Interaction Kinetics of Reversible Inhibitors and Substrates with Acetylcholinesterase and Its Fasciculin 2 Complex*

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Fasciculin 2 (Fas2), a three-fingered peptide of 61 amino acids, binds tightly to the peripheral site of acetylcholinesterases (AChE; EC 3.1.1.7), occluding the entry portal into the active center gorge of the enzyme and inhibiting its catalytic activity. We investigated the mechanism of Fas2 inhibition by studying hydrolysis of cationic and neutral substrates and by determining the kinetics of interaction for fast equilibrating cationic and neutral reversible inhibitors with the AChE:Fas2 complex and free AChE. Catalytic parameters, derived by eliminating residual Fas2-resistant activity, reveal that Fas2 reduces \( k_{cat}/K_m \) up to \( 10^6 \)-fold for cationic substrates and less than \( 10^2 \)-fold for neutral substrates. Rate constants for association of reversible inhibitors with the active center of the AChE:Fas2 complex were reduced about \( 10^2 \)-fold for both cationic and neutral inhibitors, while dissociation rate constants were reduced \( 10^5 \)-to \( 10^7 \)-fold, compared with AChE alone. Rates of ligand association with both AChE and AChE:Fas2 complex were dependent on the protonation state of ionizable ligands but were also markedly reduced by protonation of enzyme residue(s) with \( pK_a \) of 6.1–6.2. Linear free energy relationships between the equilibrium constant and the kinetic constants show that Fas2, presumably through an allosteric influence, markedly alters the position of the transition state in the reaction pathway. Since Fas2 complexation introduces an energetic barrier for hydrolysis of substrates that exceeds that found for association of reversible ligands, Fas2 influences catalytic parameters by a more complex mechanism than simple restriction of diffusional entry and exit from the active center. Conformational flexibility appears critical for facilitating ligand passage in the narrow active center gorge for both AChE and the AChE:Fas2 complex.

The interaction between acetylcholinesterase (EC 3.1.1.7) (AChE) and fasciculin 2 (Fas2) is a well studied example of a tight binding peptide-protein complex. The bound “three-fingered” peptide toxin, isolated from venom of Elapidae snakes, resides at the opening of the AChE active center gorge, thus sealing off the entry to the gorge and resulting in virtually complete inhibition of hydrolysis of the neurotransmitter acetylcholine. Static snapshots of the complex structure revealed in crystals of mouse AChE:Fas2 (1), Torpedo californica AChE:Fas2 (2) and human AChE:Fas2 (3) complexes indicate that the “solvent-accessible” surfaces of two molecules overlap in a nearly continuous area of more than 1000 Å² around the entry to the AChE active center gorge. The complex, however, retains residual catalytic activity whose magnitude was reported to vary between species, being about 3% in human AChE (4) and about 0.09% in mouse AChE (5), when measured with acetylthiocholine (ATCh). Inhibition of residual catalytic activity of both complexes by conjugation of the active center serine by either organophosphate or trifluoroacetophenone (TFK) inhibitors, showed only a 1-order of magnitude reduction in reaction rates of cationic inhibitors for the AChE:Fas2 complex when compared with uncomplexed AChE (5). Rates of association and dissociation of reversible ligand N-methylacridinium were found, however, to be reduced by 3–4 orders of magnitude in the AChE:Fas2 complex (6).

Recently, Mallender et al. (7) found that human AChE contained Fas2-resistant esterase activity in purified preparations. Monitoring the phosphorylation rates of fluorescent organophosphates, which exhibit negligible dephosphorylation of the enzyme, enabled these investigators to show that reactions of organophosphates with AChE:Fas2 were 2–3 orders of magnitude slower than those for uncomplexed AChE, a reduction greater than observed in earlier studies (4). The predominant residual catalytic activity of the human AChE:Fas2 complex appeared to be due to contaminating Fas2-resistant activity (7). Although mouse AChE:Fas2 complex appeared to contain only 0.09% activity resistant to Fas2 inhibition (5, 8), comparatively high turnover rates of a fasciculin-resistant enzyme may also complicate our analysis of ligand entry into the active center gorge.

By employing an irreversible inhibitor to inhibit selectively the residual Fas2 resistant activity, we demonstrate here that mouse AChE:Fas2 complex retains only about 0.004% catalytic activity at 1 mM ATCh instead of the previously reported 0.09% (5). Stopped-flow techniques with fluorescent detection enabled us to determine rates of association and dissociation of several reversible inhibitors that equilibrate with AChE in a subsecond time frame. Furthermore, we have developed a competitive binding assay utilizing a fluorescent carbamate, MTC, to measure binding rates of nonfluorescent inhibitors to both mouse AChE and the AChE:Fas2 complex. Utilizing these procedures, we present a quantitative characterization of interaction of 4-methylcoumarin-7-ylphosphate; MTC, N,N-dimethyl carbamoyl N-methyl-7-quinolinium; M7T, N-methyl-7-hydroxy-quinolinium.
Ligands and the Acetylcholinesterase-Fasciculin 2 Complex

Materials and Methods

Enzyme—Wild-type mouse AChE was expressed in HEK-293 cells stably transfected with cDNA encoding a monomeric form of the enzyme truncated at its carboxyl terminus at position 548. The enzyme secreted into the medium was purified to homogeneity on affinity columns as described previously (9).

