the wild-type enzyme. The ratio of k_{cat}/K_m is often used as an index of maximal catalytic efficiency, and this value for the Glu^{199} mutant is reduced to ~2% of that measured for the wild-type enzyme.

Substitution of Asp for Glu^{199} also reduces k_{cat}, but with no change in K_m (Figure 1). Interestingly, substrate inhibition is no longer observed, indicating that K_{ss} becomes infinitely large and Michaelis–Menten kinetics describe the substrate concentration dependence. Catalytic activity of this mutant at high substrate concentration actually exceeds that for the wild-type enzyme.

Reversible Inhibition. Reversible inhibition of AChE can result from ligand binding to either the active center or a peripheral anionic site (Changeux 1966; Aldridge & Reiner, 1972; Taylor & Lappi, 1975; Rosenberry, 1975). Active center ligands typically exhibit competitive kinetics while peripheral site ligands show complex patterns of inhibition which are highly dependent on ionic strength (Taylor & Lappi, 1975; Berman et al., 1985). Edrophonium has often been used as a prototype ligand for the active center, while propidium has been used as peripheral anionic site selective ligand.

Measurements of inhibition reveal no differences between the recombinant DNA-derived and native enzymes from *Torpedo* (data not shown). Early studies have shown that *m*-hydroxyl groups markedly enhance binding of phenyltrimethylammonium congeners (Wilson & Quan, 1958), and this behavior is clearly evident in the comparison of dissociation constants between edrophonium and its nonhydroxylated analogue, phenyltrimethylammonium (Table II). Moreover, the tolerance for the interaction of the *m*-hydroxyl group to stabilize the complex appears very small since the corresponding *m*-hydroxy-*N*-methylpyridinium shows a marked reduction in affinity compared to edrophonium.

Analyses of the inhibition kinetics for the mutant enzymes show that the isosteric modification of the Glu^{199} to Gln with the elimination of charge influences edrophonium binding to a greater extent than propidium binding, as might be anticipated for an active site versus a peripheral site-selective ligand. However, the Glu to Asp mutation where charge is retained affects both edrophonium and propidium affinities. An examination of ligand specificity suggests that the carboxylate side chain of residue 199 plays a specific role in stabilization of the high-affinity, active center complexes for the different congeners of phenyltrimethylammonium and *N*-methylpyridinium.

Irreversible Inhibition. It has long been known that many inhibitors of cholinesterase act by serving as hemisubstrates in which deacylation of the active site serine becomes a slow reaction. A wide variety of inhibitors that either carbamoylate, phosphorylate, or sulfonylate the serine have been described [cf. Wilson (1959)]. Tables III–V tabulate the inhibition constants for a series of representative acylating inhibitors on the native and mutant enzymes. It is perhaps simplest to analyze the carbamoylating agents (Tables III and IV) since both acylation and deacylation steps occur in a time frame for convenient kinetic measurements. Additionally, the complexities of stereospecificity inherent to the tetrahedral phosphate inhibitors is not an issue.

The very different rates of carbamoylation and decarbamoylation allow one to separate the rate constants, as shown in eq 1. As previously shown with *Electrophorus* AChE (Wilson et al., 1961), decarbamoylation is more rapid for the dialkyl (eserine) than the monoalkyl carbamates (neostigmine and pyridostigmine) for both the mouse and *Torpedo* enzymes (Table IV). Virtually no effect of the 199 mutation on decarbamoylation kinetics is evident. By contrast, the mutation of Glu^{199} to Gln or Asp markedly affect carbamoylation rates (Table III). The bimolecular rate constant k_a for carbamoylation is most comparable to the ratio of k_{cat}/K_m for substrate. As in the case of acetylthiocholine as a substrate,

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.

the Gln^{199} mutation has the most marked effect on carbamoylation rates (Table III). In the case of alkyl phosphates, slow dephosphorylation and aging preclude a direct analysis of dephosphorylation rates. As with carbamoylation, the Gln^{199} mutation has the most dramatic effects on rates of phosphorylation by a series of inhibitors (Table V).

DISCUSSION

Acetylcholinesterase Expression in Baculovirus. Our earlier study employing transient transfection of a *Torpedo* AChE cDNA into Cos cells showed that activity was retained with the Glu^{199} to Gln or to Asp substitution, whereas substitution of His^{199} resulted in loss of activity (Gibney et al., 1990b). However, the transient transfection system yields insufficient protein for routine purification and titration of active centers of the enzyme. Hence, one was limited in the precise determination of all catalytic parameters.

