

## Conversion of acetylcholinesterase to butyrylcholinesterase: Modeling and mutagenesis

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**ABSTRACT** *Torpedo* acetylcholinesterase (AcChoEase, EC 3.1.1.7) and human butyrylcholinesterase (BtChoEase, EC 3.1.1.8), while clearly differing in substrate specificity and sensitivity to inhibitors, possess 53% sequence homology; this permitted modeling human BtChoEase on the basis of the three-dimensional structure of *Torpedo* AcChoEase. The modeled BtChoEase structure closely resembled that of AcChoEase in overall features. However, six conserved aromatic residues that line the active-site gorge, which is a prominent feature of the AcChoEase structure, are absent in BtChoEase. Modeling showed that two such residues, Phe-288 and Phe-290, replaced by leucine and valine, respectively, in BtChoEase, may prevent entrance of butyrylcholine into the acyl-binding pocket. Their mutation to leucine and valine in AcChoEase, by site-directed mutagenesis, produced a double mutant that hydrolyzed butyrylthiocholine almost as well as acetylthiocholine. The mutated enzyme was also inhibited well by the bulky, BtChoEase-selective organophosphate inhibitor (tetraisopropylpyrophosphoramidate, iso-OMPA). Trp-279, at the entrance of the active-site gorge in AcChoEase, is absent in BtChoEase. Modeling designated it as part of the “peripheral” anionic site, which is lacking in BtChoEase. The mutant W279A displayed strongly reduced inhibition by the peripheral site-specific ligand propidium relative to wild-type *Torpedo* AcChoEase, whereas inhibition by the catalytic-site inhibitor edrophonium was unaffected.

In vertebrates, two enzymes efficiently catalyze acetylcholine (AcCho) hydrolysis: acetylcholinesterase (AcChoEase; EC 3.1.1.7) and butyrylcholinesterase (BtChoEase; EC 3.1.1.8) (1). The principal role of AcChoEase is termination of impulse transmission at cholinergic synapses (2). Although BtChoEase is widely distributed, its biological role is unknown (3); it is so called because it hydrolyzes butyrylcholine (BtCho) at rates similar to or faster than AcCho, whereas AcChoEase hydrolyzes BtCho much more slowly (4). BtChoEase is also known as serum cholinesterase, due to its high concentration in vertebrate serum (3). The two enzymes are further distinguished by their different susceptibility to various inhibitors (5). For example, some bisquaternary compounds, which are more potent inhibitors of AcChoEase than their monoquaternary counterparts, bind poorly to BtChoEase (6). Human BtChoEase (h-BtChoEase) is of interest to anesthesiologists and geneticists because it is responsible for breakdown of the short-term muscle relaxant succinylcholine (7) and because numerous genetic variants exist in which the rate of succinylcholine hydrolysis is reduced (8).

Cloning and sequencing reveal striking sequence homology between AcChoEase and BtChoEase (1, 8–11). There is 53% identity and 73% similarity between h-BtChoEase and the phylogenetically distant *Torpedo* AcChoEase (T-AcChoEase). The three intrachain disulfides are conserved (12, 13), and no deletions or insertions occur in the first 535 amino

acids, including all those involved in catalytic activity. This marked structural similarity encouraged us to use the three-dimensional structure of T-AcChoEase (14) to model h-BtChoEase. We hoped, thereby, to gain an understanding of how structural differences between the two might account for known differences in specificity and, also, to attempt to confer BtCho-hydrolyzing activity upon AcChoEase by limited site-directed mutagenesis.

### MATERIALS AND METHODS

**Materials.** Tetraisopropylpyrophosphoramidate (iso-OMPA), BW284C51, acetylthiocholine iodide, butyrylthiocholine iodide, acetylcholine chloride, and propidium iodide were from Sigma; edrophonium chloride was from Hoffmann–La Roche; and [<sup>3</sup>H]acetylcholine iodide (90 mCi/mM; 1 Ci = 37 GBq) was from NEN (Paris).