Inhibitors and Substrates—Purified Fas2 was kindly provided as lyophilized material by Dr. Pascale Marchot (CNRS, University of Mar- seille, France). Concentrations of Fas2 stock solutions were determined by absorbance (ε276 = 4900 m−1 cm−1) (20). m-Terbutyl trifluoroacetophenone (TFK) and m-trimethylammonium trifluoroacetophenone (TFK+) were kindly provided by Dr. Daniel Quinn (University of Iowa, Iowa City, IA). Nonfluorescent inhibitors BW286C51, edrophonium, and decamethonium were obtained from Sigma. (−)-Huperzine A was obtained from Calbiochem. M7C was obtained from Molecular Probes, Inc. (Eugene, OR). The chiral organophosphonate enantiomer S5,3,3-dimethylbutyl methylphosphonothioic acid was kindly provided by Dr. Harvey Berman (State University of New York at Buffalo, Buffalo, NY). The substrates, ATCh, propionylthiocholine iodide (PTCh), butryrylthiocholine iodide (BTCh), and p-nitrophenyl acetate (pNPAC), were obtained from Sigma, while thiophenylacetate (S-PhAc) was a product of Aldrich.

Enzyme Activity—Hydrolysis of ATCh, PTCh, BTCh, S-PhAc, and pNPAC was measured spectrophotometrically at 412 nm for released thiocholine (10) or at 405 nm for p-nitrophenol. Activities of AChE and Fas2 complexes were measured following a 10-min incubation of 640 nM AChE with 1.3 μM Fas2 to form the complex and 30-min incubation of the complex with 530 nM S5,3,3-dimethylbutyl methylphosphonothioic acid to inhibit hydrolytic activity not originating from the complex. The very slow reaction of the charged organophosphate with the active center of the Fas2AChE complex gives rise to selective inhibition. Kinetic constants for hydrolysis of the above substrates by AChE and AChE-Fas2 complex were determined as described previously (5).

Enzyme Inhibition—The time course of AChE inhibition by trifluoroacetophenones was monitored by measuring ATCh hydrolysis in aliquots from the reaction mixture. Typically, 0.96-ml aliquots were drawn from 10 ml of the reaction mixture and mixed with 30 μl of DTNB (10 mM) and 10 μl of ATCh (100 mM) at specified time intervals to stop the reaction and determine residual enzyme activity. Both the kinetic constants for the M7C reaction resulting from prior covalent reaction with the nonfluorescent inhibitor. Forty μl of 1.0 mM M7C was added to 0.4-ml aliquots drawn at different time intervals from 4 ml of reaction mixture containing buffered solution of an ATCh (10–70 nM) with or without Fas2 (650 nM) and the nonfluorescent inhibitor. Aliquots were quickly transferred into a spectrofluorometric cuvette, and the fluorescence increase was followed at λemission = 510 nm (λexcitation = 410 nm) for 20 s in the absence of Fas2 or for 100 s in the presence of Fas2. The fluorescence signal exponentially approached a maximum value, which was diminished with increasing times of prior exposure to the nonfluorescent inhibitor, indicating progress of its reaction. The first order rate constant of binding of nonfluorescent inhibitors was determined by fitting the time dependence of relative change in maximal fluorescence to Equation 1.

Equilibrium Binding Measurements—Equilibrium dissociation constants for several fluorescent reversible ligands were determined by titration with increasing concentrations of AChE or AChE-Fas2 complex, resulting in quenching of ligand fluorescence. Concentrations of free fluorophores during titrations were determined from corresponding calibration curves constructed for each fluorophore, limiting fluorescence quenching in the bound state. Corrections were made for small changes in reaction volume due to the addition of titrant. Equilibrium dissociation constants (Kd) were determined from the reciprocal of the slopes of Scatchard plots.

Determination of Ligand pKa Values—UV-visible spectra of fast equilibrating nonfluorescent inhibitors with ionizable functional groups were recorded in buffers of varying pH. Changes of peak absorbances as a function of buffer pH were analyzed using the following equations (rearranged from Ref. 14),

\[
A = A_{\text{max}} + (A_{\text{max}} - A_{\text{min}})/(1 + K_\text{a}[\text{H}^+])
\] (Eq. 2)

or

\[
A = A_{\text{max}} + (A_{\text{max}} - A_{\text{min}})/(1 + [\text{H}][K_\text{d}])
\] (Eq. 3)

where Amax and Amin are minimal and maximal peak absorbances and Kd is the equilibrium proton dissociation constant. The following buffers were used: 0.10 M citric acid plus 0.20 M sodium phosphate (pH 2.6–7.8) and 0.050 M sodium phosphate plus sodium pyrophosphate (pH 5.0–12).