Homologous recombination of AChE cDNA's into baculovirus and infection of *Spodoptera* cells with these viruses provide an expression system whereby sufficient recombinant enzyme can be purified. The stoichiometry of active centers may be determined by direct titration with a high-affinity alkyl phosphate rather than inferred from antibody precipitation of [^{35}S]methionine incorporated into protein. High levels of expression also enable one to employ sufficient enzyme concentrations to extend the range of substrate concentrations. In particular, substrate inhibition is only evident at substrate concentrations exceeding millimolar. Since the rate of general-base catalysis of esters increases with concentration, higher enzyme concentrations must be employed to monitor substrate inhibition in the mutant enzymes.

A third advantage of the baculovirus expression system stems from the lower expression efficiency of *Torpedo* AChE (Gibney et al., 1990b) and *Torpedo* acetylcholine receptor (Claudio et al., 1988) at 37 °C. It is likely that certain proteins from poikilotherms experience less than optimal folding at the higher ambient temperatures. Expression in mammalian culture systems at low temperature compromises cell viability and overall protein synthesis. *Spodoptera* cells grow well at lower temperatures, which may be a factor enhancing functional expression of the recombinant protein. Since recent crystallographic work was done on AChE from *Torpedo californica* (Sussman et al., 1991), this system carries an intrinsic advantage for the interpretation of mutagenesis experiments.

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.

The enzyme purified from baculovirus expression appears to be glycosylated since all of the enzyme exhibits an increase in electrophoretic migration in SDS after treatment with endoglycosidase F. After treatment the band sharpens, and in fact, the diffuse band for the glycosylated enzyme often appears as two very closely migrating bands, suggesting different extents or processing of glycosylation. Nevertheless, we have found no consistent differences in kinetic properties in terms of substrate K_m, K_{ss}, and k_{cat} (Table I) between recombinant-derived and tissue-derived enzymes. Hence, at this level of characterization, glycosylation processing does not appear to affect kinetic properties of AChE.

Glutamate 199 within the Active Center. The selection of Glu^{199} as a residue for mutation was initially based solely on its proximity to the catalytic serine (Ser^{200}) and conservation of amino acid residues in the active center. The cholinesterases from at least 15 sources contain Phe-Glu-Ser-Ala-Gly at the active center extending between positions 198 and 202 (using the *Torpedo* numbering system). Moreover, other homologous esterases in the family contain the conserved Glu at 199, Ser at 200, Ala at 201, and Gly at 202 (Gibney et al., 1990b;

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

inhibitor	AChE	inhibition constants[a] 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.

Gentry & Doctor, 1991). Notable exceptions are the Drosophila Est 6 in which a His was found at the 199 position (Oakshott et al., 1988) and a Geotrichium lipase isoform which contains an Asp at this position (Schrag et al., 1991). Although the serine hydrolases of the chymotrypsin family show no global homology with AChE, their active center conserved sequence of Asp-Ser-Gly-Gly is similar to the above AChE sequence. Asp replaces Glu, and the importance of Asp orientation on the active center conformation is clearly revealed by the formation of an ion pair between this Asp and the free amino group of Ile^{16}. Formation of the free amino group on Ile is associated with cleavage of a 15 amino acid peptide in the chymotrypsinogen to chymotrypsin activation (Blow & Steitz, 1970).

The three-dimensional structure of *Torpedo* AChE also uncovers a potentially unique role for Glu^{199} for it appears to be the only charged residue residing close to the substrate and deep within a hydrophobic cavity some 20 Å in depth (Sussman et al., 1991). As such, one might anticipate that it is involved in stabilization of the bound quaternary substrate or in influencing orientation of a loop containing the active center serine. However, its presence in the homologous lipases which hydrolyze uncharged fatty acid esters (Schrag et al., 1991; Brzozowski et al., 1991) suggests that either the side chain of Glu^{199} has considerable conformational flexibility or it plays a limited role in substrate specificity. The AChE and Geotrichium lipase crystal structures suggest Glu^{199} may interact with Glu^{443} and perhaps Ser^{226} through an entrapped water molecule (Sussman et al., 1991; Schrag et al., 1991). Whether the water is important for catalysis or maintenance of active center conformation remains to be ascertained.

We observed that the 199 mutation has dramatic effects on activity and quite unexpectedly affects substrate inhibition. Both the Gln and Asp mutants have diminished catalytic turnover as reflected in their k_{cat}'s being reduced to 20–28% of the wild-type enzyme. Only the Gln mutation affects K_m, which is considerably increased. The ratio of k_{cat}/K_m reflects catalytic throughput at low substrate concentrations and is perhaps the best frame of reference for catalytic efficiency. Using this parameter, the catalytic efficiency of the Gln mutant is reduced to only ~2% of the wild-type enzyme (Table I). This indicates that charge plays a critical role in substrate binding and catalytic efficiency. Even with a diminution of catalytic efficiency approaching 2 orders of magnitude, the mutant enzymes remain as highly efficient hydrolases since native AChE accelerates H_2O-catalyzed hydrolysis of acetylcholine ~10^{13}-fold (Bazelyansky et al., 1986). The reductions in substrate affinity and hydrolysis are consistent with the side chain of Glu^{199} being directed toward the active center in the complex. Homologous lipases hydrolyzing neutral esters may adopt a different orientation for the anionic side chain of this residue upon substrate binding (Schrag et al., 1991; Brzozowski et al., 1991).

