Probing the Peripheral Site of Human Butyrylcholinesterase

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ABSTRACT: Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) catalyze the hydrolysis of the neurotransmitter acetylcholine and, thereby, function as coregulators of cholinergic neurotransmission. For both enzymes, hydrolysis takes place near the bottom of a 20 Å deep active site gorge. A number of amino acid residues within the gorge have been identified as important in facilitating efficient catalysis and inhibitor binding. Of particular interest is the catalytic triad, consisting of serine, histidine, and glutamate residues, that mediates hydrolysis. Another site influencing the catalytic process is located above the catalytic triad toward the periphery of the active site gorge. This peripheral site (P-site) contains a number of aromatic amino acid residues as well as an aspartate residue that is able to interact with cationic substrates and guide them down the gorge to the catalytic triad. In human AChE, certain aryl residues in the vicinity of the anionic aspartate residue (D74), such as W286, have been implicated in ligand binding and have therefore been considered part of the P-site of the enzyme. The present study was undertaken to explore the P-site of human BuChE and determine whether, like AChE, aromatic side chains near the peripheral aspartate (D70) of this enzyme contribute to ligand binding. Results obtained, utilizing inhibitor competition studies and BuChE mutant species, indicate the participation of aryl residues (F329 and Y332) in the E-helix component of the BuChE active site gorge, along with the anionic aspartate residue (D70), in binding ligands to the P-site of the enzyme.
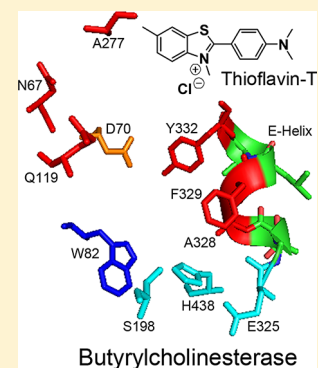
Acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BuChE, EC 3.1.1.8) are serine hydrolase enzymes that catalyze the hydrolysis of acetylcholine.[1] X-ray crystallography analysis of these cholinesterases[2,3] has established that catalysis involves a triad of amino acid residues, serine, histidine, and glutamate, located near the bottom of a 20 Å deep gorge (Figure 1). This region of the gorge has been denoted the acylation or A-site in AChE.[4,5] The efficiency of this A-site in the catalytic process has been shown to be influenced by events occurring at amino acid residues some distance away in the gorge. For example, a tryptophan residue (W86 in AChE, W82 in BuChE) near the A-site is known to facilitate catalysis by forming π-cation interactions with substrates helping align these molecules with the catalytic serine. This tryptophan residue is linked through a polypeptide segment (Ω loop) with an anionic aspartate residue (D74 in AChE; D70 in BuChE) that is one of the components of a peripheral site (P-site) that interacts with cationic substrates. At high substrate levels, the activity of AChE is decreased[6,7] while that of BuChE is increased.[8] This phenomenon of substrate inhibition of AChE is thought to occur through steric block of product release that results from the binding of a substrate molecule to the P-site.[4] Substrate activation of BuChE may be mediated by the binding of a second substrate molecule to a P-site that triggers a conformational change extending to the region near the catalytic triad in the active site.[8] In addition, this catalytic enhancement may be facilitated by stabilization of the tetrahedral intermediate.[9] Such substrate activation has also been observed for certain substrates with AChE.[5]

Mutation of the P-site aspartate residue, D74 in AChE and D70 in BuChE, to an uncharged glycine residue largely eliminates substrate inhibition in AChE and substrate activation of BuChE.[6,8] In addition to this anionic aspartate residue, other amino acid residues, especially those with aryl side chains in AChE, have been found to contribute to catalysis through interactions at the gorge periphery.

The P-site of AChE has been well mapped using mutant studies[10,11] as well as by X-ray crystallography of the enzyme bound to inhibitors that interact with various components of this site.[2,12,13] The inhibitors propidium and thioflavin T bind to the P-site of AChE while edrophonium binds to W86 and Y337, thus interfering with access to the A-site. X-ray crystallography studies corroborate a kinetic approach that determined binding site competition between these inhibitors, thereby helping to define details of the AChE P-site.[14]

Studies with a series of N-10-carbonyl derivatives of phenothiazine[15−18] as well as N-10-alkyl phenothiazines such as ethopropazine[19] also indicate the relevance of aryl residues close to the mouth of the active site gorge of BuChE that are contiguous with the catalytic triad glutamate through the E-helix.[17] This helix (E325-Y332) includes tyrosine and phenylalanine residues (F329, Y332 in BuChE) whose side chains project into the active site gorge. The residue Y332 has been implicated in the
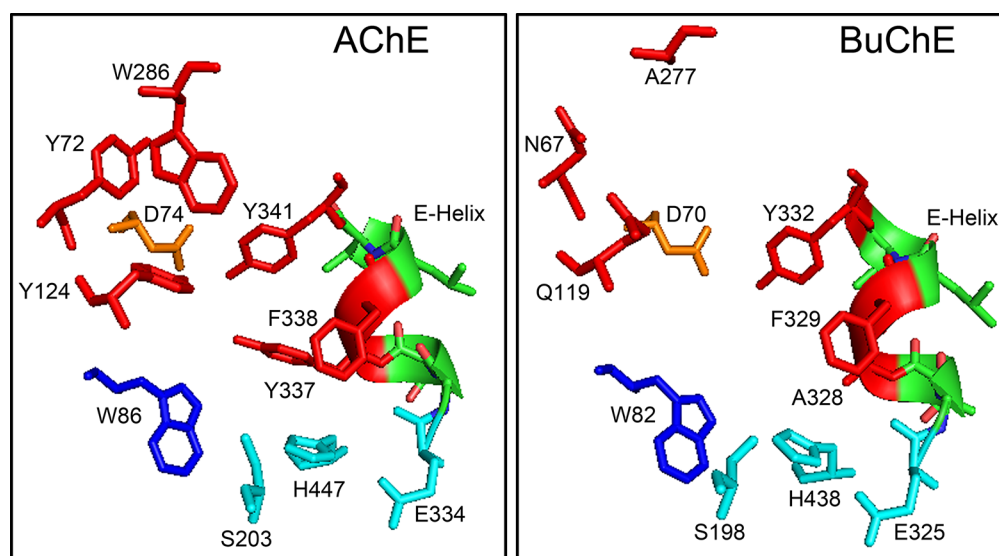
Figure 1. Active site gorge with homologous residues shown for acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). The crystal structures of human AChE (PDB ID: 1B41)[2] and BuChE (PDB ID: 1POI)[3] were obtained from the Protein Data Bank,[35] and PyMol[36] was employed to delete all amino acids save for those selected residues found in the active site.

