Substrate specificity determinants of human acetylcholinesterase (HuAChE) were identified by combination of molecular modeling and kinetic studies with enzymes mutated in residues Trp-86, Trp-286, Phe-295, Phe-297, Tyr-337, and Phe-338. The substitution of Trp-86 by alanine resulted in a 660-fold decrease in affinity for acetylthiocholine but had no effect on affinity for the isosteric uncharged substrate (3,3-dimethylbutylthioacetate). The results demonstrate that residue Trp-86 is the anionic site which binds, through cation-π interactions, the quaternary ammonium of choline, and that of active center inhibitors such as edrophonium. The results also suggest that in the noncovalent complex, charged and uncharged substrates with a common acyl moiety (acetyl) bind to different molecular environments. The hydrophobic site for the alcoholic portion of the covalent adduct (tetrahedral intermediate) includes residues Trp-86, Tyr-337, and Phe-338, which operate through nonpolar and/or stacking interactions, depending on the substrate. Substrates containing choline but differing in the acyl moiety (acetyl, propyl, and butyryl) revealed that residues Phe-295 and Phe-297 determine substrate specificity of the acyl pocket for the covalent adducts. Phe-295 also determines substrate specificity in the noncovalent enzyme substrate complex and thus, the HuAChE F295A mutant exhibits over 130-fold increase in the apparent bimolecular rate constant for butyrylthiocholine compared with wild type enzyme. Reactivity toward specific butyrylcholinesterase inhibitors is similarly dependent on the nature of residues at positions 295 and 297. Amino acid Trp-286 at the rim of the active site “gorge” and Trp-86, in the active center, are essential elements in the mechanism of inhibition by propidium, a peripheral anionic site ligand. Molecular modeling and kinetic data suggest that a cross-talk between Trp-286 and Trp-86 can result in reorientation of Trp-86 which may then interfere with stabilization of substrate enzyme complexes. It is proposed that the conformational flexibility of aromatic residues generates a plasticity in the active center that contributes to the high efficiency of AChE and its ability to respond to external stimuli.

Acetylcholinesterase (AChE, EC 3.1.1.7) is a serine hydrolase selectively reacting with its natural substrate acetylcholine (ACh) at close to diffusion control rate (Bazelyansky et al., 1966; Quinn, 1987). Kinetic studies with different substrates, as well as reversible and irreversible inhibitors, imply that the AChE active center consists of several major domains: (a) an esteratic site containing the active serine, (b) an anionic site that accommodates the positive pole of ACh, (c) hydrophobic sites which bind aryl substrates, other uncharged ligands and the alkyl portion of the acyl moiety (Quinn, 1987; Hucho et al., 1991).

The location and spatial organization of the active site serine (MacPhee-Quigley et al., 1985) and histidine (Krupka, 1966) constituting the esteratic site of AChE were determined by site-directed mutagenesis (Gibney et al., 1990; Shafferman et al., 1992a) and x-ray crystal structure studies (Sussman et al., 1991). Mutagenesis studies identified and provided biochemical evidence for the involvement of a glutamic residue in the catalytic triad (Shafferman et al., 1992a, 1992c), as also predicted by the x-ray crystal structure of Torpedo californica AChE (TeAChE; Sussman et al., 1991).

The high catalytic efficiency of AChE is attributed to the esteratic triad as well as to structural elements of recognition such as the anionic and hydrophobic subsites which confer specificity for particular alcoholic (X) and alkyl (R) groups (see Scheme 1).

Earlier studies have postulated that the anionic site contains multiple charges (Nölte et al., 1986). On the other hand, the pronounced catalytic activity of AChE toward neutral substrates (Cohen et al., 1985) led to the proposal of a hydrophobic trimethyl binding site for accommodation of the quaternary functionality (Hassan et al., 1980). Distinct binding subsites for charged and neutral alcohol moieties (X in Scheme 1 and in Fig. 1) of the substrates have also been proposed (Berman and Decker, 1986). Recently several specific amino acids were implicated in stabilization of the active center.

The abbreviations used are: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; HuAChE, human acetylcholinesterase; Tc-AChE, T. californica acetylcholinesterase; ACh, acetylcholine; BCh, butyrylcholine; ATC, acetylthiocholine; BTC, butyrylthiocholine; PTC, propionylthiocholine; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); PNPA, p-nitrophenyl acetate; iso-OMPA, tetraisopropylpyrophosphoramide; TPA, S-n-propyl thioacetate; TB, S,3,3-dimethylbutyl thiosulfate.
charged X moiety of ACh. In human AChE (HuAChE) replacement of Trp-86 (Trp-84)2 resulted in at least 100-fold reduction in the catalytic potential of the enzyme (Shafferman et al., 1992c). The same tryptophan residue in TcAChE was labeled by phenyl azidirnidine, and the reaction was blocked by edrophonium, an active center inhibitor (Kreienkamp et al., 1991; Weise et al., 1990). According to the x-ray structure data the Trp-84 residue of TcAChE is located within a suitable distance from the esteratic site for accommodation of ACh (Sussman et al., 1991). In addition to Trp-84 (Trp-86) (Shafferman et al., 1992b, 1992c), residues Tyr-337 (Phe-333) and Phe-338 (Phe-331) appear to interact with the substrate as well, since mutations Y337A and F338A in HuAChE resulted in a significant decrease in kcat of acetylthiocholine (Shafferman et al., 1992b, 1992c). Involvement of residues Phe-330 and Phe-331 in the active center of TcAChE was also indicated by labeling studies (Kieffer et al., 1986; Schalk et al., 1992).

Another aspect of AChE specificity is exemplified by the different rates of hydrolysis for ACh and butyrylcholine (BCh) (Augustinsson and Nachmansohn, 1949). Structure-activity relationship studies with acylcholine substrates were rationalized by postulating a hydrophobic pocket (Jarv, 1984) of a limited size that accommodates a methyl group better than bulkier R groups (Scheme I). Size restrictions of this pocket were also implicated in the stereoselectivity of AChE toward chiral phosphonates (Benshop and De Jong, 1988). Modeling of the butyrylcholinesterase (BChE)-BCh adduct (Harel et al., 1992a) and of the HuAChE adducts with chiral phosphonates (Barak et al., 1992) identified the size restricting elements of the acyl pocket as well as Tyr-337 (Phe-333) and Phe-327 (Phe-329). A careful analysis of the role of these residues in the formation of the initial enzyme-substrate complex or during the acylation-deacylation stages is yet to be performed.

In an attempt to further explore the structure-function relationships underlying the complex AChE substrate specificity, we employ here site directed mutagenesis and molecular modeling techniques together with examination of the kinetic behavior of the resulting mutants toward substrates and inhibitors. We identify some of the major structural elements of the hydrophobic subsite, provide evidence that this site is distinct from the anionic subsite, which is determined by HuAChE Trp-86, and demonstrate a role for Phe-295 and Phe-297 in the acyl pocket.