**Model Building.** Modeling was done interactively with FRODO (15, 16) on an Evans and Sutherland PS390 graphics system to convert the amino acid sequence of T-AcChoEase to that of h-BtChoEase. The h-BtChoEase structure was energy-minimized by the simulated hybridizing program X-PLOR (17) with the POSITIONAL refinement option.

**Site-Directed Mutagenesis.** A pEF-BOS vector, containing an insert encoding the H catalytic subunit of *Torpedo marmorata* AcChoEase (18), was introduced into *Escherichia coli* strain RZ 1032 to obtain uracil-containing template DNA (19). Single-stranded DNA was produced from the phagemid (20). The oligonucleotide, 5'-GAAGGAAACCCTGATAC-3' was used to make the double mutant F288L-F290V, and 5'-GACATTCGCCTCCACGT-3' was used to produce mutant W279A. Synthesis and ligation were achieved with T4 DNA polymerase and T4 DNA ligase in the same tube after hybridizing 1.5 pmol of 5'-phosphorylated oligonucleotide with 0.05 pmol of template. *E. coli* strain TG1 was transformed with the resulting double-stranded DNA. The coding region of a clone that presented the correct mutation was fully sequenced. DNA was purified by two CsCl gradients and transfected into COS-7 cells, as described (18).

**Cholinesterase Assays.** Cholinesterase activity was determined radiometrically on [<sup>3</sup>H]AcCho (21) or spectrophotometrically (22) with either acetylthiocholine or butyrylthiocholine.

**Active-Site Titration.** Concentration of AcChoEase active sites in COS cell extracts was determined with the organophosphate *O*-ethyl-*S*<sup>2</sup>-diisopropylaminoethyl methylphosphothionate (MPT) as titrant (23).

### RESULTS AND DISCUSSION

Residues 4–534 of T-AcChoEase can be aligned with residues 2–532 of h-BtChoEase (10) with 53% identity and no deletions or additions (Fig. 1). The catalytic triad residues are in the same

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Abbreviations: AcCho, acetylcholine; AcChoEase, acetylcholinesterase; BtCho, butyrylcholine; BtChoEase, butyrylcholinesterase; h-BtChoEase, human BtChoEase; T-AcChoEase, *Torpedo* AcChoEase; iso-OMPA, tetraisopropylpyrophosphoramidate. Single-letter amino acid code is used; substitutions are indicated by original amino acid, position number, and substituted amino acid.

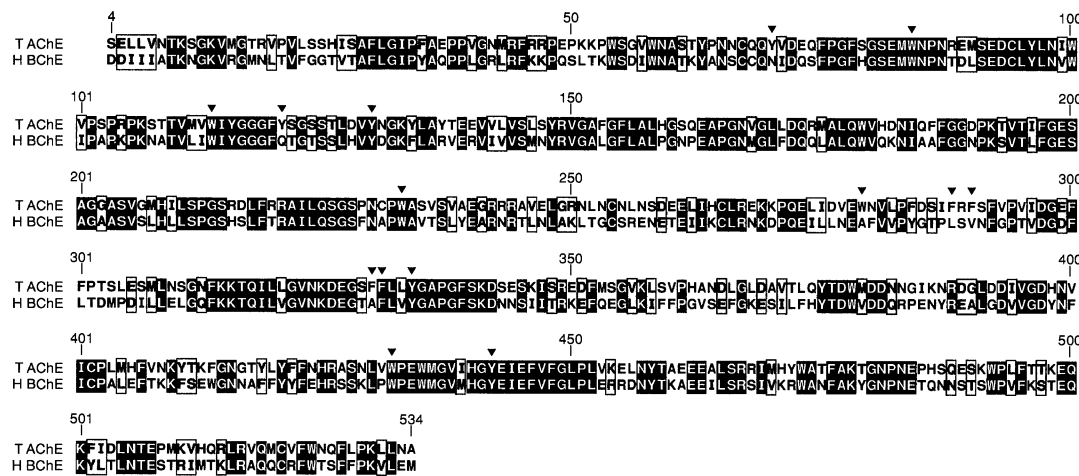


FIG. 1. Comparison of amino acid sequences of T-AcChoEase and h-BtChoEase. Residues 4–534, which are seen in the x-ray structure of T-AcChoEase, are compared. Reverse type indicates identical residues, whereas grey boxes indicate similar residues (i.e., A and G; T and S; D and E; K and R; F and Y; N and Q; L, V, I, and M). Aromatic residues in the active-site gorge are marked with ▼. Numbering is according to T-AcChoEase.