binding of substrates and inhibitors to BuChE suggesting that this amino acid residue is part of the P-site of this enzyme.[20−22] Inhibitors binding to this region may function by blocking substrate access to the active site and/or by altering hydrolytic efficiency through a conformational change that extends to the catalytic triad.

The P-site of BuChE has not been explored to the extent of the comparable AChE site. Although mutant studies have provided valuable insights,[23] lack of X-ray crystallographic data has hampered definition of BuChE P-site details. Since inhibitor binding site competition analysis and mutant studies were successful in mapping AChE P-site components, a similar approach was made to probe the BuChE P-site. To that end, studies were undertaken using wild-type and mutant BuChE species and the enzyme inhibitors thioflavin T, propidium, edrophonium and two synthetic phenothiazine derivatives.

■ EXPERIMENTAL PROCEDURES

Materials. Thioflavin T (**1**), propidium iodide (**2**), edrophonium chloride (**3**), and purified recombinant human AChE (~1500, units as determined by the supplier) were purchased from Sigma Aldrich (St. Louis, USA). AChE concentration was calculated using the assumption of 450 units/nmol (4.8 $\Delta A_{412}/$ [min × nM]).[24] N-[2-(N',N'-Diisopropylamino)ethyl]-10H-phenothiazine-10-carboxamide (**4**) and anthracen-9-yl(10H-phenothiazine-10-yl) methanone (**5**) were synthesized as described previously.[16,18] Purified human plasma wild-type BuChE and its mutants D70G, A328Y, F329A, and Y332A were a gift from Dr. Oksana Lockridge (University of Nebraska Medical Center, USA). BuChE concentration was calculated using the assumption of 62.5 unit/nmol (0.94 $\Delta A_{412}/$[min × nM]).[25] As defined previously, 0.1 unit is the amount of BuChE that gives a ΔA/min of 1.0 in the presence of 1.6×10^{-4} M butyrylthiocholine in a 1.5 mL assay.[26]

Inhibition Constant Determination. Inhibition constants of inhibitors for AChE or BuChE were determined using a modification of a described method.[14] Briefly, varying amounts of inhibitor (in 50 μL of 50% $CH_3CN_{(aq)}$) were added to 1.60 mL of buffer (0.09 M phosphate buffer, pH 8.0), 5,5′-dithio-bis (2-nitrobenzoic acid) (0.32 mM), and either acetylthiocholine

(5 μM, for AChE) or butyrylthiocholine (5 μM, for BuChE) in a quartz cuvette of 1 cm path length, and the mixture was zeroed at 412 nm. The reaction was initiated by the addition of 50 μL of AChE (to 1 nM) or BuChE (to 1 nM), in 0.1% aqueous gelatin. Assays were conducted at 23 °C. The rate of change of absorbance (ΔA/min), reflecting the rate of hydrolysis of the substrate, was recorded every 2 s for 1 min, using an Ultraspec 2100 pro UV−visible spectrophotometer (Fisher Scientific) equipped with Swift II application software. The molar extinction coefficient for the Ellman product, 5-thio-2-nitrobenzoic acid, used to convert the change in absorbance at λ = 412 nm to moles of product, was 14150 M^{-1} cm^{-1}. The second order hydrolysis rate constant (k_E) was determined at low initial substrate concentration ($[S]_0$) (i.e., $[S]_0 \leq$ ~0.2 K_{app},[27] where K_{app} is the apparent Michaelis constant) according to eq 1,[14] where $[S]$ is the concentration of substrate remaining at time (t) in the presence of the enzyme ($[E]_{tot}$). Alternatively, k_E can be determined according to eq 2 where A_{412} is absorbance, $k_{obs} = k_E[E]_{tot}$, and where $\Delta A_{412} = A_{412(final)} - A_{412(t=0)}$. Values of k_E calculated using eq 1 or eq 2 were comparable. The ratio of k_E without and with inhibitor, described by eq 3,[14] was plotted against inhibitor concentration, and the data were fitted to solve for the inhibition constant (K_I) as well as for the ratio of the relative acylation rate constant to the relative affinity of the ligands in the ternary complex (α). All experiments were performed at least in triplicate and the values were averaged. Data fitting by nonlinear regression was conducted with Excel Solver program and errors calculated using the Solver Statistics Macro.[28]

$$[S] = [S]_0 \, e^{-k_E[E]_{tot}t} \tag{1}$$

$$A_{412} = A_{412final} - \Delta A_{412} \, e^{-k_{obs}t} \tag{2}$$

$$\frac{k_{E[I]=0}}{k_{E+I}} = \frac{1 + ([I]/K_I)}{1 + (\alpha[I]/K_I)} \tag{3}$$