Results

M7C Reaction Assay—Time courses of the M7C reaction with the AChE and the AChE-Fas2 complex are shown in Fig. 1. M7C added at a concentration of 100 μM binds to AChE at a rate too fast for measurement by conventional mixing and detection by spectrophotometry. Hence, the carbamoylation step is reflected in a rapid burst of fluorescence. The reaction of M7C with the AChE-Fas2 complex was slower but was virtually complete within 100 s. The final fluorescence intensities upon carbamoylation and release of M7H were linearly dependent on AChE and AChE-Fas2 concentrations (Fig. 1, B and C).

The rapid and stoichiometric reaction of M7C with AChE or the AChE-Fas2 complex enabled us to develop a fluorescent assay for monitoring of binding rates for nonfluorescent ligands. The assay is based on consecutive, rapid M7C titrations of residual catalytically active AChE determined by counting its reaction with nonfluorescent ligands. The first order rate constants for reaction were determined by fitting to Equation 1 the fluorescence intensities obtained in subsequent titrations. Good agreement between rate constants for ligand reaction measured from the decrement in the M7C reaction and ligand inhibition of ATCh hydrolysis is evident in Table 1.
Hydrolysis of Substrates by AChE and AChE-Fas2 Complex—Catalytic hydrolysis of three cationic (ATCh, PTCh, and BTCh) and two neutral (S-PhAc and pNPAc) esters by AChE and AChE-Fas2 was compared. Substrate concentration dependences of AChE hydrolysis deviate from Michaelis kinetics at millimolar substrate concentrations, with ATCh, PTCh, and BTCh inhibiting their own hydrolysis. The kinetics of hydrolysis is characterized by substrate inhibition constants ($K_{s}$) and relative activities of enzyme-substrate ternary complexes (b), in addition to Michaelis constants ($K_m$) and maximum turnover rates ($k_{cat}$) (Table II; Refs. 16 and 17). The three cationic substrates have similar Michaelis constants (15–18), yet the commitment to catalysis (defined as a ratio of the enzyme acylation rate constant and dissociation rate constant of the Michaelis complex (cf. Ref. 19)) and the formation of the tetrahedral transition state, reflected in $k_{cat}$, appears severely restricted for BTCh and other substrates containing a large acyl group (17, 19).

Hydrolysis of neutral substrates by AChE obeyed Michaelis kinetics (Table II), but with $K_m$ values up to 2 orders of magnitude larger than for cationic substrates, thus emphasizing the importance of electrostatic interactions in formation of Michaelis complexes. $k_{cat}$ of S-PhAc was similar to $k_{cat}$ values for charged substrates and significantly greater than $k_{cat}$ for pNPAc. A similar relationship between catalytic constants for PhAc and pNPAc has been well described for Electrophorus electricus AChE (20).

Although the AChE-Fas2 complex hydrolyzed all substrates significantly more slowly than AChE, cationic substrates with high turnover rates were most affected. Hydrolysis of both cationic and neutral substrates by AChE-Fas2 obeyed Michaelis kinetics. Our earlier published curve of ATCh hydrolysis by AChE-Fas2 was biphasic, indicating substrate activation (5). The first phase, due to the presence of residual, Fas2-resistant, esterase activity, was inhibited selectively using the organophosphate, $S_{p,3.3}$-dimethylbutyl methylphosphonothioate. Hence with this correction, the ATCh hydrolysis profile for the Fas2-AChE complex shows typical Michaelis kinetics. $k_{cat}/K_m$ values for ATCh and PTCCh were reduced by 5–6 orders of magnitude, whereas $k_{cat}/K_m$ for BTCh was reduced by only 3 orders of magnitude. Neutral substrates S-PhAc and pNPAc exhibited much smaller reductions in $k_{cat}/K_m$ (680 and 96 times, respectively). The reduction in $k_{cat}/K_m$ for the five substrates by AChE-Fas2 resulted in distinct clusters of values for charged and neutral substrates. Values of $1.2–4.7 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$ for neutral substrates approach the $k_{cat}/K_m$ value for benzoylthiocholine hydrolysis by AChE (1.1 $\times 10^4 \text{ M}^{-1} \text{s}^{-1}$; Ref. 17).

$k_{cat}/K_m$ values for neutral substrates with the AChE-Fas2 complex were 1–2 orders of magnitude greater than for the charged substrates, suggesting that Fas2 association may impart a more restrictive barrier in the AChE-Fas2 complex to diffusional entry and acylation by cationic substrates. The reduction in values of $k_{cat}/K_m$ was reflected in both an increase in $K_m$ and reduction in $k_{cat}$. Whereas for neutral substrates and free AChE, the large $K_m$ values were limiting, the situation is reversed for the AChE-Fas2 complex, where large $K_m$ values primarily limit hydrolysis of cationic substrates. $k_{cat}$ values for the AChE-Fas2 complex were significantly reduced for all substrates. In addition, $k_{cat}$ values for the AChE-Fas2 complex correlate with the size of the acyl moiety in the substrate, suggesting that the steric occlusion by acyl pocket residues also limits substrate hydrolysis for the AChE-Fas2 complex. Coulombic repulsion from arginines and lysines of bound Fas2 could also interfere with the orientation of thiocholine in the transition state and its exit from the gorge. Both the reversible binding steps and turnover of two reactive cationic substrates, ATCh and PTCCh, appeared to be reduced to the greatest extent in the AChE-Fas2 complex, suggesting a similar influence of the acyl pocket on the stability of the Michaelis complexes and tetrahedral transition states. This was not the case for neutral substrates, where $k_{cat}$ alone is affected.