positions (Ser-200, Glu-327, and His-440 in T-AcChoEase) are the intrachain disulfides (12, 13). In the following, amino acid numbers will correspond to the numbering for T-AcChoEase. Computer modeling was done with the T-AcChoEase sequence and three-dimensional structure, whereas site-directed mutagenesis used *T. marmorata* AcChoEase. The two AcChoEase sequences differ by only 10 amino acids (11), none of which appear pertinent to their differences in activity.

**Modeling of h-BtChoEase.** The starting model for h-BtChoEase was the refined 2.8-Å x-ray T-AcChoEase structure (14) [Brookhaven access code 1ACE (24)]. In this structure, all residues except 1–3, 485–489, and 535–537 were seen, although 81 atoms in the side chains of some surface polar residues were not visible. To obtain the h-BtChoEase model, all residues in the T-AcChoEase sequence differing from those of h-BtChoEase were changed accordingly. In h-BtChoEase 356 side chains are either identical to those of T-AcChoEase or have the same number of dihedral angles. The initial h-BtChoEase model retained the experimentally determined torsion angles for these side chains. An additional 83 residues, with fewer side-chain torsion angles in h-BtChoEase than in T-AcChoEase, were also allowed to retain the experimental x-ray torsion angles of T-AcChoEase. The dihedral angles of 87 residues with longer side chains in h-BtChoEase than in T-AcChoEase were fixed to their most frequently found torsion angle values (25). In only 13 cases, where the most common rotamer of a side chain overlapped with neighboring atoms, the second most common rotamer was taken. Energy minimization was achieved after 110 cycles of positional refinement. At that stage the  $C_{\alpha}$  rms deviation between T-AcChoEase and the h-BtChoEase model was 0.28 Å. The largest shift, 1.4 Å, was displayed by A534M, the last C-terminal residue seen in the x-ray structure; the second largest shift, 0.9 Å, was seen in H159P, a surface residue. Positions of the catalytic triad residues S200, E327, and H440 showed very small shifts ( $C_{\alpha}$  shifts of 0.1, 0.1, and 0.14 Å, respectively).

In the T-AcChoEase structure, the catalytic triad is located near the bottom of an  $\approx 20$ -Å deep narrow cavity, which we named the aromatic gorge because  $\approx 40\%$  of its surface area is lined with the rings of 14 aromatic amino acids (14). All these residues are fully conserved in the five known vertebrate AcChoEase sequences (10, 11), except for one case in which phenylalanine is replaced by tyrosine. This result suggests that these aromatic rings are important in AcChoEase function. Indeed, there is strong evidence that Phe-330 and Trp-84, which are near the catalytic triad, are directly involved in binding the quaternary group of AcCho (14, 26) (see below). The role of other aromatic residues, more distal from the active site, remains to be clarified; we have suggested that some may facilitate catalysis by providing low-

affinity binding sites both for AcCho and for the choline produced by enzymic activity (14). Although as many as 30 residues contribute, to some extent, to the gorge lining in the T-AcChoEase structure, comparison of T-AcChoEase and h-BtChoEase sequences, as well as other known AcChoEase and BtChoEase sequences (27–32), shows that only 10 amino acids that have side chains facing the gorge differ in BtChoEase (Table 1, Fig. 2). Four of these changes are probably not associated with differences in enzymic properties of the two enzymes because they involve substitution by residues with similar side chains—i.e., V71I, S122T, L282V, and S286T. The other six cases all involve substitution of an aromatic residue in AcChoEase by a nonaromatic residue in BtChoEase—namely, Y70N, Y121Q, W279A, F288L, F290V, and F/Y330A (see Fig. 3).