The influence of the 199 mutations on substrate inhibition and peripheral site ligand binding, while complex, likely reflects the allosteric interaction between the peripheral site and active center. Several lines of evidence now suggest that the peripheral site and active center are physically distinct at a distance of 20 Å or greater (Berman et al., 1978; Weise et al., 1990). Ternary complexes with the respective ligands simultaneously bound to the two sites can also be demonstrated (Taylor & Lappi, 1975; Epstein et al., 1979). Moreover, ligand binding at one site can be shown to affect the conformation of the second site (Epstein et al., 1979). On the basis of selective affinities determined with competitive back-titrations, substrate inhibition may result from a second substrate molecule binding at the peripheral site (Radić et al., 1990). Peripheral site inhibition, whether is arises from excess substrate or inhibitors selective for that site, might affect catalysis by altering either the position of residues in the catalytic triad or other residues influencing substrate specificity. The elimination of the charge on residue 199 in the Gln mutation influences both K_m and K_{ss} to similar extents. However, shortening the side chain, as seen in the Asp^{199} mutation, only affects K_{ss} and not K_m.

Previous studies have shown that ionic strength and other external parameters affect K_m and K_{ss} differently, but the mutation reveals a direct functional dissociation between the sites. Because of substantial evidence for an allosteric action of the peripheral site on the active center and the possibility that substrate inhibition is mediated through a peripheral site, it is not necessary for Glu^{199} to be directly contained within both the active center and the substrate inhibition site. The functional linkage between the peripheral site and active center