**Kinetics of Binding of Reversible Ligands to AChE**—The second order association and the first order dissociation rate constants for two substituted trifluorocatephosphonens and 10 noncovalent reversible ligands to AChE and AChE-Fas2 complex are listed in Table III. The constants for slow equilibrating substrates have similar Michaelis constants (15–18), yet the commitment to catalysis (defined as a ratio of the enzyme acylation rate constant and dissociation rate constant of the Michaelis complex (cf. Ref. 19)) and the formation of the tetrahedral transition state, reflected in $k_{cat}$, appear severely restricted for BTCh and other substrates containing a large acyl group (17, 19).

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inhibitors such as the trifluoroacetophenones and (−)-huperzine A were determined using the M7C reaction assay, whereas for the faster equilibrating inhibitors, stopped-flow techniques with fluorescence monitoring were used (Figs. 2 and 3). Rates of interaction for BW286C51 were analyzed by both techniques. The association rate constants of the 10 ligands with AChE covered a narrow range of less than 2 orders of magnitude (4.5 × 10⁷ to 2.8 × 10⁹ M⁻¹ s⁻¹), and only (−)-huperzine A had a significantly slower association rate constant. Since several fast equilibrating ligands from Table III bear ionizable groups, their protonation states at pH 7.0 were analyzed by determining their pKₐ values (Table IV). These pKₐ values indicate that,
in addition to quaternary ligands, tacrine and 9-aminoacridine will be positively charged at pH 7.0; acridine will be neutral and coumarin exists as approximately a 3:1 ratio of neutral to anionic species. The fastest association rate constants were observed for quaternary ammonium ligands bearing a permanent cation ($k_1 = 1.3 \times 10^9$ to $2.8 \times 10^9 $ M$^{-1}$ s$^{-1}$). Protonated cationic ligands, tacrine and 9-aminoacridine ($k_1 = 1.3 \times 10^8$ to $1.5 \times 10^8 $ M$^{-1}$ s$^{-1}$), approached the lower end of the range of rates for quaternary compounds, while neutral ligands (acridine and coumarin) had the slowest association rate constants ($k_1 = 4.5 \times 10^7$ to $1.2 \times 10^8 $ M$^{-1}$ s$^{-1}$). Association rate constants for peripheral site ligands, propidium and coumarin, exhibited the same rates as those ligands associating with the active center, despite the greater surface accessibility of the peripheral site.

The rate constants for dissociation of the 12 ligands extending over 7 orders of magnitude ($1.8 \times 10^{-5}$ to $4.2 \times 10^2 $ s$^{-1}$), spanned a greater range than the association rate constants. Trifluoroacetophenones were the slowest dissociating ligands. These slowly reversible ligands interact covalently at the esteratic site and noncovalently in the choline binding site (12, 21). Comparison of quaternary, protonated, and neutral ligands binding at the active center reveals that apart from trifluoroacetophenones and (−)-huperzine A, cationic bisquaternary BW286C51 and the cationic tertiary amine, tacrine and 9-aminoacridine, had the slowest dissociation rate constants.

**Kinetics of Binding of Reversible Ligands to the AChE-Fas2 Complex**—Ligand interactions with the AChE-Fas2 complex reveal marked reductions in both association and dissociation rate constants, compared with free AChE (Table III). The most affected should be the peripheral site ligands, propidium and coumarin, whose binding is competitive with Fas2 and the bisquaternary ligands, decamethonium and BW286C51, which share a portion of the peripheral binding site with Fas2. The 8-order of magnitude slower association rate constants of BW286C51 and decamethonium accompanied with only 1–3-order of magnitude slower dissociation constants indicate that these bisquaternary ligands will form ternary complex with mAChE-Fas2.