**Substrate Fitting.** We previously suggested a plausible model for docking of AcCho, in an all-trans configuration, within the active site of T-AcChoEase (14). In this model, the acetyl group of AcCho was positioned to make a tetrahedral bond with Ser-200  $O^{\gamma}$ . This resulted in the positively charged quaternary group of the choline moiety being within van der Waals distance ( $\approx 3.5$  Å) of Trp-84, earlier suggested by affinity labeling to be within the “anionic” site (26). This assignment is confirmed by the structures of two AcChoEase–inhibitor complexes with the anionic-site-directed competitive inhibitors tacrine and edrophonium, which we have recently determined (M.H., I.S., and J.L.S., unpublished work). In the h-BtChoEase model, Trp-84, like the catalytic triad, does not move relative to its position in T-AcChoEase ( $C_{\alpha}$  shift of 0.07 Å). Hence we can model a bound BtCho molecule in the same orientation as AcCho. When we thus try to model BtCho in the active site of

Table 1. Residues with side chains facing the active-site gorge where differences are found between AcChoEases and BtChoEases

Residue	AcChoEase				BtChoEase		
	T(8)	H(29)	M(30)	B(31)	H(9)	M(30)	R(32)
70	Y	Y	Y	Y	N	N	N
71	V	V	V	V	I	I	I
121	Y	Y	Y	Y	Q	Q	Q
122	S	S	S	S	T	T	T
279	W	W	W	W	A	R	V
282	L	L	L	L	V	L	V
286	S	S	S	H	T	S	S
288	F	F	F	F	L	L	L
290	F	F	F	F	V	I	V
330	F	Y	Y	Y	A	A	A

Numbers in parentheses indicate references. T, *Torpedo*; H, human; M, mouse; B, bovine; R, rabbit.



FIG. 2. Stereo view of the 14 aromatic residues, the side chains of which face the active-site gorge, and a modeled bound BtCho molecule. T-AcChoEase is shown in yellow; h-BtChoEase is shown in blue.

AcChoEase, the bulkier butyryl moiety of BtCho clearly cannot fit into the "esteratic" locus: two residues, Phe-288 and Phe-290, are seen to be near the modeled acetyl moiety of the bound AcCho molecule (see Fig. 4a). In the h-BtChoEase model, however, substantial reduction in size of the side chains of the corresponding residues, Leu-288 and Val-290, permits the butyryl group to fit into the larger esteratic pocket of the model (see Fig. 4b).

**Butyrylthiocholine Hydrolysis by a F288L-F290V Double Mutant.** Prediction of the theoretical docking procedure was

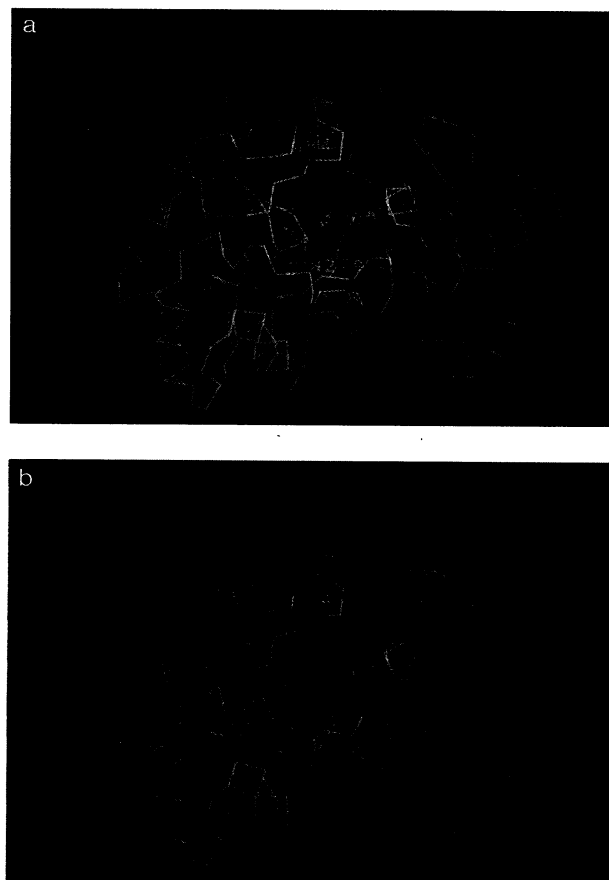


FIG. 3.  $C_{\alpha}$  tracings of enzymes and van der Waals volumes of aromatic residues in the active-site gorge of T-AcChoEase x-ray structure (a) and h-BtChoEase model (b); view is down the active-site gorge.