**Experimental Procedures**

**Mutagenesis of Recombinant HuAChE and Construction of Expression Vectors—**Mutagenesis of AChE was performed by DNA cassette replacement into a series of HuAChE sequence variants which conserve the wild type (Sreeq et al., 1990) coding specificity, but carry new unique restriction sites (Velan et al., 1991a; Kronman et al., 1992; Shafferman et al., 1992a). Generation of mutants W86A(W84), W286A(W279), Y337A(F330) Y337F, and F338A(F331) was described previously (Shafferman et al., 1992b). Substitution of residues Phe-286(Phe-288) and Phe-297(Phe-290) was carried out by replacement of the Mut-l-Narl DNA fragment of the AChE-w7 variant (Shafferman et al., 1992a) with synthetic DNA duplexes carrying the mutated codons. The TTC codon of Phe-295 was changed to CTG(Leu) or GCC(Ala) and that of Phe-297 to GTG(Val) or GCC(Ala). All the synthetic DNA oligodeoxynucleotides were prepared using the automatic Applied Biosystems DNA synthesizer. The sequences of all new clones were verified by the dieoxy sequencing method (United States Biochemical Corp. Sequenase kit). The HuAChE cDNA mutants were expressed in tripartite vectors which allows expression of the cat reporter gene and the neo selection marker (Kronman et al., 1992; Shafferman et al., 1992a).

**Transient Transfection and Quantitation of AChE—**Human embryonal 293 cells were transfected by purified plasmid preparations using the calcium phosphate method. Transient transfection was carried on as described previously (Shafferman et al., 1992a, 1992b) and stably transfected colony pools (Velan et al., 1991b) were generated by G418 selection. AChE secreted by transiently or stably transfected cells was collected by incubating cells in 2 ml of AChE-depleted medium (Shafferman et al., 1992a) (100-mm plate for 48 h). AChE activity in cell supernatant was tested as described below and AChE-protein mass was determined by a specific enzyme-linked immunosorbent assay (ELISA) (Shafferman et al., 1992a). Efficiency of transfection was monitored (Kronman et al., 1992) by levels of the coexpressed chloramphenolic acetyltransferase activity (Gorman et al., 1982).

**Substrates and Inhibitors—**Purified human serum butyrylcholinesterase (HuBChE), acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC), propionylthiocholine iodide (PTC), 5:5'-dithio-1-nitrobenzoic acid (DTNB), p-nitrophenyl acetate (PNPA), 3,4-diamino-5,5'-trimethylammoniumpropyl-6-phenyl phenanthridinium iodide (propidium), ethyl-1-hydroxymethyl-1-decyllanum chloride (edrophonium), di(p-allyl-N-methylamino)phenyl)pentane-3,1-one (BW284C51), and tetraisopropylpyrophosphoramide (iso-OMPA) were all purchased from Sigma. N-Propylthioiacteate (CH3C(O)SC3H7) (TFA) was synthesized from n-propylmercaptane and acetyclcholide with triethylamine. Distillation afforded 95% yield of the product, b.p. 53 °C/20 mmHg (b.p. 139.8 °C/760 mmHg (Wenzel and Emmel-Reid, 1937)). Structural assignment was confirmed by NMR spectroscopy. S-3,3-Dimethylbutyl thioacetate (CH3C(O)SC3H7CH2) (TB) was synthesized using the following steps: (a) 3,3-Dimethylbutanol was obtained by LiAIH reduction of 3,3-dimethylbutyric acid. Distillation resulted in 93% yield of the alcohol b.p. 68 °C/25 mmHg (b.p. 144 °C/740 mmHg (Sarel and Newman, 1956)). (b) 3,3-Dimethylbutyl bromide was obtained from the alcohol and aqueous HBr 48%. Distillation gave 91% yield of the bromide b.p. 40 °C/20 mmHg (b.p. 88.5 °C/51 mmHg (Backer et al., 1936)). (c) The final product was obtained by reacting the dimethyl butyl bromide with thiocetic acid and KOH in ethanol. Distillation gave 90% yield of the thioacetate b.p. 79 °C/20 mmHg (b.p. 165-186 °C (Bazelyansky et al., 1986)). Structural assignment was confirmed by NMR spectroscopy.

**Determination of AChE Activity and Analysis of Kinetic Data—**AChE activity with thioesters was assayed according to Ellman et al. (1961). Standard assays were performed in the presence of 0.1 mg/ml bovine serum albumin, 0.3 mM DTNB, 50 mM sodium phosphate buffer, pH 8.0, and varying substrate (ATC, BTC, PTC, TB, or TPA) concentrations. Hydrolysis of ENPA was performed in the buffer solution described above but without DTNB, and the reaction product was monitored by the absorbance of the p-nitrophenylate anion at 405 nm. The assays were carried out at 27 °C and monitored by a ThermoMax microplate reader (Molecular Devices). Michaelis-Menton constants (Km) values were obtained from the double-reciprocal Lineweaver-Burk plots, and kcat calculations were based on ELSIA quantitations (Shafferman et al., 1992a). Interactions with inhibitors were monitored by determining residual activity following preincubation of HuAChE or its mutants for 20 min with increasing concentrations of edrophonium, propidium, iso-OMPA, or BW284C51.

**SCHEME 1**

```
O
E-OH + \( h_1 \) \( \text{R} \) \( \text{R} \) \( k_1 \) \( E-O-C \) \( \text{R} \) \( \text{R} \) \( \text{H}_2 \text{O} \) \( \text{R} \) \( \text{R} \) \( k_2 \) \( E-O-C \) \( \text{R} \) \( \text{R} \) \( \text{R} \) \( \text{R} \) \( \text{C-OX} \)
```

2 Amino acids and numbers in parenthesis refer to the position of analogous residues in TeAChE, according to the recommended nomenclature (Massoulié et al., 1992).
$K_e$ values for the reversible competitive inhibitor edrophonium were calculated from the equation $K_e = IC_{50}/[1 + ([S]/K_m)]$ as described by Hobbiger and Peck (1969), where $IC_{50}$ is the concentration resulting in 50% inhibition and [S] the substrate concentration.

Kinetic data for inhibition by propidium were analyzed according to the kinetic treatment developed by Barnett and Roseneberry (1977) and Berman and Leonard (1980) for the reaction described in the following scheme.

In this scheme $K$ is the competitive inhibition constant and $K_c$ the noncompetitive inhibition constant. A kinetic solution for the dependence of the reciprocal rate on the inverse concentration of substrate is provided in Equation 1. The expressions of relative slopes ($R_i$) and relative intercepts ($R_o$), in presence and absence of inhibitor, are provided by Equations 2 and 3, respectively. Values of $R_o$ and $R_i$ can be computed from the double-reciprocal plots of rate versus substrate concentration.

If replots of $R_o$ versus inhibitor concentration ([II]) are linear, $\alpha/K_m = 0$; and $K_c$ is given by the reciprocal of the slope of $R_o$ versus [I]. In such cases $K_m$ is derived from the reciprocal of the slope of $R_o$ versus [I]. When replots of $R_o$ are not linear, the enzyme cannot be completely inhibited, $K_c$ can then be derived from the double-reciprocal plots of ($R_o - 1$) versus [I] (Barnett and Rosenberry, 1977). In such cases an independent $K_m$ value can not be derived.

### Structure Analysis and Molecular Graphics—Building and analysis of the three-dimensional molecular models was performed on Silicon Graphics work station IRIS70/GT, using the SYBYL modeling software (Tripos Inc.). Construction of models for HuAChE mutants and the corresponding adducts with substrates and inhibitors were based on a model structure of the enzyme obtained by comparative modeling (Barak et al., 1992) from coordinates of TcAChE (Sussman et al., 1991). Structural refinement by molecular mechanics was done using the MAXMIN force field and zone refinement procedure Anneal, both included in SYBYL. The zone of refinement included 33 amino acids within the active site gorge and in its close vicinity.

