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Hydrolysis of low concentrations of the acetylthiocholine analogs acetyl(*homo*)thiocholine and acetyl(*nor*)thiocholine by acetylcholinesterase may be limited by selective gating at the enzyme peripheral site

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Abstract

Hydrolysis of acetylcholine by acetylcholinesterase (AChE) is extremely rapid, with a second-order hydrolysis rate constant k_E (often denoted k_{cat}/K_M) that approaches $10^8 \text{ M}^{-1}\text{s}^{-1}$. AChE contains a deep active site gorge with two sites of ligand binding, an acylation site (or A-site) containing the catalytic triad at the base of the gorge and a peripheral site (or P-site) near the gorge entrance. The P-site is known to contribute to catalytic efficiency with acetylthiocholine (AcSCh) by transiently trapping the substrate in a low affinity complex on its way to the A-site, where a short-lived acyl enzyme intermediate is produced. Here we ask whether the P-site does more than simply trap the substrate but in fact selectively gates entry to the A-site to provide specificity for AcSCh (and acetylcholine) relative to the close structural analogs acetyl*homo*thiocholine (Ac-*h*SCh, which adds one additional methylene group to thiocholine) and acetyl*nor*thiocholine (Ac-*n*SCh, which deletes one methylene group from thiocholine). We synthesized Ac-*h*SCh and Ac-*n*SCh and overcame technical difficulties associated with instability of the *nor*thiocholine hydrolysis product. We then compared the catalytic parameters of these substrates with AChE to those of AcSCh. Values of k_E for Ac-*h*SCh and Ac-*n*SCh were about 2% of that for AcSCh. The k_E for AcSCh is close to the theoretical diffusion-controlled limit for the substrate association rate constant, but k_E values for Ac-*h*SCh or Ac-*n*SCh are too low to be limited by diffusion control. However, analyses of kinetic solvent isotope effects and inhibition patterns for P-site inhibitors indicate that these two analogs also do not equilibrate with the A-site prior to the initial acylation step of catalysis. We propose that k_E for these substrates is partially rate-limited by a gating step that involves the movement of bound substrate from the P-site to the A-site.

Keywords

Acetylcholinesterase; peripheral site; active site gating; acetylthiocholine analogs; thioflavin T; enzyme mechanism

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1. Introduction

A better understanding of the AChE catalytic pathway may identify new mechanistic opportunities for AChE inhibition that could be therapeutically beneficial. Kinetic and thermodynamic studies have revealed that substrates and inhibitors can interact with either or both of two binding sites in AChE (1–4), and X-ray crystallography has provided information about the location of these sites (5–8). A narrow active site gorge some 20 Å deep penetrates nearly to the center of the ~65 kDa catalytic subunits. Near the base of the gorge is the acylation or A-site where H447, E334, and S203² participate in a triad that catalyzes the transient acylation and deacylation of S203 during each substrate turnover. The peripheral or P-site, spanned by residues W286 near the mouth of the gorge and D74 near a constriction at the boundary between the P-site and the A-site, specifically binds certain ligands like the neurotoxin fasciculin (9, 10) and the fluorescent probes propidium (2) and thioflavin T (3, 7). The P-site thus far has been shown to contribute to catalytic efficiency by insuring that most substrate molecules that collide with and transiently bind to the P-site proceed on to the A-site (11–13) and, with certain bound cationic substrates, by providing a modest allosteric activation of the acylation step (14). Here we ask whether the P-site does more than simply trap the substrate but in fact selectively gates entry to the A-site to provide specificity for AcSCh (and acetylcholine) relative to the close structural analogs acetyl(*homo*)thiocholine (Ac-*homo*SCh, which adds one additional methylene group to thiocholine) and acetyl(*nor*)thiocholine (Ac-*nor*SCh, which deletes one methylene group from thiocholine) (Fig. 1).