Inhibitor Competition Determination. When a single inhibitor binds to cholinesterase, the rate of substrate hydrolysis is reduced. If a second inhibitor targets the same site as the first, competition between the two inhibitors will ensue and this will
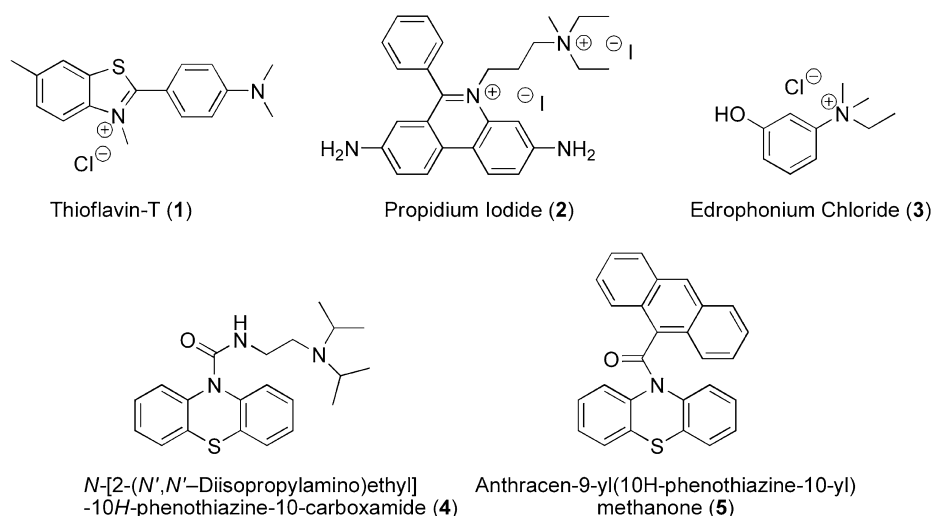
Figure 2. Structures of cholinesterase inhibitors used.

Table 1. Inhibition Constants (K_I) for AChE and BuChE; Binding Site Competition Ratios (K_{12}/K_1) for AChE and BuChE for Each Inhibitor in the Presence of Thioflavin T[a]

		AChE		BuChE	
	compound	K_I (μM)	K_{12}/K_1	K_I (μM)	K_{12}/K_1
1	thioflavin T	4.99 ± 0.13		3.62 ± 0.18	
2	propidium	9.73 ± 0.04	93 (C)	2.03 ± 0.30	1.1 (NC)
3	edrophonium	1.01 ± 0.10	1.5 (NC)	198 ± 1	1.6 (NC)
4	N-[2-(N′,N′-diisopropylamino)ethyl]-10H-phenothiazine-10-carboxamide	1.64 ± 0.10	70 (C)	0.021 ± 0.004	72 (C)
5	anthracen-9-yl(10H-phenothiazine-10-yl) methanone	no inhibition		0.016 ± 0.006	1.1 (NC)

[a]A K_{12}/K_1 ratio of ~1 indicates no competition at binding sites (NC), whereas values ≫1 denotes competition at the same binding site (C).

result in a further alteration in substrate hydrolysis rate. Determining the change in the rate of substrate hydrolysis in the presence of two inhibitors provides information as to whether these are interacting with the same or different enzyme binding sites.[14] On the basis of this principle, assays were conducted as described above in the presence of a fixed concentration of one inhibitor [I1], here being one of propidium iodide (**2**), edrophonium chloride (**3**), N-[2-(N′,N′-diisopropylamino)ethyl]-10H-phenothiazine-10-carboxamide (**4**), or anthracen-9-yl(10H-phenothiazine-10-yl) methanone (**5**) and varying concentrations of a second inhibitor [I2], namely, thioflavin T (**1**). The ratio of k_E in the presence of a fixed concentration of inhibitor, [I1], and varying concentrations of another inhibitor, [I2], to k_E in the presence of only I1 was plotted against [I2] and fitted to eq 4 with Excel Solver as described above. In this equation, K_1 and K_2 are the known equilibrium dissociation constants for the inhibitors I1 and I2 with enzyme, respectively, having been determined as K_I values with eq 3 as detailed above. K_{12} is the fitted equilibrium dissociation constant for I1 with the binary complex of enzyme and I2. The ratio [I1]/K_{12} reflects whether the two inhibitors are interacting with the same or different enzyme binding sites. Thus, as this ratio approaches zero, it signifies that the inhibitors are in competition for a common binding site on the enzyme. By setting this ratio to zero, a theoretical data set representing complete competition between the inhibitors was generated with eq 4. The curve for this theoretical data set along with that for the fitted data set was graphed using GraphPad Prism (California, USA). A numeric value for such competition was calculated as the ratio of K_{12}/K_1 where a value of ~1 indicates no binding

competition and values ≫1 indicate competition between inhibitors.

$$\frac{k_{E+I2}}{k_{E[I2]=0}} = \frac{K_2(1 + ([I1]/K_1))}{K_2(1 + ([I1]/K_1)) + [I2](1 + ([I1]/K_{12}))} \tag{4}$$

Butyrylcholinesterase Mutant Inhibition Studies. Inhibitor potency toward wild type BuChE and toward each of the BuChE mutants, D70G, A328Y, F329A, and Y332A, was determined. The esterase activity was determined by a modification[29] of the Ellman[30] spectrophotometric method. Briefly, one of the inhibitors (compounds **1**−**5**) or blank (in 50 μL of 50% $CH_3CN_{(aq)}$) was added to 1.35 mL of buffer (0.09 M phosphate buffer, pH 8.0), 5,5′-dithio-bis(2-nitrobenzoic acid) (0.32 mM), and BuChE (~0.035 units) in 0.1% aqueous gelatin in a stoppered cuvette of 1 cm path length. The mixture was zeroed at 412 nm, and the reaction was initiated by the addition of 50 μL of butyrylthiocholine in an aqueous solution at a final concentration of 1.6 × 10⁻⁴ M. The reactions were performed at 23 °C. The rate of change of absorbance (ΔA/min), reflecting the rate of hydrolysis of butyrylthiocholine, was recorded every 5 s for 1 min, using a Milton-Roy 1201 UV−vis spectrophotometer (Milton-Roy, Ivyland, PA) set at λ = 412 nm. The amount of activity with and without inhibitor was determined for wild type BuChE and the BuChE mutants.