By contrast, the interaction of coumarin with the AChE-Fas2 complex was altered in a different manner than for propidium,
BW286C51, and decamethonium. The small reduction in the coumarin association rate constant is characteristic of active center ligands. Coumarin’s dissociation rate constant was, however, reduced significantly more than those for the above ligands. Taken together, the comparative kinetics indicate the existence of a secondary binding site for coumarin in the active center gorge of the AChE. Previous mutagenesis and molecular modeling studies also suggest coumarin binding to the active center gorge in AChE. Previous results with AChE and the AChE-Fas2 complex typically by 1 order of magnitude but in some cases by 2 orders. The Kd for active center ligands in the AChE-Fas2 complex were about 4 orders of magnitude smaller than the corresponding constants for AChE, irre- 

**Scheme 1**

It can be shown that for association between ligand and enzyme in the above scheme, the following equation holds,

$$ k_1 = k_1^{\text{max}}(1 + bK_E[H^+])/(1 + K_E[H^+])/(1 + [H^+]/K_E^L) \quad (\text{Eq. 4}) $$

where $k_1$ and $k_1^{\text{max}}$ are second order association rate constants for ligand and enzyme at a given pH and a maximal rate constant of the reactive species, respectively. Factor $b$ is a measure of the difference in the second order rate constants for reaction between enzyme and protonated versus nonprotonated forms of ligand. The species $EH^-$ is assumed not to bind ligand. $K_E$ and $K_E^L$ are equilibrium dissociation constants for a proton from ionizable groups in the enzyme and ligand, respectively.

The pH dependence of reaction rate constants of four ligands with AChE was studied in the range of pH 4.5 to 11.0 (Table VI). Association rate constants ($k_1$) for all ligands decreased at
leaving group (25). The reduction of association constants for cationic ligands in titration with 25–3000 nM AChE (closed symbols) and the AChE:Fas2 complex (open symbols). Concentration of both ligands was 10 nM in titration with 5–500 nM AChE and was 50 nM (tacrine) or 100 nM (9-aminoacridine) in titration with 25–3000 nM AChE:Fas2 complex. Inset, decreases in fluorescent emission spectrum of 9-aminoacridine during titration with increasing concentrations of AChE and AChE:Fas2 complex. Shown are 10 nM 9-aminoacridine titrated with concentrations between 0 and 500 nM AChE and 100 nM 9-aminoacridine titrated with concentrations between 0 and 3000 nM AChE:Fas2 complex.

pH values below 6.0 by 1–3 orders of magnitude; the greatest reduction was observed for quaternary cation edrophonium. Analysis of dependences of association rate constants for edrophonium and 9-aminoacridine on pH, using the model from Scheme 1 and the corresponding Equation 4, is shown in Fig. 5A. Association of both ligands in the low pH range depends on ionization of the same group, presumably in AChE, with a $K_a$ value of 6.1–6.2. The AChE group with $p_{Ka}$ of 6.2 was implicated as critical for carbamoylation and phosphorylation of the AChE active center gorge.

The pH dependence of 9-aminoacridine association with AChE:Fas2 complex at low pH is very similar to the dependence observed for association with AChE alone, and reflected in $K_{a,b}$ value of 6.1 (Fig. 5B). At the high pH, the association rate constants were only slightly reduced, much less than observed for association with AChE alone. Thus, both protonated and neutral 9-aminoacridine appear to associate with AChE:Fas2 at similar association rates. In addition, protonation of a basic AChE residue(s), with a presumed $p_{Ka}$ of −10.3 (Fig. 5A), may be affected by bound Fas2 or is no longer a rate-limiting factor in ligand association. The deviations of experimental points from the calculated curve observed in Fig. 5B at intermediate and high pH appeared reproducible and are possibly an indication of the interacting species being more complex than described in Scheme 1. Rates of 9-aminoacridine dissociation from AChE:Fas2 complex were largely independent on pH.

**TABLE V**

| Ligand                  | $K_{a,b}$ AChE | $K_{a,b}$ AChE:Fas2 |
|-------------------------|----------------|---------------------|
| N-Methylacridinium      | 260            | ND                  |
| 9-Aminoacridine         | 38             | 760                 |
| Tacrine                 | 44             | 800                 |
| Coumarin                | 14,000         | ND                  |

**DISCUSSION**

Our comparison of the interaction kinetics of five substrates and 12 reversible inhibitors between the AChE:Fas2 complex and free AChE (Fig. 6) showed second order reaction rate constants for substrates and association rate constants for reversible ligands to be markedly reduced for the AChE:Fas2 complex. When the contribution of the fasciculin-resistant enzyme species is accounted for, the magnitude of the rate reductions approaches 4–6 orders of magnitude.

**Fas2-resistant Esterase**—A Fas2-resistant esterase in quantities consisting of 0.09% of the total activity is retained and eluted from the affinity columns. Characterization of this species is problematic due to its comparatively small quantities. Its specificity for cationic organophosphates differs from mouse butyrylcholinesterase and suggests that it may be a proteolysis product or a spontaneous modification of AChE. Allowing for the contaminant, we now estimate the catalytic efficiency for ATCh with the AChE:Fas2 complex to be 0.004% of uncompromised AChE activity instead of the previously reported 0.09% (2). Mallender et al. (7) found that a large fraction of catalytic activity of the human AChE:Fas2 complex, that represented 2–31% of uncompromised human AChE activity, was in fact contaminating, Fas2-resistant esterase activity. Their findings prompted us to develop multiple approaches to examine whether mouse AChE, despite its low residual activity in the presence of Fas2, also contained a Fas2-resistant component.