2. Experimental methods

2.1. Synthesis of 3-(acetylthio)-N,N,N-trimethylpropanaminium iodide (Ac-*homo*TCh)

Thiolacetic acid (5.68 mL, 79.0 mmol) was added to a solution of 3-(dimethylamino)propyl chloride hydrochloride (10.0 g, 63.3 mmol) in *N,N*-dimethylformamide (135 mL). This was followed by portionwise addition of cesium carbonate (51.5 g, 158 mmol). The resulting suspension was stirred under nitrogen for 10 min and then heated to 70° C in an oil bath overnight. After cooling to room temperature, water (300 mL) was added and the reaction mixture was poured into a separatory funnel and extracted with ether (4 × 100 mL). The combined organic extracts were washed with water, dried over MgSO₄, filtered, and concentrated in vacuo to afford the product, *S*-3-(dimethylamino)propyl ethanethioate (I), as a light yellow oil: Yield 10.1 g (99%). This material was used in the next step without further purification. ¹H NMR (CDCl₃, 300 MHz) δ 2.90 (t, 2H, *J* = 7.2 Hz), 2.32 (m, 2H), 2.21 (s, 6H), 1.74 (m, 2H). MS *m/z* (ESI) 162.16 (M+1)⁺.

Compound I (9.0 g, 56 mmol) in diethyl ether (295 mL) was placed in a clean, dry round bottom flask containing sodium carbonate (8.87 g, 84.0 mmol). The solution was stirred and iodomethane (17.45 mL, 279.0 mmol) was slowly introduced to the resulting suspension at room temperature under nitrogen. The flask was covered with a black cloth and the reaction was allowed to proceed in the dark overnight. During this time, a solid precipitated that was separated by filtration. Drying of this solid produced a lumpy material that was pressed into a fine powder. This powder was first washed with a (1:1) mixture of diethyl ether and hexane, and then thoroughly leached and extracted with several fresh portions of dichloromethane. The combined dichloromethane fractions were evaporated under reduced pressure to afford a light brownish solid that was dissolved in water and decolorized by stirring with activated carbon for 30 min at 25 °C. The product Ac-*homo*TCh was filtered through Celite and lyophilized. The residue was further purified by recrystallization with

²Throughout this paper we number amino acid residues according to the human AChE sequence unless otherwise noted.

dichloromethane to afford a white solid: Yield 13.5 g (80%). ^1H NMR (CDCl_3 , 300 MHz) δ 3.74 (m, 2H), 3.46 (s, 9H), 3.00 (t, 2H, $J = 6.9$ Hz), 2.39 (s, 3H), 2.12 (m, 2H). MS m/z (ESI) 176.32 (M^+) $^+$.

2.2. Synthesis of 1-(acetylthio)-N,N,N-trimethylmethanaminium iodide (Ac-norSCh)

N-methyl-*N*-methylenemethanaminium chloride (3.00 g, 32.1 mmol) and potassium thioacetate (4.39 g, 38.5 mmol) were suspended in diethyl ether (33 mL) and stirred under nitrogen overnight. After filtering through Celite, the filtrate was concentrated in vacuo to afford the adduct, *S*-(dimethylamino)methyl ethanethioate (II), as a light yellow oil: Yield 3.75 g (88%). This material was carried to the next step without further purification. ^1H NMR (CDCl_3 , 300 MHz) δ 4.56 (s, 2H), 2.40 (s, 3H), 2.22 (s, 6H). MS m/z (ESI) 134.19 ($\text{M} + 1$) $^+$.