■ **RESULTS AND DISCUSSION**

In AChE, in addition to the anionic residue D74, there are several amino acid residues that constitute the P-site.[2,10−13] Of the substrate and inhibitor binding sites at the BuChE active site

gorge, D70 has previously been determined to be part of the P-site.[8] The present work was undertaken to determine whether, in addition to D70, BuChE has other amino acid residues that constitute this site. Determining the P-site of BuChE will facilitate elucidation of the role of these components in the functioning of the enzyme and aid in development of inhibitors that might have therapeutic value. To that end, several compounds (**1**−**5**) (Figure 2) were evaluated using both inhibitor competition and BuChE mutant studies to identify amino acid residues that may contribute to P-site function in BuChE.

Inhibition Constants. The affinity constants (K_I values) for the cholinesterase inhibitors used in this study are summarized in Table 1. Plots of the ratio of second-order hydrolysis rates
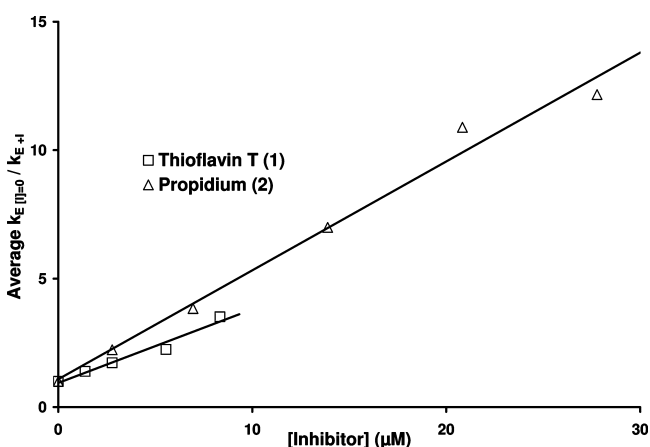

Figure 3. Plots of average second-order substrate hydrolysis ratios by BuChE in the absence ($k_{E[I]=0}$) and presence (k_{E+I}) of thioflavin T (**1**) or propidium (**2**). These plots show a linear relationship between the reciprocal of the second-order hydrolysis rate and inhibitor concentration.

($k_{E[I]=0}/k_{E+I}$, eq 3) demonstrated a linear relationship with increasing inhibitor concentrations, indicating that the constant α in eq 3 assumed a value close to zero as observed previously for rapidly hydrolyzed substrates[14] such as acetylthiocholine and butyrylthiocholine. Examples of such plots for thioflavin T (**1**) and propidium (**2**) are presented in Figure 3. Edrophonium (**3**) and the phenothiazine urea (**4**) and amide (**5**) also showed similar linear relationships. Thioflavin T (**1**), the known AChE P-site inhibitor,[14] was observed to also inhibit BuChE with comparable potency (Table 1). This fluorescent inhibitor probe has been shown in earlier kinetic and X-ray crystallographic studies to bind to the P-site of the AChE active site gorge.[12,24]

Another fluorescent inhibitor probe, propidium (**2**), also interacts with the AChE P-site,[31] but it is thought to be able to enter the larger active site gorge of BuChE and bind closer to the catalytic triad.[8,23] In the present study, propidium (**2**) inhibited BuChE with about 5-fold higher potency than for AChE (Table 1). Edrophonium (**3**), which interacts with the catalytic site of AChE,[31] was about 200-fold weaker as an inhibitor of BuChE (Table 1), consistent with results described previously.[23]

As observed earlier,[18] the phenothiazine urea derivative (**4**) was 80-fold more potent as an inhibitor of BuChE relative to AChE (Table 1). Similarly, the phenothiazine amide derivative (**5**)[16] was a robust inhibitor of BuChE (Table 1) but did not inhibit AChE under the same conditions. Evidence was presented earlier[16,18] that phenothiazines interact with residues such as Y332 near the periphery of the BuChE active site gorge. It may be significant that such aryl residues are part of a polypeptide segment (E-helix, Figure 1) that also contains the glutamate (E325) of the catalytic triad and thus may influence the catalytic activity of the enzyme. No X-ray crystallographic analyses are yet available to establish the peripheral binding sites of inhibitors to BuChE. However, indirect kinetic evidence, as with the phenothiazine
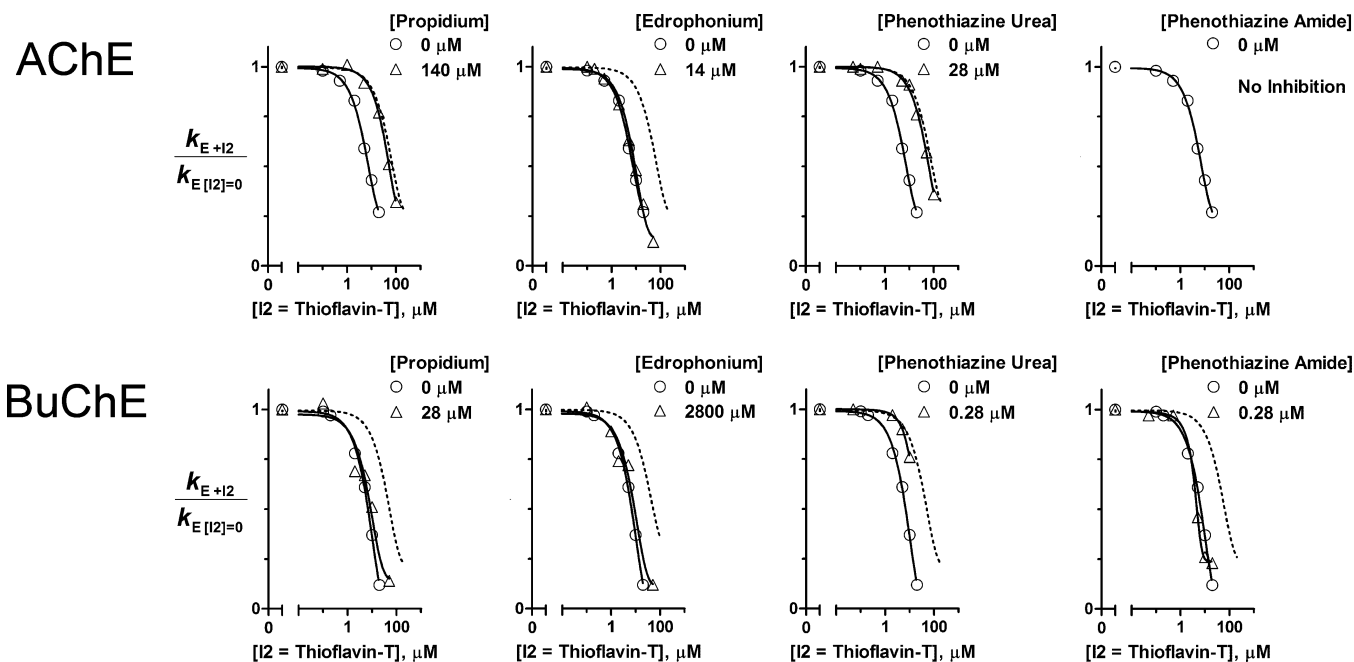