tested experimentally by generating a double mutant, F288L-F290V, of T-AcChoEase, which was expressed in COS cells. Active-site titration with the organophosphate *O*-ethyl-*S*<sup>2</sup>-diisopropylaminoethyl methylphosphothionate (23) showed that the double mutant retained  $\approx 10\%$  of the specific activity of the wild type toward AcCho. Comparison of its activity toward butyrylthiocholine and acetylthiocholine (Table 2) showed that it hydrolyzed the butyryl ester at a substantial rate compared with the acetyl ester, whereas no detectable activity on butyrylthiocholine was displayed by wild-type T-AcChoEase. Thus, removal of the bulky phenyl rings of F288 and F290 appears, as predicted, to permit BtCho to enter the active site readily and to be hydrolyzed efficiently.

The organophosphate iso-OMPA, which contains four isopropyl groups, is a selective inhibitor of BtChoEase, inhibiting AcChoEase much more slowly than BtChoEase from the same species (5). Although iso-OMPA is a rather poor inhibitor of wild-type AcChoEase, it inhibits the F288L-F290V mutant much more effectively (Table 3). Presumably the double mutation provides space for entrance of the bulky organophosphate inhibitor, just as for BtCho.

**Mutation Involving the Peripheral Anionic Site.** Two other changes involving substitution of aromatic by nonaromatic residues are of amino acids the aromatic side chains of which undergo localized conformational changes upon binding of the competitive inhibitors tacrine and edrophonium, as shown by our crystallographic data (M.H., I.S., and J.L.S., unpublished work). These changes are in Trp-279 and Phe-330. Phe-330 is near the ligand-binding site, and in both complexes, the conformational change seen involves an aromatic-aromatic or an aromatic-quaternary nitrogen interaction with the bound ligand (M. Verdonk, private communication). Trp-279 is, however, at least 8 Å away from the "anionic" site; any direct contact with a small inhibitor bound can thus be precluded. Bisquaternary ligands, such as decamethonium (33), are more potent inhibitors of AcChoEase than the corresponding monoquaternary ligands; this has been ascribed to their binding simultaneously to the anionic subsite of the catalytic site and to the "peripheral" anionic site (6). Binding studies of several series of *n*-alkyl bisammonium ions (34, 35) have shown an optimal separation of 14–15 Å between the two quaternary groups, whereas the distance between the two indole moieties of Trp-84 and Trp-279 is  $\approx 16$  Å in AcChoEase. These data can be rationalized by assuming that Trp-279, at the mouth of the active-site gorge, is an important component of the peripheral anionic site. Our recent study of the complex of T-AcChoE-