### RESULTS

#### Reactivity of HuAChE and Selected Mutants toward Substrates Containing Charged and Uncharged Alcohol Moieties

To study enzyme specificity toward the alcoholic domain (X in Scheme 1 and Fig. 1) several substrates with a common acyl moiety (acetyl) were analyzed. Besides the charged choline moiety of ATC, three uncharged X residues (Fig. 1) with different properties were selected for study: a reactive alkyl acetate (TB), a relatively unreactive alkyl acetate (TPA), and an aryl ester (PNPA). TB contains a t-butyl moiety which is isosteric with the trimethyl ammonium group of ATC and should therefore allow examination of the net effect of charge on reactivity. The aromatic amino acids in the active center that were previously implicated (see Introduction) in interactions with the choline moiety of ACh, namely Trp-86, Phe-338, and Tyr-337, were selected for study. In addition to wild type HuAChE and to the active center mutants, W86A, Y337A, and F338A, we also included an alanine mutant of Trp-286, an amino acid located at the rim (Barak et al., 1992) of the HuAChE active center "gorge." We have shown previously that Trp-286 may be involved in substrate inhibition by triggering a conformational change of Tyr-337 in the active center (Shafferman et al., 1992b). According to Scheme 1, the Michaelis-Menten constant ($K_m$) corresponds to $k_3/(k_2 + k_3)$. The apparent catalytic first order rate constant ($k_{cat}$) corresponds to the ratio of first order rate constants for acylation ($k_2$) and deacylation ($k_3$) and is equal to $k_2/k_3$. Using the Lineweaver-Burk analysis, $K_m$, $K_{cat}$, and the apparent molecular rate constants ($k_{cat}/K_m$) were calculated for the hydrolysis of the various substrates by HuAChE and its mutants (Table I). The $K_m$ value of the wild type enzyme for ATC was about 2 orders of magnitude higher than that for TPA and PNPA and about 7-fold higher than that for TB (Table I). Considering the differences in enzyme source and assay conditions, these values are in good agreement.

### SUBSTRATES

<table>
<thead>
<tr>
<th>Chemical formula</th>
<th>Substrates Containing Charged and Uncharged Alcohol Moieties</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTC</td>
<td>PTC</td>
</tr>
<tr>
<td>PTC</td>
<td>ATC</td>
</tr>
<tr>
<td>ATC</td>
<td>TB</td>
</tr>
<tr>
<td>TB</td>
<td>TPA</td>
</tr>
<tr>
<td>TPA</td>
<td>PNPA</td>
</tr>
</tbody>
</table>

### INHIBITORS

<table>
<thead>
<tr>
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<th>INHIBITORS</th>
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</thead>
<tbody>
<tr>
<td>BW284C51</td>
<td>Propidium</td>
</tr>
<tr>
<td>Edrophonium</td>
<td>iso-OPMA</td>
</tr>
</tbody>
</table>

**FIG. 1.** Chemical formula of substrates and inhibitors used in this study.
Determinants of AChE Substrate Specificity

**Table I**

Kinetic constants for the hydrolysis of ATC, TB, TPA and PNPA by HuAChE and selected HuAChE mutants

<table>
<thead>
<tr>
<th>Substrate</th>
<th>kcat</th>
<th>kcat/Km (ATC)</th>
<th>kcat/Km (TB)</th>
<th>kcat/Km (TPA)</th>
<th>kcat/Km (PNPA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATC</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>W86A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W286A</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Y337A</td>
<td></td>
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<tr>
<td>F338A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W86A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
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<td></td>
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<tr>
<td>AChE</td>
<td></td>
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</tr>
</tbody>
</table>

Values represent mean of triplicate determinations, with standard deviations not exceeding 20%.

**Table II**

Edrophonium inhibition of hydrolysis of various substrates by HuACh and selected mutants

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K_i (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATC</td>
<td></td>
</tr>
<tr>
<td>TB</td>
<td></td>
</tr>
<tr>
<td>TPA</td>
<td></td>
</tr>
<tr>
<td>PNPA</td>
<td></td>
</tr>
</tbody>
</table>

Enzyme inhibition by edrophonium was determined as described under "Experimental Procedures." Values represent mean of triplicate determinations, with standard deviations not exceeding 20%.

*Wild type recombinant HuAChE.

**Inhibition by Edrophonium**—Inhibition of the hydrolysis of various substrates by edrophonium, a reversible active center inhibitor, for HuAChE and the four mutants is summarized in Table II. We have shown previously that an aromatic amino acid at position 337 is required for better interaction of edrophonium with the active center of AChE (Shafferman et al., 1992b, 1992c). Indeed, irrespective of the substrate used, the Y337A mutant exhibits about 10–40 increase in edrophonium inhibition constants compared with the values for the wild type enzyme. The effects of W86A were even more pronounced. Edrophonium at 150 μM, a concentration 100-fold higher than that required to achieve 50% inhibition (IC50) of wild type enzyme, was insufficient to inhibit the catalysis of any of the substrates tested by the W86A mutant. These results suggest that Trp-86 is a part of the edrophonium binding site, which is in agreement with x-ray crystal data (Sussman et al., 1992), and with our assignment of the anionic subsite to this residue.
propidium inhibition patterns obtained with the active center but actually accelerates the catalysis (Fig. 3). Altogether, the  

eral anionic site at the exterior and the Trp-86 residue which  

crease in 

propidium, respectively. 

The most striking observation is the inefficiency of propidium to inhibit catalysis in the W86A mutant for all the  

substrates. In the case of ATC hydrolysis, in the W86A mutant  

only 35% inhibition could be achieved at very high 

acetylthiocholine concentration in presence and absence of propidium, respectively. 

Accordingly, iso-OMPA ICso values for the F295L, F295A, F297V, F297A, F295L/F297V, Y337F, Y337A, and F338A. The substrates ATC, BTC, and PTC, which have a common X (choline) residue but differ in their R substituent (see Scheme 1 and Fig. 1), were selected for probing the effect of different acyl moieties on substrate specificity. Results from kinetic studies, summarized in Table IV, demonstrate that any of the substitutions of Phe-295 by leucine or alanine and of Phe-297 by valine or alanine resulted in a significant increase in kcat for BTC relative to wild type (Fig. 4). The same substitutions hardly affected the kcat for PTC but reduced to some extent the kcat for ATC. These results clearly demonstrate the importance of both Phe-295 and Phe-297 in determining substrate specificity. In contrast to the effect of these mutations on kcat, their effect on Km differentiate between Phe-295 and Phe-297 mutations. HuAChE F295L and F295A mutants had lower Km for BTC than the wild type HuAChE or the Phe-297 mutants (Table IV and Fig. 4). Moreover Km values for ATC and PTC were somewhat higher for Phe-297 mutant (relative to wild type). The double mutant F295L/F297V and the F295L mutant had similar Km values for BTC, suggesting that residue 295 is the major contributor to the improved interaction of BTC with the mutein enzyme. We note that BChE has the Leu-295/Val-297 configuration and the Km for butyrylthiocholine is similar to that of the HuAChE double mutant (F295L/F297V) (Table IV). Moreover, comparison of the Km values between the HuAChE F295A mutant and BChE suggests that an engineered AChE mutant could be more specific for BTC than BChE. Recently the analogous double mutant in TcAChE (F288L/290V) was reported to have increased hydrolytic activity toward BTC (Harel et al., 1992b).