Figure 4. Plots of second-order substrate hydrolysis rates by BuChE or AChE with thioflavin T and in the presence (triangle) or absence (circle) of another inhibitor with lines fitted or calculated according to eq 4. Dotted lines represent the theoretical plot that denotes complete competition between the inhibitor and thioflavin T for that enzyme. For example, for BuChE, propidium and thioflavin T do not compete as exemplified by overlap in the plots with and without propidium. In contrast, for AChE, propidium competes with thioflavin T as indicated by absence of overlap in the plots with and without propidium, and overlap between the presence of propidium and complete competition plots.
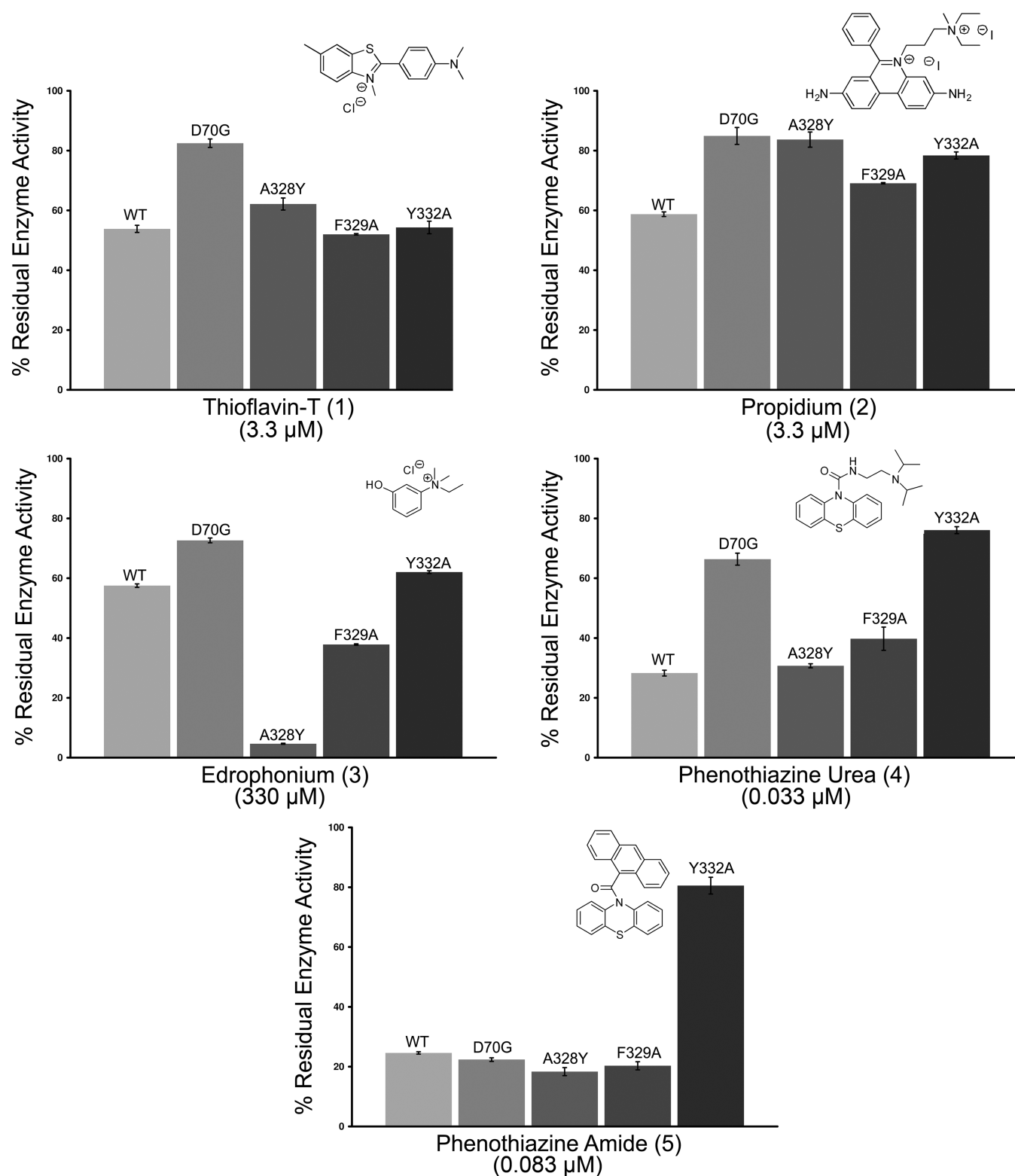
Figure 5. Enzyme activity of wild type BuChE and its mutants in the absence and presence of compounds **1−5**. The % residual enzyme activity indicates the activity in the presence of inhibitor relative to the activity in the absence of inhibitor for each mutant. Note that D70 mediates, in part, inhibition by all cationic inhibitors (compounds **1−4**). In addition, residues of the E-helix, F329 and Y332, are involved in ligand binding (compounds **2−5**). Thus, D70, Y332, and F329 are components of the P-site of BuChE.