Development of a novel competitive binding assay based on
fluorescent product of the carbamoyl ester, M7C, enabled us to determine rate constants for binding of nonfluorescent ligands to mouse AChE:Fas2 complex and to unliganded mouse AChE. The carbamoylation reaction weights each active center equivalently and avoids amplification from the high turnover by residual enzyme not inhibited by Fas2. In addition, incubation of a mixture of the AChE:Fas2 complex and Fas2-resistant esterase with a bulky organophosphate selectively inhibits the latter, enabling one to account for the disparity of findings with previous studies. Finally, employing stopped-flow kinetics with direct fluorescence detection of the complexes allowed us to separate association and dissociation rate constants for binding of several rapidly equilibrating reversible inhibitors to both free AChE and the AChE:Fas2 complex.

Influence of Fasciculin on Hydrolysis of Substrates—Analysis of the kinetics indicates that inhibition of AChE activity by Fas2 is not due solely to a diminished rate of substrate entry into the active center in the AChE:Fas2 complex. The commonly considered reaction steps in substrate hydrolysis by AChE (14, 28, 29) are shown in Scheme 2,

\[
E + S \rightarrow ES \rightarrow EA \rightarrow E + P_2
\]

Scheme 2

where enzyme (E) and substrate (S) associate to form a Michaelis complex (ES). In turn, the complex is converted to an unstable acyl enzyme (EA) with loss of the substrate leaving group (P1). EA is hydrolyzed to free enzyme and acyl group of substrate (P2). The corresponding catalytic parameters, \( K_m \), \( k_{cat} \), and \( k_{cat}/K_m \) are described as follows.

\[
K_m = (k_{-1} + k_2)k_b/(k_1k_2 + k_b)
\]

(Eq. 5)

\[
k_{cat} = (k_1k_2)(k_1 + k_2)
\]

(Eq. 6)

\[
k_{cat}/K_m = (k_1k_2)(k_{-1} + k_b)
\]

(Eq. 7)

Second order rate constants for reaction of cationic substrates with AChE:Fas2, reflected in \( k_{cat}/K_m \) values between 60 and 100 \( M^{-1} s^{-1} \), are slower than rate constants for entry of reversible ligands into the AChE:Fas2 complex. The exceptionally slow association rates of BW286C51 and decamethonium are most easily explained by partial overlap of their binding site with the site of Fas2 residence due to their extended conformation (cf. Fig. 6). Thus, additional conformational constraints apply to their association rates. Furthermore, the reduction of nearly 6 orders of magnitude in \( k_{cat}/K_m \) for ATCh and PTCh exceeds the reduction in association rate constants for the reversible cationic inhibitors with the AChE:Fas2 complex. Catalyzed hydrolysis by AChE:Fas2 for various substrates yielded sets of limiting values for \( k_{cat} \) and for \( k_{cat}/K_m \). The similarity of \( k_{cat} \) values for charged (ATCh) and two neutral acetate esters (S-PhAc and pNP4Ac) suggests that the rate of deacylation of EA (k2) may be involved in rate limitation for the chemical part of the catalytic reaction under the influence of bound Fas2.

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**TABLE VI**

Effect of pH on association and dissociation rate constants for selected reversible ligands and mouse AChE

Reactions were performed in duplicate.

| pH  | 9-Aminoacridine | Acridine | Edrophonium | Coumarin |
|-----|-----------------|----------|-------------|----------|
|     | \( k_1 \)       | \( k_{-1} \) | \( k_1 \)   | \( k_{-1} \) | \( k_1 \) | \( k_{-1} \) |
|     | \( \mu M^{-1} s^{-1} \) | \( s^{-1} \) | \( \mu M^{-1} s^{-1} \) | \( s^{-1} \) | \( \mu M^{-1} s^{-1} \) | \( s^{-1} \) |
| 4.5 | 2.7            | 1.4      | 5.8         | 20       | 0.14   | 46       | –         |
| 5.0 | 7.5            | 2.5      | –          | –        | –      | –        | –         |
| 5.5 | 25             | 3.9      | 130        | 78       | 17     | 64       | –         |
| 6.0 | 70             | 3.3      | –          | –        | 47     | 26       | 150       |
| 7.0 | 120            | 4.2      | 180        | 200      | 55     | 27       | 45        |
| 8.0 | 140            | 4.9      | –          | –        | 48     | 28       | –         |
| 9.0 | 140            | 3.4      | –          | –        | 26     | 34       | 8.0       |
| 10.0| 100            | 3.2      | –          | –        | –      | –        | 21        |
| 10.6| 41             | 5.5      | –          | –        | –      | –        | –         |
| 11.0| 17             | 4.4      | –          | –        | –      | –        | –         |