Replacement of Tyr-337 or Phe-338 by alanine reduced the kcat for ATC, PTC, and BTC (Table IV). When the tyrosine at position 337 was replaced by another aromatic amino acid as in the Y337F mutant values similar to those of wild type were obtained. The results suggest that, the aromatic side chains, Tyr-337 and Phe-338, play some role in the stabilization of the acyl moiety during acylation or deacylation. The Phe-338 mutant, unlike the Tyr-337 mutant, appears to have an effect on the Km values with the substrates tested. Thus the Phe-338 residue could be involved in stabilizing the acyl moiety of the substrate in the noncovalent enzyme-substrate complex. We note that Phe-338 had similar effects on the X moiety of the compound (see above and Table I).

Interaction with Reversible and Irreversible Inhibitors—The inhibition of the 295 and 297 mutants by selective BChE and AChE inhibitors further supports the kinetic data, suggesting the involvement of these residues in determining substrate specificity. Although iso-OMPA, a selective BChE inhibitor, is a slow irreversible inhibitor, we used the IC50 values as a crude indicator for relative inhibition. Accordingly, iso-OMPA IC50 values for the F295L, F295A, F297V, F297A mutants and for the double F295L/297V mutant are all lower by 5-30-fold than that for wild type AChE. Interestingly, the specificity of the AChE F295A and F297A mutants toward

The nature of the tested substrate. On the other hand the inhibition constants and kinetic behavior of the ternary complexes (ESI, Scheme 2) are substrate dependent (Table III and Fig. 3). Since ATC, TB, and PNPA should generate a common acyl intermediate, the differential effect of propidium should be related to interaction of inhibitor with the different enzyme-substrate complexes.

The most striking observation is the inefficiency of propidium to inhibit catalysis in the W86A mutant for all the tested substrates. In the case of ATC hydrolysis, in the W86A mutant only 35% inhibition could be achieved at very high propidium concentrations (0.15 mM) and plots of double-reciprocals of rate versus substrate concentration (Fig. 2C) manifest an almost pure uncompetitive inhibition pattern. Accordingly, a 700-fold increase in Kc and only 10-fold increase in Ks value relative to the wild type is observed (Table III). The above observations clearly demonstrate that mutation in position Trp-86 reduces the ability of propidium to inhibit the mutant enzyme and that the residual inhibition proceeds through the ternary complex ESI (see Scheme 2; Ks >> Kc). In the cases of TB and PNPA hydrolysis by the W86A mutant, propidium is not only inefficient as inhibitor but actually accelerates the catalysis (Fig. 3). Altogether, the propidium inhibition patterns obtained with the active center mutant W86A strongly suggest coupling between the peripheral anionic site at the exterior and the Trp-86 residue which is buried deeply within the protein.

Reactivity of HuAChE and Selected Mutants toward Substrates Containing Different Acyl Moieties

Molecular modeling of HuAChE adducts with chiral methyl phosphonates (Barak et al., 1992) and modeling of the BChE-BCh complex (Harel et al., 1992a) showed that the two phenylalanine residues at positions 295 and 297, which are replaced by leucine and valine, respectively, in BChE, may prevent accommodation of large acyl moieties. Based on our modeling studies with HuAChE (see below), the Phe-338 and Tyr-337 residues may also take part in accommodation of the acyl moiety. Accordingly we studied the following AChE mutants: F295L, F295A, F297V, F297A, F295L/F297V, Y337F, Y337A, and F338A. The substrates ATC, BTC, and PTC, which have a common X (choline) residue but differ in their R substituent (see Scheme 1 and Fig. 1), were selected for probing the effect of different acyl moieties on substrate specificity. Results from kinetic studies, summarized in Table IV, demonstrate that any of the substitutions of Phe-295 by leucine or alanine and of Phe-297 by valine or alanine resulted in a significant increase in kcat for BTC relative to wild type (Fig. 4). The same substitutions hardly affected the kcat for PTC but reduced to some extent the kcat for ATC. These results clearly demonstrate the importance of both Phe-295 and Phe-297 in determining substrate specificity. In contrast to the effect of these mutations on kcat, their effect on Km differentiate between Phe-295 and Phe-297 mutations. HuAChE F295L and F295A mutants had lower Km for BTC than the wild type HuAChE or the Phe-297 mutants (Table IV and Fig. 4). Moreover Km values for ATC and PTC were somewhat higher for Phe-297 mutant (relative to wild type). The double mutant F295L/F297V and the F295L mutant had similar Km values for BTC, suggesting that residue 295 is the major contributor to the improved interaction of BTC with the mutein enzyme. We note that BChE has the Leu-295/Val-297 configuration and the Km for butyrylthiocholine is similar to that of the HuAChE double mutant (F295L/F297V) (Table IV). Moreover, comparison of the Km values between the HuAChE F295A mutant and BChE suggests that an engineered AChE mutant could be more specific for BTC than BChE. Recently the analogous double mutant in TcAChE (F288L/290V) was reported to have increased hydrolytic activity toward BTC (Harel et al., 1992b).

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Inhibition constants of propidium for hydrolysis of various substrates by HuAChE and selected mutants

Table 111

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ki (mM)</th>
<th>Ki (mM)</th>
<th>Ki (mM)</th>
<th>Ki (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATC</td>
<td>1.4</td>
<td>1.8</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>TB</td>
<td>14.3</td>
<td>28.3</td>
<td>12.3</td>
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</tr>
<tr>
<td>PNPA</td>
<td>870.0</td>
<td>13.5</td>
<td>c</td>
<td>c</td>
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</tbody>
</table>

WT: Wild type recombinant HuAChE.
W286A: Nonlinear inhibition pattern was observed (see Fig. 3). The values of the competitive inhibition constant (Ki) were calculated from the slopes and intercepts of the double reciprocal plots of (1/R - 1) versus inhibitor concentration, whereas Ki values could not be extracted in these cases (see methods and text).
W86A: For the substrates TB and PNPA kinetic behavior did not follow mixed linear or nonlinear inhibition pattern and at high inhibitor concentrations indication of acceleration was observed (see Fig. 3 and text).

Substitution of the HuAChE aromatic residues Tyr-337 and Phe-296/Phe-297 by the corresponding residues of HuBChE (Y337A and F295L/F297V) increased the Ki values for inhibition of the mutated enzymes by edrophonium by a factor of 30 and 7, respectively, as compared with wild type HuAChE. This observation is in accordance with the overall 178-fold higher Ki for edrophonium in BChE than in AChE. Mutations at position 295 and 297 appear to have a small effect on susceptibility to propidium (data not shown).