derivatives cited above and propidium,[8,23] provide some reference for determining binding site locations from inhibitor competition studies.

Thioflavin T (**1**) was chosen as the reference inhibitor for competition studies because of its known binding to the P-site of AChE

and because it also inhibits BuChE with comparable potency (K_I, Table 1). The other inhibitors (compounds **2−5**, Figure 2) were chosen to test for inhibitor competition with thioflavin T (**1**) because of their varied putative sites of interaction with BuChE.

Inhibition Competition Studies. The results of competition studies between thioflavin T (**1**) and compounds **2**–**5** are summarized in Table 1 and related plots are presented in Figure 4. Competition between thioflavin T (**1**) and propidium (**2**) with AChE confirmed the earlier observation that these two compounds bind to the same site of this enzyme. [14] In contrast, the lack of competition between thioflavin T (**1**) and propidium (**2**) with BuChE indicates that these inhibitors bind at different sites on this enzyme. Propidium has been suggested to bind closer to the catalytic triad in the BuChE active site,[23] implying that thioflavin T (**1**) may bind to a more peripheral region of the enzyme active site gorge. Consistent with a previous observation,[14] competition studies between thioflavin T (**1**) and edrophonium (**3**) with AChE (Table 1, Figure 4) indicated different binding sites for these inhibitors. Both observations are in keeping with earlier conclusions that edrophonium (**3**) binds at the A-site of AChE[31,32] while thioflavin T (**1**) binds at the P-site.[24] With BuChE, competition studies between thioflavin T (**1**) and edrophonium (**3**) (Table 1, Figure 4) suggest that these two inhibitors bind at different sites on this enzyme as well. Similar experiments using thioflavin T (**1**) and the cationic phenothiazine urea derivative (**4**), which is thought to interact with the E-helix and D70,[18] demonstrate overlapping binding sites. In contrast, lack of competition between thioflavin T and the neutral phenothiazine amide derivative (**5**) with BuChE (Table 1, Figure 4) suggests that this phenothiazine derivative has a binding site distinct from that of thioflavin T (**1**) on this enzyme.

In these inhibitor studies with BuChE, lack of competition between thioflavin T (**1**) and inhibitors that are thought to bind near the catalytic triad in the active site, such as propidium (**2**) and edrophonium (**3**), suggests that thioflavin T (**1**) binds to a P-site of this enzyme. This conclusion is further supported by the observation that thioflavin T (**1**) competes with inhibitors such as the phenothiazine urea (**4**), which has multiple binding sites on the enzyme, but not with the phenothiazine amide (**5**), that is thought to bind to the E-helix of the active site gorge. [16]

Butyrylcholinesterase Mutant Studies. The extent of inhibition by compounds **1**–**5** (Figure 2) was determined for wild type BuChE and BuChE mutants D70G, A328Y, F329A, and Y332A (Figure 5). Although some caution is required to interpret the results from mutation studies because of potential complex indirect effects on the conformation of the active site gorge, such studies do provide insights into the importance of a particular amino acid residues in ligand binding.[21,33,34] For thioflavin T (**1**), replacement of the peripheral anionic site residue D70 with the neutral residue glycine in the BuChE D70G mutant reduced inhibition by this compound compared to wild type and other mutants (Figure 5). The similar inhibition constants for AChE and BuChE and the attenuated inhibition toward BuChE D70G suggest that thioflavin T (**1**) binds to D70 in the P-site of BuChE to effect inhibition. Although thioflavin T (**1**) inhibition of the corresponding AChE mutants has not been studied, these results are unexpected based on the X-ray crystal structure of the thioflavin T–AChE complex.[12] This structure shows no direct interaction between thioflavin T (**1**) and D74 and close contacts of this ligand with the residues corresponding to Y337 (A328 in BuChE) and Y341 (Y332 in BuChE). The P-site localization of thioflavin T (**1**) in BuChE thus may differ somewhat from that of thioflavin T (**1**) in AChE.

Propidium (**2**) had reduced inhibition potency for all BuChE mutants D70G, A328Y, F329A, and Y332A compared to wild type (Figure 5). This indicates that propidium (**2**) interacts with D70 and its inhibitory action is influenced by aryl residues on the

E-helix, F329 and Y332, in BuChE. Thus, even though this inhibitor may compete with substrate at the active site,[8] residues located above the catalytic triad toward the periphery of the active site gorge (D70, F329, and Y332) influence the inhibitory mechanism, perhaps through multipronged electrostatic and π–π interactions in the active site gorge. The apparent disruption of the propidium (**2**) inhibition by the AChE-like mutation A328Y (Figure 5) also supports the notion of propidium (**2**) interaction with the E-helix.