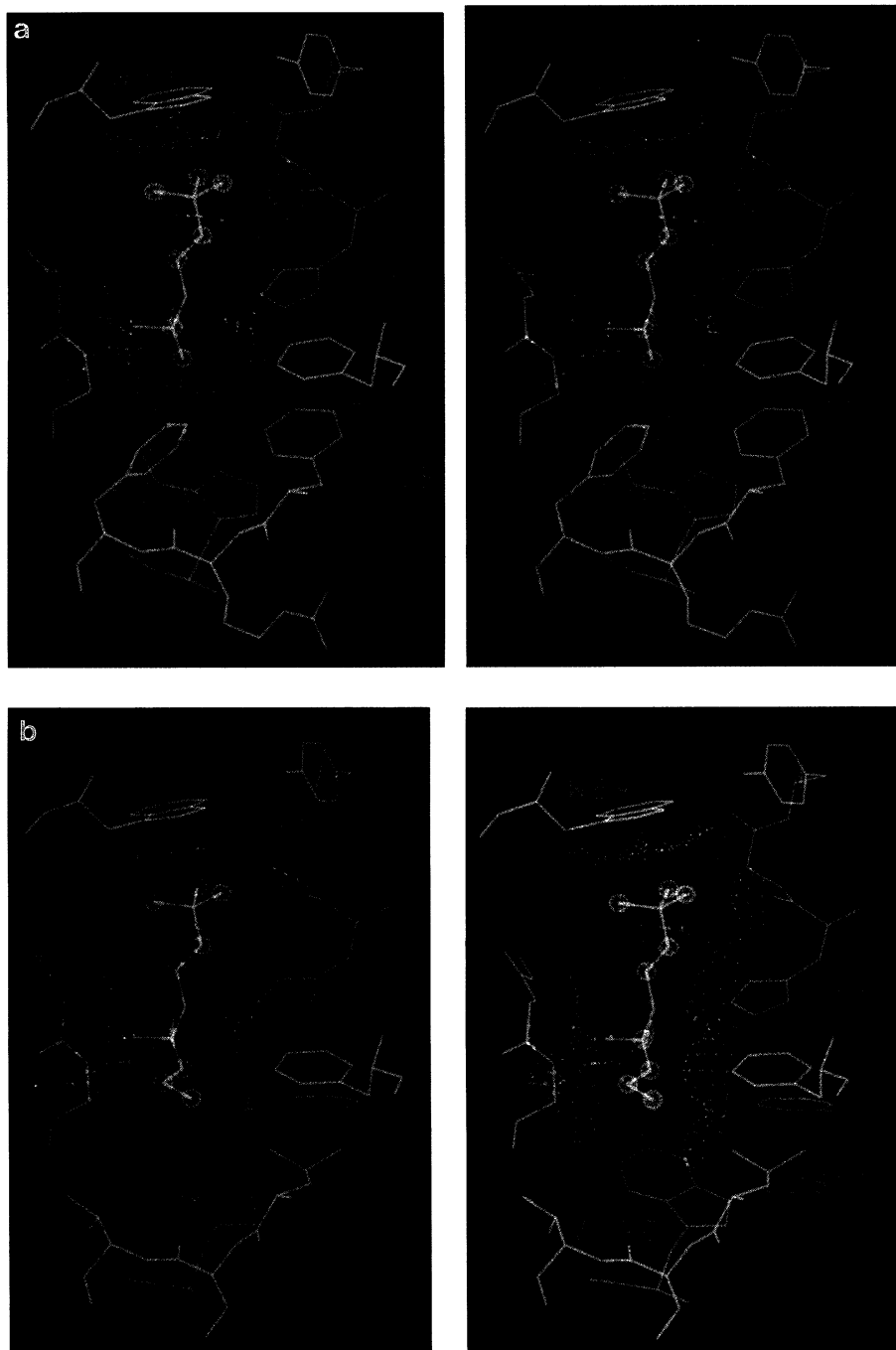


FIG. 4. Stereo view of van der Waals surface of atoms with 3 Å of AcCho in the T-AcChoEase x-ray structure (a) and BtCho in the h-BtChoEase model (b).

ase with the potent AcChoEase-selective bisquaternary anticholinesterase agent BW284C51 (5) clearly implicates Trp-279 as the binding site for the distal quaternary group (M.H., I.S., and J.L.S., unpublished work). The enhanced potency

Table 2. Activity of wild-type (WT) AcChoEase and F288L-F290V double mutant on acetylthiocholine and butyrylthiocholine

	Activity, % on 1.2 mM acetylthiocholine			
	Acetylthiocholine		Butyrylthiocholine	
	1.2 mM	25 mM	1.2 mM	25 mM
WT	100	142	ND	ND
F288L-F290V	100	120	40	105

Relative activities on acetylthiocholine and butyrylthiocholine were determined by the Ellman procedure. ND, not detectable.

of bisquaternary compounds relative to the corresponding monoquaternary ligands does not hold for BtChoEase (6). Replacement of Trp-279 by alanine in BtChoEase might

Table 3. Inhibition of wild-type (WT) AcChoEase and F288L-F290V double mutant by iso-OMPA

	Activity, % residual on [ <sup>3</sup> H]AcCho	
	110 min	200 min
WT	91	73
F288L-F290V	4	2

Inhibition was done by incubation with 1 mM iso-OMPA for the indicated times in 0.1 M phosphate buffer, pH 7.0, at room temperature.