Molecular Modeling of HuAChE, Its Mutants, and Their Substrate Adducts

Models of the HuAChE mutants and enzyme-substrate adducts were constructed by replacing the appropriate amino acid and ligand followed by zone refinement (see "Experimental Procedures"). Since, in all cases considered here, the mutants retained some of the original catalytic activity, we assumed that the structure of the molecule and in particular the spatial architecture of the active site gorge was only locally perturbed (Bone and Agard, 1991). The initial model for docking ACh within the active site of HuAChE (Barak et al., 1992), which is analogous to that reported for TcAChE-ACh adduct (Sussman et al., 1991), included positioning of a tetrahedral carbon within bonding distance of Or-S203. The model places the quaternary ammonium group within an interaction distance from Trp-86 and Glu-202. The main stabilizing interaction is that of the acyl oxygen nested within the "oxyanion hole" (hydrogen bonds to the amide nitrogens of Gly-121, Gly-122, and Ala-204). Additional stabilization is conferred by the choline oxygen bound to Nε-His-447 and the
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**TABLE IV**

Kinetic constants for hydrolysis of ATC, PTC, and BTC by HuAChE, selected HuAChE mutants, and HuBChE

Values represent mean of triplicate determinations with standard deviations not exceeding 20%.

<table>
<thead>
<tr>
<th></th>
<th>(k_m^{\text{ATC}})</th>
<th>(k_m^{\text{PTC}})</th>
<th>(k_m^{\text{BTC}})</th>
<th>(k_{cat}^{\text{ATC}})</th>
<th>(k_{cat}^{\text{PTC}})</th>
<th>(k_{cat}^{\text{BTC}})</th>
<th>(k_{cat}/K_m)</th>
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<tr>
<td>WT</td>
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<td>0.9</td>
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<td>HuBChE</td>
<td>0.035</td>
<td>0.055</td>
<td>0.05</td>
<td>0.5</td>
<td>1.0</td>
<td>1.10</td>
<td>14.3</td>
</tr>
</tbody>
</table>

* Wild type recombinant HuAChE.
* Values for HuBChE were determined using purified human serum butyrylcholinesterase.

![Fig. 4](image-url)

**Fig. 4.** The ratio of kinetic constants for butyrylthiocholine hydrolysis by HuAChE mutants and the wild type enzyme. Relative kinetic constants were calculated from values presented in Table IV.

methyl group of the acyl moiety forming close contacts (hydrophobic interactions) with the aromatic rings of Phe-295 and Phe-297 (Fig. 5a). This basic model served as a template for modeling experiments of HuAChE-substrate adducts, since the interactions involving the three oxygen substituents of the tetrahedral carbon are assumed to provide most of the stabilization energy. Such models reflect better the tetrahedral intermediate of the acylation reaction than the product of noncovalent association (e.g. Michaelis-Menten complex) and therefore are more relevant to the molecular events influencing \(k_{cat}\) rather than those influencing \(K_m\).

**Models of HuAChE-ATC Adducts—**The constructed model (Fig. 5a) closely resembles our basic HuAChE-ACh structure. The only noticeable difference is in the shorter distance (4.45 Å versus 4.05 Å) of the quaternary ammonium nitrogen from the centroid of the indole ring of Trp-86 due to the longer S-C bond compared with the O-C bond.

**Models of HuAChE-PNPA Adducts—**For initial modeling the atomic positions of the tetrahedral carbon attached to Oyat-203 and its substituents were retained as in the HuAChE-ACh model and then the dihedral angles around bonds C-O and O-C aromatic, determining the orientation of the aryl moiety relative to the fixed tetrahedral carbon, were changed systematically. The most energetically probable orientation was achieved when the p-nitrophenyl ring was stacked against the phenyl moiety of Phe-338, whereas in the latter the dihedral angle \(\chi_2\) (\(\chi_1\) and \(\chi_2\) denote dihedral angles for bonds \(C_{\alpha}-C_{\beta}\) and \(C_{\beta}-C_{\gamma}\) of amino acid side chains, respectively) changed from \(-77^\circ\) to \(-101^\circ\) (Fig. 5d). Further zone refinement of the model did not result in significant changes of the structure. The two interesting features of this model are first, the identification of Phe-338 as an important structural element interacting with aryl acetates in the active site and second, the demonstration that the aryloxy moiety cannot assume the spatial orientation with respect to Trp-86, that was postulated for choline esters (compare Fig. 5, a and d). Indeed, our kinetic data show that although replacement of Phe-338 by alanine reduces \(k_{cat}\) for PNPA, the replacement of Trp-86 by alanine actually resulted in a small increase in \(k_{cat}\) for this substrate (Table I). The edgewise interaction (Burley and Petsko, 1988) of the p-nitrophenyl ring with the \(x\) electrons of Phe-337 in the final model was marginal, again in accordance with the minimal effect of the Y337A mutation on the PNPA kinetic parameters (Table I).

**Models of HuAChE-TR and HuAChE-TPA Adducts—**Adducts were constructed following a procedure similar to that described for HuAChE-ATC, but without the constrain of positioning the quaternary ammonium group. In both cases the conformational search, results in the trans-conformation for the alkylthio moiety. This conformation is different from...
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Fig. 5. Stereo view of adducts of HuAChE with charged and uncharged acetate-esters. Only the amino acids in the immediate vicinity of the ligand are displayed and hydrogen atoms are omitted for clarity. The ligand is marked by a heavy line. a, HuAChE-ATC adduct: distances of the negatively charged oxygen from amide nitrogens of Gly-121, Gly-122, and Ala-204 are 2.64, 2.60, and 2.82 Å, respectively; distances of the terminal methyl from aromatic C, of Phe-297 and from aromatic Cs of Phe-295 are 4.07 and 3.65, respectively. The above distances are similar for all other adducts (b–d). Distances of the quaternary ammonium N-methyl carbons from the centroids of corresponding pyrol and phenyl parts of the indole moiety are 3.41 and 3.37 Å, respectively; b, HuAChE-TB adduct: relative position of choline moiety is added for comparison (light line). Rotation around C-S bond places the three methyl carbon group in a hydrophobic environment created by aryl rings of Tyr-337 and Phe-338, the participation of Trp-86 in this stabilization was also considered (see text). c, HuAChE-TPA adduct: conformation of the S-alkyl chain is similar to that observed for TB adduct; an alternative conformation of Trp-86 side chain ($x_1 = 84^\circ; x_2 = 150^\circ$) is indicated by a light line. d, HuAChE-PNPA adduct: side chain positions of residues Phe-337, Phe-338, and Trp-86 are shifted compared with ATC adduct (light line). Relative position of choline moiety is added for comparison (light line). Position of W86 changed slightly during energy optimization.

determination of Trp-86 side chain ($x_1 = 84^\circ; x_2 = 150^\circ$) is indicated by a light line. d, HuAChE-PNPA adduct: side chain positions of residues Phe-337, Phe-338, and Trp-86 are shifted compared with ATC adduct (light line). Relative position of choline moiety is added for comparison (light line). Position of W86 changed slightly during energy optimization.

the one found for the choline part of ATC adduct and suggests a different spatial orientation of the possible interaction of Trp-86 with the alcohol portion of substrates like TB and TPA (Fig. 5, b and c). Stabilization energies for these alkylthio residues originate mainly from hydrophobic interactions which are a consequence of the complementarity of surface areas between the ligand and the active site gorge. The observed kinetic differences ($k_{cat}$) between TB and TPA support this conclusion, and the proposed models are in agreement with related correlations of volume activity for the alkylthio moieties (Cohen et al., 1985).