Edrophonium (**3**) is a potent inhibitor of AChE compared to its effect on BuChE (Table 1). [23] This has been attributed to the ability of the compound to interact with Y337 in AChE, which is an alanine residue (A328) in BuChE. This was confirmed here (Figure 5) by the greatly increased inhibition of BuChE mutant A328Y over wild type BuChE and by the lack of competition between edrophonium (**3**) and thioflavin T (**1**) for both AChE and BuChE (Table 1). Similar to propidium (**2**), the D70G BuChE mutant is more resistant to edrophonium (**3**) inhibition compared to wild type (Figure 5). This signifies that, for cationic ligands, binding to D70 partially mediates inhibition potency. Also, replacing F329 with alanine increases inhibition for this mutant over wild type BuChE, suggesting that this aryl residue normally interferes with edrophonium binding to some other residue, such as W82, that now becomes available.

N-[2-(N',N'-Diisopropylamino)ethyl]-10H-phenothiazine-10-carboxamide (**4**) inhibition was decreased with the D70G, F329, and Y332A BuChE mutants compared to wild type BuChE, as previously reported.[18] It has been suggested that the cationic nitrogen of this compound, present at pH 8.0, spans the gorge between BuChE E-helix residues and D70 and contributes to the high potency of this compound to disrupt substrate hydrolysis.[18]

Anthracen-9-yl(10H-phenothiazine-10-yl) methanone (**5**) inhibition was significantly decreased only by the Y332A BuChE mutant. This neutral phenothiazine derivative, unlike the cationic amino urea (**4**), would not be expected to bind to the anionic D70. Thus, both these phenothiazine derivatives (**4** and **5**) associate with the E-helix, contributing to their inhibitor potency.

■ CONCLUSION

Determination of inhibition constants, binding site competition values and mutant studies suggest that D70, F329, and Y332 are amino acid residues important for binding inhibitors at the P-site of BuChE.

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Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

■ REFERENCES

(1) Silver, A. (1974) *The Biology of Cholinesterases*, Elsevier, Amsterdam.

(2) Sussman, J. L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L., and Silman, I. (1991) Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein. *Science 253*, 872−879.

(3) Nicolet, Y., Lockridge, O., Masson, P., Fontecilla-Camps, J. C., and Nachon, F. (2003) Crystal structure of human butyrylcholinesterase and of its complexes with substrate and products. *J. Biol. Chem. 278*, 41141−41147.

(4) Szegletes, T., Mallender, W. D., and Rosenberry, T. L. (1998) Nonequilibrium analysis alters the mechanistic interpretation of inhibition of acetylcholinesterase by peripheral site ligands. *Biochemistry 37*, 4206−4216.

(5) Johnson, J. L., Cusack, B., Davies, M. P., Fauq, A., and Rosenberry, T. L. (2003) Unmasking tandem site interaction in human acetylcholinesterase. Substrate activation with a cationic acetanilide substrate. *Biochemistry 42*, 5438−5452.

(6) Mallender, W. D., Szegletes, T., and Rosenberry, T. L. (2000) Acetylthiocholine binds to Asp74 at the peripheral site of human acetylcholinesterase as the first step in the catalytic pathway. *Biochemistry 39*, 7753−7763.

(7) Nachmansohn, D., and Wilson, I. B. (1951) The enzymic hydrolysis and synthesis of acetylcholine. *Adv. Enzymol. Relat. Subj. Biochem. 12*, 259−339.

(8) Masson, P., Froment, M. T., Bartels, C. F., and Lockridge, O. (1996) Asp70 in the peripheral anionic site of human butyrylcholinesterase. *Eur. J. Biochem. 235*, 36−48.

(9) Tormos, J. R., Wiley, K. L., Seravalli, J., Nachon, F., Masson, P., Nicolet, Y., and Quinn, D. M. (2005) The reactant state for substrate-activated turnover of acetylthiocholine by butyrylcholinesterase is a tetrahedral intermediate. *J. Am. Chem. Soc. 127*, 14538−14539.

(10) Radic, Z., Pickering, N. A., Vellom, D. C., Camp, S., and Taylor, P. (1993) Three distinct domains in the cholinesterase molecule confer selectivity for acetyl- and butyrylcholinesterase inhibitors. *Biochemistry 32*, 12074−12084.

(11) Barak, D., Kronman, C., Ordentlich, A., Ariel, N., Bromberg, A., Marcus, D., Lazar, A., Velan, B., and Shafferman, A. (1994) Acetylcholinesterase peripheral anionic site degeneracy conferred by amino acid arrays sharing a common core. *J. Biol. Chem. 269*, 6296−6305.

(12) Harel, M., Sonoda, L. K., Silman, I., Sussman, J. L., and Rosenberry, T. L. (2008) Crystal structure of thioflavin T bound to the peripheral site of *Torpedo californica* acetylcholinesterase reveals how thioflavin T acts as a sensitive fluorescent reporter of ligand binding to the acylation site. *J. Am. Chem. Soc. 130*, 7856−7861.

(13) Harel, M., Quinn, D. M., Nair, H. K., Silman, I., and Sussman, J. L. (1996) The X-ray structure of a transition state analog complex reveals the molecular origins of the catalytic power and substrate specificity of acetylcholinesterase. *J. Am. Chem. Soc. 118*, 2340−2346.

(14) Auletta, J. T., Johnson, J. L., and Rosenberry, T. L. (2010) Molecular basis of inhibition of substrate hydrolysis by a ligand bound to the peripheral site of acetylcholinesterase. *Chem. Biol. Interact. 187*, 135−141.

(15) Darvesh, S., McDonald, R. S., Penwell, A., Conrad, S., Darvesh, K. V., Mataija, D., Gomez, G., Caines, A., Walsh, R., and Martin, E. (2005) Structure-activity relationships for inhibition of human cholinesterases by alkyl amide phenothiazine derivatives. *Bioorg. Med. Chem. 13*, 211−222.