**FIG. 5** The pH dependence for association of edrophonium (C), and 9-aminoacridine (A), with AChE (panel A) and AChE:Fas2 complex (panel B). The second order rate constants of association were fitted to Equation 4 with the following parameters. \( k_1^{max} = (1.2 \pm 0.3) \times 10^{6} \ M^{-1} s^{-1} \), \( pK_a^E = 6.2 \), \( pK_a^L = 7.6 \), \( b = 0.19 \pm 0.03 \) (for edrophonium) and \( k_1^{max} = (1.4 \pm 0.1) \times 10^{6} \ M^{-1} s^{-1} \), \( pK_a^E = 6.1 \), \( pK_a^L = 10.2 \), \( b = 0 \) (for 9-aminoacridine). In \( k_1^{max} = (5.2 \pm 0.2) \times 10^{6} \ M^{-1} s^{-1} \), \( pK_a^E = 6.1 \), \( pK_a^L = 11.9 \), \( b = 0 \) (for 9-aminoacridine).
Parallel reductions in $k_2$ by Fas2 are also likely, since the inhibition rates of several organophosphates were shown to be reduced in the AChE-Fas2 complex by several orders of magnitude more than the rates of association of reversible inhibitors (6). Furthermore, a second clustering of $k_{cat}/K_m$ values for hydrolysis of charged acyl thiocelaines by AChE-Fas2 ($-83 \text{ M}^{-1} \text{s}^{-1}$) as opposed to neutral acetates ($-3000 \text{ M}^{-1} \text{s}^{-1}$) indicates that the corresponding $k_1$ and $k_2$ constants are of the same magnitude and, consequently, together with $k_1$ both influence values of $k_{cat}/K_m$. This follows because the reduction of $k_{cat}/K_m$ for ATCh, imposed by Fas2, is larger than reductions observed in rates of association of reversible inhibitors. Charge on the leaving group influences both reactions, described by $k_1$ and $k_2$, which both participate in limiting the rates of hydrolysis by the AChE-Fas2 complex. The absence of the selective steric constraints of the acyl pocket in the AChE-Fas2 complex (Table II) further suggests that the leaving group of the substrate is limiting for catalytic throughput ($k_{cat}/K_m$) in the AChE-Fas2 complex. Thus, in addition to the pronounced effects of bound Fas2 on rates of ligand entry into AChE, reflected in $k_1$, an allosteric interaction between the Fas2 binding site and the active center appears to affect the efficiency of AChE catalysis primarily through reduction of both $k_1$ and $k_2$.

**Influence of Fasciculin on the Binding of Reversible Ligands**—Our compilation of reaction rate constants for diverse reversible inhibitors of AChE permits one to dissect the factors controlling ligand entry to the gorge and steric constraints within the gorge. The affinities of these inhibitors are governed by two factors. First, the association rate constants for all but one ligand studied approach the diffusion-limited range, ensuring rapid formation of inhibitory complexes. Similar rates of association for ligands binding at the peripheral site and active center reflect minimal steric constraints for entry into the narrow, 18–20 Å in depth active center gorge (cf. Fig. 6) for those ligands not exceeding a critical volume (propidium) or shape ((-)huperzine A).

Second, the residence times of ligands in the stabilized complexes with AChE varied over 7 orders of magnitude. Previously reported rate constants for ligand association with AChE, limited to slow dissociating fasciculins, huprines, trifluoroacetophenones, huperzine A, ambenonion, and bisquaternary o-CIB-BQ (Table VII), and only three fast equilibrating ligands, N-methylacridinium, 7-hydroxyquinolinium, and propidium, were in good agreement with the constants reported here (cf. Tables III and VII). To date, N-methylacridinium is the only reversible inhibitor characterized for interaction with the AChE-Fas2 complex ($k_1 = 1.0 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$, $k_2 = 0.40 \text{ s}^{-1}$) for human AChE; Ref. 6).

The binding of several ligands, such as edrophonium and decamethonium, that lack a spectral overlap with tryptophan fluorescence emission nonetheless results in diminished AChE fluorescence in the ligand-enzyme complex. Since fluorescence resonance energy transfer is not possible in this situation, the quantum yields of several tryptophans have been affected by ligand occupation of the enzyme active center. This observation not only increases the range of ligands whose association with the enzyme can be measured directly but also indicates that the microenvironments of several aromatic residues are perturbed in the complex. The implications of this finding will be addressed in subsequent studies.

**Linear Free Energy Relationships**—Reduced association and dissociation rate constants for ligands interacting with the AChE-Fas2 complex reveal significant steric hindrance for entry of ligands into the gorge. The different magnitude of the steric intervention on association and dissociation steps, however, suggests that Fas2 alters the position of the transition state relative to the free and bound species. This is evident from the strikingly different linear free energy relationships between association or dissociation rate constants of the ligands and their equilibrium dissociation constants for AChE and AChE-Fas2 complex (Fig. 7).