Optimization of the surface complementarity for the TB and TPA adducts included examination of the side chain flexibility of Trp-86, since the indole ring could provide part of the active site gorge surface in contact with the ligand. Surprisingly, the results indicated a relatively unrestrained movement of the indole moiety within a certain range of dihedral angles $\chi_1$ and $\chi_2$ for the adducts of uncharged substrates. Furthermore, and not less important, such motion could be observed also for the unoccupied enzyme. The torsional strain resulting from Trp-86 side chain reorientation is counterbalanced by van der Waals interactions with elements of the active site gorge and with the ligands when present. The feasibility of such movement is evident particularly in the case of TPA adduct. In the latter the thiopropyl group is too short to interact with the indole moiety in its unmodified conformation unless Trp-86 is allowed to rotate as described above (see Fig. 5c). Comparison of the extent of Trp-86 side chain movement, for the adducts examined, indicates that in the TPA adduct, as in the unoccupied enzyme, such motion can occur over the $\chi_2$ range 183–150°, while $\chi_1 = 84^\circ$ within 2.5 kcal/mol (the unmodified conformation is $\chi_1 = 183^\circ; \chi_2 = 120^\circ$). For TB and PNPA adducts this motion is restricted by the size of the ligand ($\chi_2$ range 183–165°),
whereas for ATC no such motion is possible. Indeed, the experimental data \( k_{\text{cat}} \) clearly demonstrate the involvement of Trp-86 in stabilization of the transition state for acylation in the cases of TB and TPA (Table I).

**Modeling of Adducts of PTC and BTC with HuAChE and Mutants**—The modeling of these adducts (Fig. 6) corroborates our previous observations regarding the steric interference for bulky substituents equivalent to the alkyl part of the acyl moiety (Barak et al., 1992). Residues Phe-338, Phe-295, Phe-297, and the main chain of Gly-122 form a hydrophobic pocket the size of which allows for accommodation of a methyl group (Fig. 6a). To accommodate the ethyl group of the propionyl moiety, a nearly eclipsed conformation of PTC has to be assumed. For the BTC adduct, the lowest energy conformation for the alkyl group within hydrophobic pocket indicates a less stable adduct than that for ATC and PTC (Fig. 6a).

Most of the destabilization in this conformation comes from interactions with Phe-297. Other orientations of the alkyl group, in the BTC adduct, are blocked by either Phe-295 and Phe-338 or by Trp-236. Models of HuAChE(F297A)-BTC (Fig. 6b) and of HuAChE(F297V)-BTC (not shown) indicate that removal of the phenyl 297 group from the immediate vicinity of the propyl group removes most of the interference. Indeed, replacement of Phe-297 by either alanine or valine improves \( k_{\text{cat}} \) for BTC hydrolysis (Table IV, Fig. 4). One of the alternative conformations examined for the HuAChE-BTC adduct was energetically unfavorable mainly due to strong interference from the phenyl portion of Phe-295 (Fig. 6a). Modeling of BTC adducts with HuAChE mutated at the 295 position by alanine or leucine predicted that the alkyl group will assume an energetically probable conformation (Fig. 6c), as is indeed demonstrated by the increase in \( k_{\text{cat}} \) values for the two mutants F295A and F295L (Table IV). Although rigorous comparison of conformational energies for the different BTC adducts (of the F297A versus the F295A mutant) could not be carried out by the methods employed, the relative stabilizations of the alkyl group in adducts with 295 and with 297 mutants appeared to be similar. Again, this conclusion from the models is supported by the observed similar \( k_{\text{cat}} \) values for the 295 and 297 mutants as well as for the double mutant F295L/F297V (Fig. 4, Table IV). Thus, modeling of BTC adducts with wild type HuAChE and mutants at positions 295 or 297 could predict the higher turnover numbers for the mutants. However one of the more intriguing results from our kinetic studies is the differential effect of mutation in position 295 and in position 297 on \( k_{\text{cat}} \) values. Yet we note that our model is based on a covalent adducts and therefore is not expected to predict the stabilities of Michaelis-Menten complexes.

**DISCUSSION**

The structure-function relationship underlying AChE sub- strate specificity was investigated by employing site-directed mutagenesis, kinetic studies of the mutants toward substrates and inhibitors, and molecular modeling. Each of these approaches can provide useful insights into the nature of enzymatic activity, and each has been used to probe the catalytic activity of AChE. It is however the combination of these methods that is recently emerging as a most powerful tool for investigation of structure-property relationships in biological systems. We have recently applied this approach to the study of substrate inhibition in AChE (Shafferman et al., 1992b). Here we describe the identification and the mode of action of some structural elements within the architecture of the AChE active center which determine specificity toward: (a) the positive charge of the quaternary ammonium, (b) the structural determinants of the alcoholic part \( (X, \text{ see Scheme I}), \) and \( (c) \) the structure of the acyl moiety.

**Trp-86 Constitutes the Classical Anionic Subsite**—Based on
the interaction of AChE with various types of positively charged compounds, the active center of AChE was suggested to contain a negatively charged anionic subsite (Nolte et al., 1980). This hypothesis was criticized on the basis of correlations between AChE reactivity and the partial molal volume of substituent X (Scheme 1) for a series of charged and uncharged substrates (Hassan et al., 1980). Accordingly, an alternative hypothesis was advanced which postulated a hydrophobic trimethyl binding site that accommodates the quaternary functionality of ACh as well as the X groups of uncharged substrates and inhibitors. Yet, by comparing the kinetic properties of the covalent adducts of AChE with isosteric charged and uncharged methyl phosphonates (Berman and Decker, 1986), it was concluded that the anionic and the hydrophobic subsites are not equivalent but constitute two partially overlapping regions.

The three-dimensional models (Sussman et al., 1991; Barak et al., 1992) indicate that the only potential negative charge in the vicinity of the esteratic site is that of Glu-202(Glu-199), but this residue has been shown to affect the acylation-deacylation steps and not the noncovalent complex formation (Shafferman et al., 1992b, 1992c; Radic et al., 1992). On the other hand results from labeling studies (Weise et al., 1990), X-ray crystallography (Sussman et al., 1991), and site-directed mutagenesis studies (Shafferman et al., 1992b) implicate Trp-86 as the locus of interaction with the positively charged "head group" of the substrate. The charge distribution in the indole ring is characterized by a partial negative charge in the \( \pi \) system (Schneider, 1992). Such "diffuse" negative pole might be better suited to interact with a tetraalkyl ammonium cation as suggested previously (Satow et al., 1986; Dougherty and Stauffer, 1990).