(16) Darvesh, S., McDonald, R. S., Darvesh, K. V., Mataija, D., Conrad, S., Gomez, G., Walsh, R., and Martin, E. (2007) Selective reversible inhibition of human butyrylcholinesterase by aryl amide derivatives of phenothiazine. *Bioorg. Med. Chem. 15*, 6367−6378.

(17) Darvesh, S., Darvesh, K. V., McDonald, R. S., Mataija, D., Walsh, R., Mothana, S., Lockridge, O., and Martin, E. (2008) Carbamates with differential mechanism of inhibition toward acetylcholinesterase and butyrylcholinesterase. *J. Med. Chem. 51*, 4200−4212.

(18) Darvesh, S., Pottie, I. R., Darvesh, K. V., McDonald, R. S., Walsh, R., Conrad, S., Penwell, A., Mataija, D., and Martin, E. (2010) Differential binding of phenothiazine urea derivatives to wild-type human cholinesterases and butyrylcholinesterase mutants. *Bioorg. Med. Chem. 18*, 2232−2244.

(19) Sinko, G., Kovarik, Z., Reiner, E., Simeon-Rudolf, V., and Stojan, J. (2011) Mechanism of stereoselective interaction between butyrylcholinesterase and ethopropazine enantiomers. *Biochimie 93*, 1797−1807.

(20) Masson, P., Legrand, P., Bartels, C. F., Froment, M. T., Schopfer, L. M., and Lockridge, O. (1997) Role of aspartate 70 and tryptophan 82 in binding of succinyldithiocholine to human butyrylcholinesterase. *Biochemistry 36*, 2266−2277.

(21) Nachon, F., Ehret-Sabatier, L., Loew, D., Colas, C., van Dorsselaer, A., and Goeldner, M. (1998) Trp82 and Tyr332 are involved in two quaternary ammonium binding domains of human butyrylcholinesterase as revealed by photoaffinity labeling with [3H]DDF. *Biochemistry 37*, 10507−10513.

(22) Masson, P., Xie, W., Froment, M. T., Levitsky, V., Fortier, P. L., Albaret, C., and Lockridge, O. (1999) Interaction between the peripheral site residues of human butyrylcholinesterase, D70 and Y332, in binding and hydrolysis of substrates. *Biochim. Biophys. Acta 1433*, 281−293.

(23) Saxena, A., Redman, A. M., Jiang, X., Lockridge, O., and Doctor, B. P. (1997) Differences in active site gorge dimensions of cholinesterases revealed by binding of inhibitors to human butyrylcholinesterase. *Biochemistry 36*, 14642−14651.

(24) De Ferrari, G. V., Mallender, W. D., Inestrosa, N. C., and Rosenberry, T. L. (2001) Thioflavin T is a fluorescent probe of the acetylcholinesterase peripheral site that reveals conformational interactions between the peripheral and acylation sites. *J. Biol. Chem. 276*, 23282−23287.

(25) Darvesh, S., McDonald, R. S., Darvesh, K. V., Mataija, D., Mothana, S., Cook, H., Carneiro, K. M., Richard, N., Walsh, R., and Martin, E. (2006) On the active site for hydrolysis of aryl amides and choline esters by human cholinesterases. *Bioorg. Med. Chem. 14*, 4586−4599.

(26) Darvesh, S., Kumar, R., Roberts, S., Walsh, R., and Martin, E. (2001) Butyrylcholinesterase-Mediated enhancement of the enzymatic activity of trypsin. *Cell Mol. Neurobiol. 21*, 285−296.

(27) Eastman, J., Wilson, E. J., Cervenansky, C., and Rosenberry, T. L. (1995) Fasciculin 2 binds to the peripheral site on acetylcholinesterase and inhibits substrate hydrolysis by slowing a step involving proton transfer during enzyme acylation. *J. Biol. Chem. 270*, 19694−19701.

(28) Billo, E. J. (2011) *Excel for Chemists*, 3rd ed., John Wiley and Sons, Hoboken, NJ.

(29) Darvesh, S., Walsh, R., Kumar, R., Caines, A., Roberts, S., Magee, D., Rockwood, K., and Martin, E. (2003) Inhibition of human cholinesterases by drugs used to treat Alzheimer disease. *Alzheimer Dis. Assoc. Disord. 17*, 117−126.

(30) Ellman, G. L., Courtney, K. D., Andres, V., Jr., and Feather-Stone, R. M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol. 7*, 88−95.

(31) Taylor, P., and Lappi, S. (1975) Interaction of fluorescence probes with acetylcholinesterase. The site and specificity of propidium binding. *Biochemistry 14*, 1989−1997.

(32) Harel, M., Schalk, I., Ehret-Sabatier, L., Bouet, F., Goeldner, M., Hirth, C., Axelsen, P. H., Silman, I., and Sussman, J. L. (1993) Quaternary ligand binding to aromatic residues in the active-site gorge of acetylcholinesterase. *Proc. Natl. Acad. Sci. U. S. A. 90*, 9031−9035.

(33) Shafferman, A., Ordentlich, A., Barak, D., Stein, D., Ariel, N., and Velan, B. (1996) Aging of phosphylated human acetylcholinesterase: catalytic processes mediated by aromatic and polar residues of the active centre. *Biochem. J. 318*, 833−840.

(34) Masson, P., Cléry, C., Guerra, P., Redslob, A., Albaret, C., and Fortier, P. L. (1999) Hydration change during the aging of phosphorylated human butyrylcholinesterase: importance of residues aspartate-70 and glutamate-197 in the water network as probed by hydrostatic and osmotic pressures. *Biochem. J. 343*, 361−369.

(35) Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N., and Bourne, P. E. (2000) The Protein Data Bank. *Nucleic Acids Res. 28*, 235.

(36) DeLano, W. L. (2002) The PyMOL Molecular Graphics System, DeLano Scientific, SanCarlos, CA, USA.

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