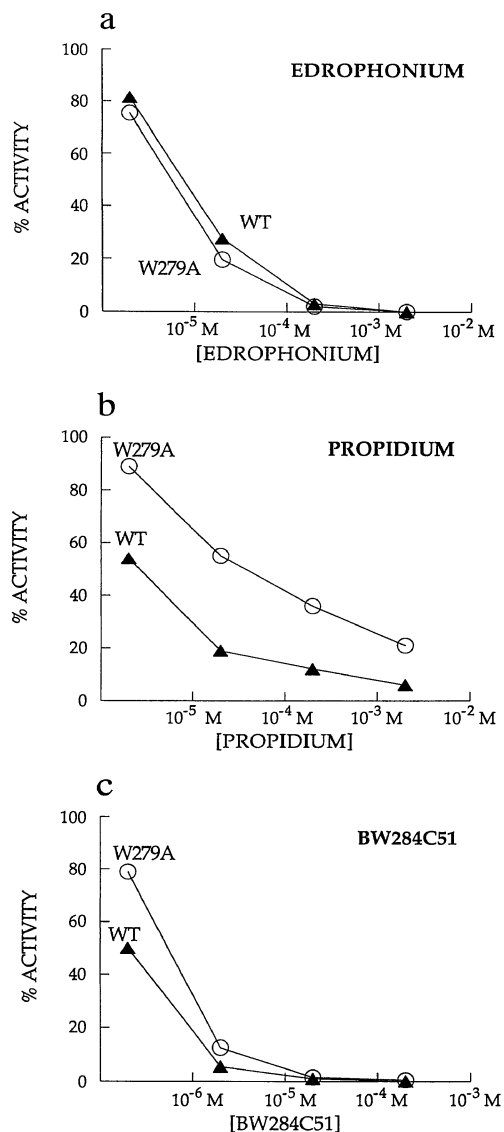


FIG. 5. Inhibition of wild-type (WT) AcChoEase and the W279A mutant by edrophonium (a), propidium (b), and BW284C51 (c). Activity was measured radiometrically.

explain its lack of a peripheral site and, thus, its failure to display enhanced sensitivity to bisquaternary inhibitors.

The mutation W279A was designed and expressed to test the above hypothesis. Active-site titration shows that it retains  $\approx 80\%$  of the specific activity of wild-type AcChoEase toward AcCho. Fig. 5a shows that inhibition of W279A by edrophonium, which binds specifically to the anionic subsite of the catalytic site, is similar to that of the wild type. In contrast, inhibition by the peripheral-site ligand propidium is reduced at least 10-fold (Fig. 5b). The inhibitory capacity of the bisquaternary inhibitor BW284C51, which is believed to span the two sites, is reduced (Fig. 5c) but far less than that of propidium.

AcChoEase is inhibited by excess AcCho, whereas BtChoEase is not (4). Recent evidence (36) suggests that the peripheral anionic site and the AcCho-binding site involved in substrate inhibition may be identical. Measurement of the degree of substrate inhibition obtained for W279A at 50 mM acetylthiocholine showed, however, only slightly less substrate inhibition than for the wild type (30% versus 40%), relative to the activity seen at 1.2 mM substrate. It should be borne in mind that BtChoEase displays substrate inhibition with benzoylcholine (37) and that the mutation of Glu-199, adjacent to Ser-200, abolishes substrate inhibition in T-AcCho-

Ease (38). Thus the molecular basis for substrate inhibition in both AcChoEase and BtChoEase remains to be clarified.

The studies outlined above show that, for the pair of enzymes under consideration, modeling was of powerful predictive capacity. Following the predictions, it was possible to convert AcChoEase to a BtCho-hydrolyzing enzyme and to strongly reduce the affinity of a peripheral ligand-binding site that is absent in BtChoEase.

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