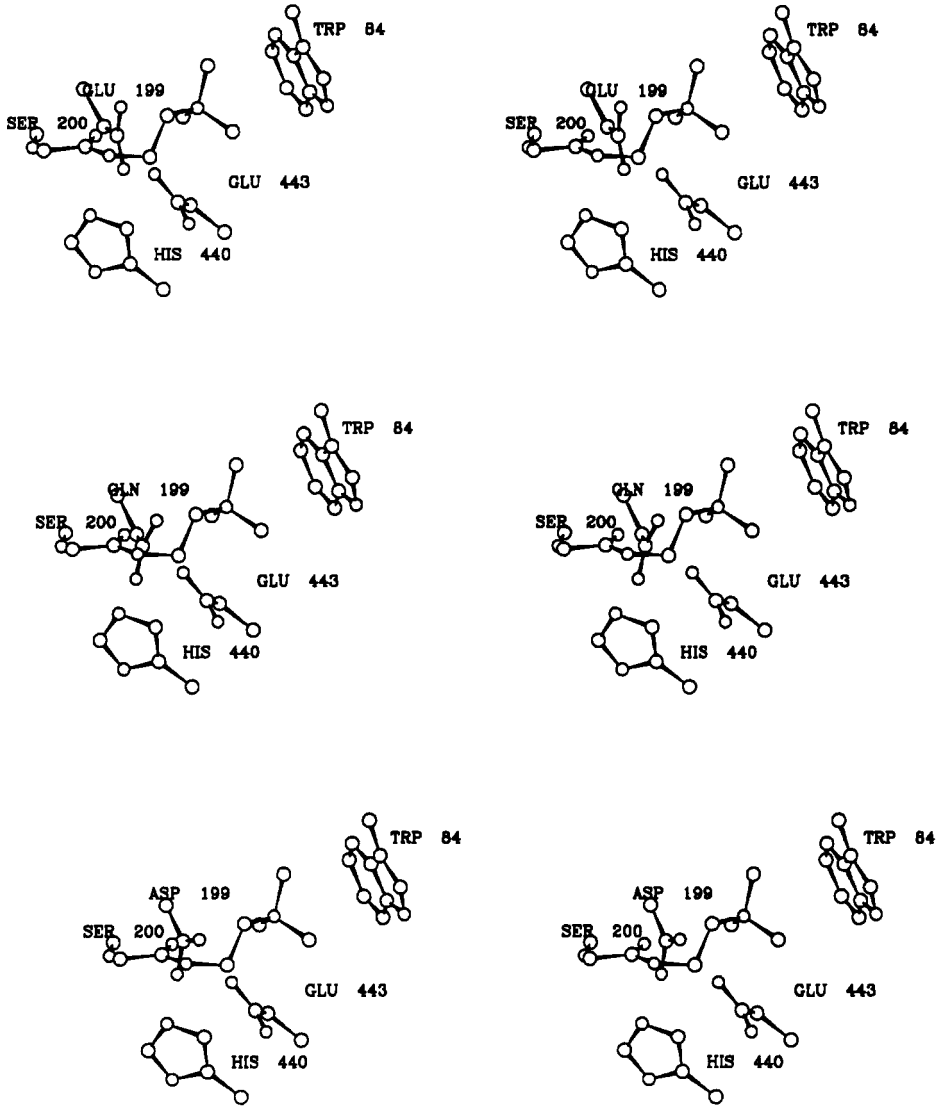


FIGURE 5: Stereoview of the position of the 199 side chain in relation to other functional residues established by X-ray crystallography of acetylcholinesterase. The positions of the side chains are taken directly from the coordinates of Sussman et al. (1991). Substrate position and conformation and side-chain orientation of the 199 residue were determined by an energy minimization docking program (Biosym Technologies) Insight II on a Silicongraphics Personal Iris 4D/35.

is decoupled when the side chain of residue 199 is shortened. This points to the critical role of the 199 residue in the conformation of the active center and suggests that the peripheral site occupation induces changes in active center conformation. The latter conclusion is supported by fluorescence studies which revealed changes in quantum yield of an active center fluorophore upon binding of a peripheral site ligand (Epstein et al., 1979).

Influence of Residue 199 Mutants on Reactions with Other Substrates. The carbamoylating substrates clearly demonstrate the predominant influence of the 199 mutation on the acylation rather than the deacylation step where the latter is virtually unaffected by the two mutations. Acylation by phosphorylating and sulfonylating agents is also affected by the mutations, with the greatest effect occurring with charge removal in the Gln mutation. While it may be dangerous to extrapolate these data to the kinetics of carboxylate ester hydrolysis where rates of deacylation are rapid, our findings would suggest that the 199 mutations affect primarily the acylation step of the reaction.

Reversible Inhibitors. Eliminating charge by substitution of Glu at 199 has a large effect on edrophonium binding at the active center. By contrast, the 199 mutations only marginally affect the binding of the weaker inhibitors. As shown many years ago (Wilson & Quan, 1958), the meta hydroxyl moiety greatly enhances phenyltrimethylammonium potency. The enhanced influence of charge removal on edrophonium binding suggests that Glu^{199} is critical to the orientation for such inhibitors when bound (Sussman et al., 1991).

The Asp^{199} mutant appears to affect the binding of the peripheral site and active center ligands similarly. This is surprising since the peripheral site ligand is not expected to overlap with the active site. The finding likely reflects allosteric coupling between the sites, which is diminished following mutation from Glu to Asp. Consistent with substrate inhibition also emanating from occupation of the peripheral site, the Asp mutation most markedly affects substrate inhibition.

Functional Role of Residue 199 and Aromatic Residues in Inhibitor Binding. As noted by Sussman and colleagues (1991), Trp^{84} and other aromatic residues may be the primary contributing residues to the stabilization of the quaternary moiety in acetylcholine and various inhibitors. In fact, the role played by aromatic side chains in the stabilization of complexes of quaternary ligands appears widespread (Segal et al., 1984; Cohen et al., 1989; Dougherty & Stauffer 1990; Abramson et al., 1989; Galzi et al., 1990). Nevertheless, the

complex may be stabilized by both longer range electrostatic (Coulombic) and shorter range hydrophobic (π electron) forces. In fact, the reduction of k_{cat}/K_m by 2 orders of magnitude in the Gln^{199} mutation clearly reveals the balance of both forces in dictating catalytic potential. The projected orientations of the side chains, with respect to the position of the substrate, is shown in Figure 5 for the native and Gln^{199} mutant enzymes.

The reduction of substrate acylation rates associated with the mutation of residue 199 is dependent on the leaving group of the carbamylating agent (Table III). It appears that ligands with leaving groups in which the cationic charge is delocalized in the pyridinium ring are affected to a greater extent by elimination of the charge on Glu^{199}. Charge delocalization may enable the positive charge to be in closer proximity to residue 199.

A comparison of the influence of the mutations on acylation by carbamates and organophosphates shows the latter to be more sensitive to the charge at 199. This likely reflects the tetrahedral configuration of the organophosphates and their close mimicry of the transition state for carboxyl or carbamoyl ester hydrolysis. The spatial disposition of the leaving group can be expected to differ for the organophosphates since the transition state should be pentacoordinate. The larger size of the phosphate group and the necessity to accommodate two substituents in addition to the ester oxygen in the active center may result in the 199 carboxyl group having a greater direct inductive effect on the phosphorus.

Further analyses of organophosphate inhibition will be strengthened by more exhaustive structure–activity studies and crystal structures of the enzyme–phosphoryl conjugates. It will also be of interest to see if residue 199 appears in any naturally occurring insect mutations (Morton & Singh, 1982).

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