Relating free energy changes in the transition state to those in the ground states for the various ligands enables one to better describe the energetic pathway for ligand entry and exit from the gorge for AChE and the AChE-Fas2 complex (37–39). Thus, for a series of ligands, $L$, the following linear free energy

\[ AChE-Fas2 + L \rightarrow AChE-Fas2L \]

\[ AChE + L \rightarrow AChE-L \]

\[ k_{cat}/K_m = k_{cat}/K_m^0 + \Delta G^* \]

where $k_{cat}/K_m^0$ is the rate constant for the reaction in the absence of the inhibitor, $\Delta G^*$ is the free energy change associated with the binding of the inhibitor, and $L$ is the ligand. This relationship suggests that the energetic pathway for ligand entry and exit from the gorge is affected by the presence of Fas2, which alters the transition state position relative to the ground state. This finding is consistent with previous reports suggesting that Fas2 alters the position of the transition state relative to the ground state for ligand entry and exit from the gorge. The implications of this finding will be addressed in subsequent studies.
relationships can be developed.

\[ \Delta G^{\dagger}_{A,L} = \Phi \Delta G^{\dagger}_{L} + \Delta G^0 \]  
(Eq. 8)

\[ \Delta G^{\dagger}_{D,L} = (\Phi - 1)\Delta G^{\dagger}_{L} + \Delta G^0 \]  
(Eq. 9)

\( \Delta G^{\dagger}_{L} \) and \( \Delta G^0 \) are the activation free energies for association and dissociation, \( \Delta G^0 \) is the respective equilibrium free energy for each ligand, and \( \Delta G^0 \) is a constant, intrinsic activation barrier. The slope of the relationships is related to \( \Phi \), an
Ligands and the Acetylcholinesterase-Fasciculin 2 Complex

Factors Controlling Kinetics of Ligand Interaction—By using stopped-flow instrumentation, we examined interaction kinetics for rapid and slow equilibrating ligands with selectivity for the active and the peripheral sites. It has been suggested (7, 40–42) that binding of cationic substrates to peripheral site of mammalian, fish, or insect AChE effectively increases the local substrate concentration available for binding to the active center and thus enhances catalytic efficiency. Such a mechanism seems plausible where substrates would have to traverse an energy barrier imposed by a narrow gorge to reach to the site of catalysis in the active center. As would be expected, the kinetic barriers imposed by the pre-Michaelis complex are minimal, since active center ligands and peripheral site ligands associate with AChE at comparable rates. The modular analysis of molecular traffic through the AChE active center (41) suggested distinct limits for diffusion of neutral and cationic ligands, differing by about 1 order of magnitude. Our determined average second order association rate constants of $8.9 \times 10^7$ M$^{-1}$ s$^{-1}$ (for cationic ligands) and $8.2 \times 10^7$ M$^{-1}$ s$^{-1}$ (for neutral ligands) are in accord with the prediction.

Protonation of neutral ligands (9-aminoacridine, tacrine) only modestly affects association rates with AChE and the AChE-Fas2 complex, perhaps reflecting a balance between rate acceleration by the addition of a charge and deceleration from the increased hydrated molecular volume. The hydrated cation structure, however, decreases the dissociation rates, as evidenced by a comparison of N-methylacridinium and acridine with 9-aminoacridine and tacrine as well as by pH-dependent decrease of the dissociation rate of acridine. Net neutralization of charged edrophonium by turning it into zwitterion, as well as the addition of negative charge to neutral coumarin markedly reduced their association rate constants, as reported for 1-methyl 7-hydroxyquinolinium (36). We find that protonation of enzyme group(s) of $K_a$ 6.1–6.2 critically decreases association rates of positively charged ligands with both AChE and the AChE-Fas2 complex in addition to reduction of phosphorylation and carbamylation rates of the AChE active serine (25).

Rapid conformational isomerizations may control the opening times of the active center gorge to admit ligands (43). In addition to pathway restrictions imposed by charge and steric constraints of Fas2, these isomerizations could be far less frequent in AChE-Fas2 due to restrictions in the breathing motions of domains of AChE immobilized by Fas2. Fas2 at the gorge entry can also be expected to influence allosterically the conformation of residues controlling catalysis and ligand dissociation at the base of the gorge.

Comparative analysis of the kinetics of ligand entry and exit from the active center gorge for free AChE and AChE-Fas2 complex reveals that the steric influences of Fas2 at the gorge entry are insufficient to explain the differences in linear free energy relationships between the kinetic and equilibrium constants for free AChE and the Fas2-AChE complex (Fig. 7).

![Graph](image-url)

**Fig. 7.** Linear free energy relationships for interaction of small reversible inhibitors with AChE (open symbols) and the AChE-Fas2 complex (closed symbols). The numbers on the symbols indicate their order in Table III. Quaternary ammonium, cationic inhibitors are noted by circles, protonated cationic inhibitors by diamonds, and uncharged inhibitors by squares. Lines were generated by linear regression of Equations 8 and 9. (-)-Huperzine A (inhibitor number 4) was not included in the calculation. A, linear free energy relationship for dissociation from AChE and AChE-Fas2. Calculated slopes were $(1.0 \pm 0.1)$ for AChE, and $(0.074 \pm 0.13)$ for AChE-Fas2. B, linear free energy relationship for association with AChE and AChE-Fas2. Calculated slopes were $-(0.093 \pm 0.056)$ for AChE, and $-(0.93 \pm 0.13)$ for AChE-Fas2.

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