The function of Trp-86 in orienting and accommodating the charged part of the substrate is clearly demonstrated in the kinetic behavior of the W86A mutant. The dramatic 60-fold decrease in the value of \( K_m \) for ATC in the W86A mutant, as compared with the value of the wild type enzyme (Table I), attests to the loss of stabilization of the enzyme-substrate complex. On the other hand the \( k_{cat} \) for this mutant is less than 5-fold lower than that for the wild type, indicating that other functions of the active center are relatively unaffected. The fact that for the W86A mutant the \( K_m \) values of all the uncharged substrates, including TB the isosteric substrate of ATC, are comparable with those of the wild type demonstrate that Trp-86 does not participate in accommodation of uncharged X groups in the noncovalent complexes. It also shows that the integrity of the hydrophobic binding sites, occupied by the uncharged substrates in the noncovalent complexes, is not affected by the replacement of Trp-86. In the above discussion it is assumed that \( K_m \) for the W86A mutant reflects the affinity of the enzyme toward the substrate which in turn implies that \( k_\text{cat} \sim k_\text{a} \) (see Scheme 1). Alternatively, if \( k_\text{cat} \sim k_\text{a} \) then the effect in W86A on \( K_m \) should be related to a decreased in \( k_\text{a} \) (or the apparent bimolecular rate constant \( k_\text{a}/K_m \)). However, the latter is not compatible with the apparent bimolecular rate constant for reaction of the uncharged substrate TB with W86A mutant and the fact that the same constant for the isosteric ATC in this mutant is over 50-fold lower. Comparison of \( K_m \) values for ATC and TB, in the wild type and the W86A mutant (Table I), underscores the fact that although the site accommodating TB remains unchanged, it does not bind ATC. Thus, at the stage of noncovalent complex formation, the charged and the uncharged substrates bind to two different molecular environments.

The role of Trp-86 as the main interaction locus for orienting the positive charge in the active center is further demonstrated by inhibition experiments with edrophonium. The low affinity of the W86A mutant for edrophonium correlates well with the low affinity toward ATC. A recently published x-ray structure of TeAChE-edrophonium complex shows that the trialkylammonium moiety is positioned within interaction distance from the indole plane of the TeAChE Trp-84 (Sussman et al., 1992). The pronounced effects on \( K_m \) for ATC and on \( K_m \) for edrophonium, of the W86A mutation (Tables I and II) may indicate that the region for the noncovalent interaction for the two molecules is similar and operates through cation-\( \pi \) interaction (McCory et al., 1992).

Amino acids Trp-86, Tyr-337, and Phe-338 Are Elements of the Hydrophobic Subsite for the Covalent Adduct in the Active Center—Mutation of Trp-86 resulted in a decrease of \( k_{\text{cat}} \) for hydrolysis of ATC, TB, and TPA but not of PNPA (Table I). This effect of Trp-86 replacement on the acylation rates should depend upon the nature of X group (Fig. 1), since all four substrates are acetates. For ATC, TB, and TPA the effect of the mutation on \( K_m \) is comparable (4–8-fold), indicating a participation of Trp-86 in stabilization of the corresponding transition intermediates for the acylation reactions. The manner in which Trp-86 can interact with uncharged substrates was suggested by the modeling experiments. The feasibility of Trp-86 side chain rotation, in the adducts of uncharged substrates, provides a mechanism for the adjustment of its distance from the X group as is best demonstrated by the movement of the Trp-86 side chain in the TPA adduct (Fig. 5c). The fact that replacement of Trp-86 by alanine did not affect \( K_m \) for PNPA may be explained by the different orientation of residue X (p-nitrophenyl) through stacking interaction with the aromatic residue Phe-338 (Fig. 5d).

This conclusion is supported by the marked effect of F338A mutation on the value of \( K_m \) for PNPA. The significance of the conformational flexibility of Trp-86 aromatic side chain will be discussed further in the context of the cross-talk between the peripheral anionic site and the active center.

Molecular modeling indicates that, besides Trp-86, residues Tyr-337 and Phe-338 can also interact with group X in the covalent adduct. However, the change of the \( K_m \) values upon substitution of Tyr-337 by alanine is much less significant than that resulting from a similar substitution of Phe-338. This discrepancy between the kinetic data and the prediction of the model could suggest that the positioning of the aryl moiety of Tyr-337 in the model needs further refinement. In this context it is relevant to mention that conformational flexibility of Tyr-337 has been proposed and demonstrated before (Sussman et al., 1992; Shafferman et al., 1992b). Kinetic data for the F338A mutant are in good agreement with the prediction of the model as mentioned above regarding the PNPA adduct (Fig. 5d). Replacement of Phe-338 by alanine affects the stability of the TB adduct to a greater extent than that of the isosteric ATC, which also is in good agreement with the different covalentizations of the two X moieties in the model (compare Fig. 5, a with b). All these observations support the notion that the hydrophobic subsite is distinct from the anionic subsite. This conclusion helps to clarify the ambiguity in the literature regarding the structure and function of these two subsites and supports previous interpretation of kinetic data, suggesting the nonequivalence of the two subsites (Berman and Decker, 1986).

In summary, the hydrophobic interactions in the covalent adducts appear to be different from those in the noncovalent complexes. This conclusion is supported by the fact that mutations in HuAChE at positions 338 and 86 have marginal effects on \( K_m \) but a substantial effect on \( K_m \) with all the three uncharged substrates TB, TPA, and PNPA. Furthermore the
results indicate that for uncharged substrates the noncovalent adducts are formed in a different hydrophobic environment from the one referred to as the hydrophobic subsite of covalent adducts (tetrahedral intermediate) in the active center.

Residues Phe-295 and Phe-297 Confer Specificity for the Acyl Moiety—Molecular modeling of the adducts of ATC, PTC, and BTC with HuAChE and the relevant mutants (Fig. 6) predict that replacement of the aromatic moieties in either the 295 or the 297 position removes most of the steric strain imposed upon the propyl group in the HuAChE-BTC adduct. The increase in values of $k_{\text{cat}}$ for BTC in the Phe-295 and Phe-297 mutants as well as in the Fhe-295/Fhe-297 mutant (Table IV, Fig. 4) supports this conclusion. The prediction based on the model was also tested by examination of the relative stabilization of PTC adducts. In this case, mutations in positions 295 and 297 were not expected to affect the stability of the enzyme-PTC adduct, as was indeed found from the corresponding values of $k_{\text{cat}}$ (Table IV). The model predicts further that due to hydrophobic interactions the stabilization of the methyl group of ATC is optimal in the wild type. Indeed, replacement of either one of the residues, Phe-295 or Phe-297, results in a decrease of the $k_{\text{cat}}$ value for ATC. The value for ATC in the double mutant F295L/F297V is close to that in BChE, indicating that the difference in the $k_{\text{cat}}$ values for AChE and BChE is due to the loss of stabilization of the methyl group. The prediction concerning the size of the methyl binding pocket can be extended also to suggest that replacement of Phe-297 by a bulkier residue would lower the reactivity toward ACh. Indeed this appears to be valid, since replacement of Phe-368 in Drosophila melanogaster AChE (a position analogous to 297 in HuAChE) by tyrosine lowers the reactivity toward ACh and increases the resistance to organophosphorous inhibitors (Fournier et al., 1992). The influence of residues Phe-295 and Phe-297 on ligand selectivity was also demonstrated for the BChE-specific organophosphorous inhibitor iso-OMPA in the corresponding mutants. In the case of BW284C51, a selective inhibitor for AChE, replacement of either Phe-295 or Phe-297 indicates that the pronounced selectivity of BW284C51 toward AChE does not depend on these residues.

A surprising observation was the differential effect of mutation in positions 295 and 297 on the values of $k_{\text{cat}}$ for the various substrates (Table IV). This effect becomes more pronounced as the size of the acyl moiety increases from ATC to BTC. The fact that no such difference was observed in the $k_{\text{cat}}$ values could be interpreted as a difference in the stabilization of the acyl moiety in the noncovalent complex as compared with that in the covalent adduct. This may be due to the difference in the planar versus the tetrahedral carbon configurations in the two states. The enhanced affinity for BTC in the F295A and F295L mutants provides the major contribution to the increase in the values of the apparent bimolecular rate constants (Table IV and Fig. 4). For the F295A mutant this value is 130-fold higher than the value for wild type AChE and 1.5 times higher than that of BChE. In fact comparison of the apparent bimolecular rate constants for F295A, F295L, F297A, F297V, and F295L/F297V mutants with the corresponding value for BChE shows that the main difference in the catalytic efficiency between AChE and BChE toward BChE can be traced to a difference in the side chain of a single residue, Phe-295.

Although residue Phe-338 appears to participate in the stabilization of both the noncovalent and the covalent complexes (Table 4), the effects of its replacement are similar for ATC, PTC, and BTC. Therefore it appears that this residue does not contribute to specificity for the alky group of the acyl moiety (we note that in HuBChE the analogous position to Phe-338 of HuAChE is also a phenylalanine).

Plasticity of the Active Center and Substrate Specificity—Conformational changes in the active center of AChE induced by inhibitors or even by the natural substrate have been invoked in the past to account for the complex kinetic behavior of the enzyme (Quinn, 1987). It was suggested that, at least for the neutral substrates, an induced-fit conformation change of AChE occurs after substrate binding but before chemical catalysis (Rosenberry, 1975). In addition, allosteric regulation of AChE activity by ligand binding to an anionic site(s) physically removed from the active site was suggested (Changeux, 1966). Allosteric changes were shown experimentally by measurements of emission maxima shifts for fluorescent alkyl phosphonates attached to the active site serine (Berman et al., 1981). Thus, the concept that AChE possesses conformational flexibility of catalytic significance is not new; however, demonstration of this plasticity through the individual motions of participating residues is only beginning to emerge.

In a previous study we have proposed that substrate inhibition operates via a sequence of changes leading to the motion of residue Tyr-337, induced by substrate binding to the peripheral anionic site (Shafferman et al., 1992b). Residue Tyr-337, together with residues Trp-86, Phe-398, and Phe-297, is also involved in stabilizing the complex of HuAChE with the inhibitors edrophonium and BW284C51 (Table II). Stabilization of these interactions with inhibitors by Tyr-337 is probably achieved through stacking interactions of the aromatic moieties of the inhibitors. Residue Trp-86 should function in accommodating the charged tetraalkylammonium group of edrophonium and BW284C51 (Table II) in a way similar to that in the HuAChE-ATC adduct. To account for both the interaction of the cationic head with Trp-86 and the stacking interaction with Tyr-337, our model suggests relative motions of the two residues out of their original position in the unoccupied enzyme (Fig. 7). In fact, for TrAChE-edrophonium complex in the solid state, reorientation of Phe-330 (analogous to HuAChE Y337) was observed (Sussman et al., 1992).

As mentioned in the preceding sections, movements of

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Determinants of AChE Substrate Specificity

Aromatic residues in the active site center were also implicated in stabilization of covalent enzyme-substrate adducts and in determination of substrate specificity. The contribution of W86 to the stabilization of the covalent adducts for TB and TPA probably involves motion of the side chain of this residue. The feasibility of such motion was shown by molecular modeling for the adducts with uncharged substrates (Fig. 5c) as well as for the unoccupied enzyme (Fig. 7). Motion of residue Phe-338 was suggested as a part of the conformational adjustment in the active center, for accommodation of the aryl moiety of PNPA (Fig. 1d). Accommodation of TPA involves slight motions of Phe-337 and Phe-338 apart from that of Trp-86. Movement of Phe-297 was suggested by molecular modeling of the HuAChE-ETC adduct (Fig. 6a). Thus, the plasticity of the active site center, exemplified here through the motions of aromatic residues Trp-86, Phe-297, Tyr-337, and Phe-338, is an intrinsic element of the HuAChE active center dynamics and is directly involved in determining substrate specificity for both the noncovalent and for the covalent adducts.

A Possible “Cross-talk” between the Peripheral Anionic Site and the Active Center Trp-86—The conformational flexibility of Trp-86 side chain described above may also be related to the observed resistance of the W86A mutant to inhibition by the peripheral anionic site ligand propidium. Compared with the wild type enzyme the hydrolysis of ATC by W86A mutant is almost completely refractive to inhibition by propidium. Moreover, for hydrolysis of TB and PNPA, we find that the W86A mutant is not only resistant to inhibition but that hydrolysis is actually accelerated by propidium. Certain aromatic center ligands were reported (Barnett and Rosenberry, 1977; Sepp and Jarv, 1991) to accelerate catalysis, but to our knowledge this is the first report of acceleration of hydrolysis of AChE substrates by a peripheral anionic site ligands. These observations are quite surprising in view of the location of W86A deep inside in the active site gorge, 15 Å away from the surface of the enzyme. To account for this and other results presented here, we propose a functional cross-talk between Trp-86 and residue Trp-286 which constitutes part of the peripheral anionic site (Shafferman et al., 1992c and Table III).

In this cross-talk between Trp-86 and Trp-86, we assume that the conformational state of Trp-86 depends on the interaction of ligands with peripheral anionic site. In view of the architecture of the active site gorge, this inhibitor-induced conformational state should effect differently the various substrates. Thus PNPA, which, as discussed above, has a minimal interaction with Trp-86 in the wild type enzyme, should be less efficiently inhibited by propidium, and a non-linear inhibition pattern is expected as is indeed the case (Fig. 3). Also consistent with this idea is the observation that inhibition of TB hydrolysis becomes non-linear in both W86A and W286A mutants, whereas it is linear in the wild type enzyme. In these cases there is no efficient coupling between Trp-286 and Trp-86, and the TB ternary complexes may proceed to the acyl enzyme, in spite of the presence of propidium. From the kinetic behavior of the various mutants with ATC, TB, and PNPA in the presence of propidium, we may therefore conclude that a functional cross-talk operates whereby the differential state of occupation of the peripheral anionic site induces distinct conformations of Trp-86 in the active center. This conclusion is also consistent with spectroscopic studies by Berman et al. (1981) and Berman and Nowak (1992) that demonstrated that occupation of the peripheral anionic anion site affects the conformation of the active center.

In summary the dissection of the HuAChE active center by site-directed mutagenesis begins to unravel a complex array of interactions that modulate the activity of the enzyme. We have identified several aromatic amino acids that determine substrate specificity for both the noncovalent and for the covalent adducts. These results suggest that a functional cross-talk between the peripheral and the active center may be the key to understanding the catalytic efficiency of AChE. We have suggested previously (Shafferman et al., 1992b) that substrate inhibition is modulated by cross-talk between the periphery and the active center through Asp-74 and Tyr-341 as well as other aromatic residues along the active site gorge terminating in Tyr-337. Another manifestation of the cross-talk between the periphery and the active center is suggested here, terminates in Trp-86 and again affects the activity of the enzyme. The sensitivity of the residues on the surface and the plasticity of the active center are probably the result of evolutionary design aimed to confer optimal catalytic activity under a wide variety of conditions that are characteristic for the operation of AChE in the synaptic cleft.

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Addendum—After submission of this manuscript, a study analyzing amino acid residues controlling mouse AChE and BChE specificity was published (Vellom et al., 1993). The findings reported there are in good agreement with the results presented above regarding the role of Phe-295 and Phe-297 in determining acyl pocket selectivity.

REFERENCES

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