Iodomethane (14.55 mL, 233.0 mmol) was added to a solution of II (6.2 g, 46.5 mmol) in diethyl ether (243 mL). Sodium carbonate (7.40 g, 69.8 mmol) was added portionwise. The flask was covered with a black cloth and the turbid suspension was stirred under nitrogen at room temperature overnight. The precipitated solid generated during the reaction was filtered and washed with a 1:1 mixture of diethyl ether and hexanes. The residue was scraped with a spatula and leached with several portions of dry dichloromethane. The combined dichloromethane extracts were filtered and evaporated under reduced pressure to furnish the product as a light brownish solid. Double recrystallization, first with isopropanol and then with dichloromethane, furnished a white solid that was judged pure by ^1H NMR. Yield 8.20 g (64%). ^1H NMR (CDCl_3 , 300 MHz) δ 5.2 (s, 2H), 3.46 (s, 9H), 2.57 (s, 2H). MS m/z (ESI) 148.21 (M^+) $^+$.

2.3. Assays of substrate hydrolysis

Recombinant human AChE was expressed as a secreted, disulfide-linked dimer in *Drosophila* S2 cells and purified as outlined previously (15). Thioflavin T (Sigma) was recrystallized from water, and concentrations were assigned by absorbance at 412 nm with $\epsilon_{412\text{ nm}} = 36,000\text{ M}^{-1}\text{cm}^{-1}$. For AcSCh and the two new thioester substrates, the hydrolysis rates v were measured in a coupled Ellman reaction in which the thiol generated in the presence of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (2.0 mM unless otherwise noted) was determined by formation of the thiolate dianion of DTNB from the absorbance (A) at 412 nm ($\Delta\epsilon_{412\text{ nm}} = 14,150\text{ M}^{-1}\text{cm}^{-1}$) (14). Total AChE concentrations (E_{tot}) were calculated assuming 450 units/nmol (which, under the assay conditions here, corresponds to $4.8\text{ }\Delta A_{412}/\text{min}$ with 0.5 mM acetylthiocholine substrate and 1 nM AChE) (3). Assays were conducted at 25°C in 20 mM sodium phosphate buffer (pH 7.0) and 0.01% bovine serum albumin (BSA) (assay buffer), and to maintain constant ionic strength NaCl was added so that the sum of the substrate and NaCl concentrations was 60 mM. AChE concentrations were varied to optimize measured rates, and non-enzymatic hydrolysis rates were deducted from all enzymatic hydrolysis rates.

2.4. Kinetic analysis of substrate hydrolysis rates

When the concentration of substrate (S) is low ($[S] \ll K_D$, where K_D is defined in Eq. (2) below), the most accurate way to measure the AChE-catalyzed second-order hydrolysis rate constant k_E is from Eq. (1) (16).

$$[S] = [S]_0 e^{-zt} \quad (1)$$

In Eq. (1), $z = k_E[E]_{\text{tot}}$, where t is the time after initial mixing of enzyme (E) and S at $t = 0$. To fit hydrolysis data, the following substitutions were made in Eq. (1) and the equation was

solved for A_{412} at time t : $[S] = (A_{412} - A_{412(\text{final})})/\Delta\epsilon_{412 \text{ nm}}$, where $A_{412(\text{final})}$ is A_{412} at the conclusion of the exponential time course; and $[S]_0 = (A_{412(\text{final})} - A_{412(t=0)})/\Delta\epsilon_{412 \text{ nm}}$.

When an inhibitor specific for the P-site is present during second order substrate hydrolysis (e.g., Scheme 1 below), the ratio of k_E in the absence of inhibitor ($k_E [I]=0$) to k_E in the presence of inhibitor (k_{E+I}) can be fitted to Eq. (2) (16, 17).

$$\frac{k_{E[I]=0}}{k_{E+I}} = \frac{\left(1 + \frac{[I]}{K_I}\right)}{\left(1 + \frac{\alpha[I]}{K_I}\right)} \quad (2)$$

The fitted parameter α can be interpreted in mechanistic terms regardless of whether the substrate equilibrates with the enzyme (16).

Over a wider range of $[S]$, AChE-catalyzed hydrolysis of several substrates has been shown to deviate from classical hyperbolic Michaelis-Menten kinetics (14). Eq. (3) includes terms that can account for these deviations.

$$\frac{v}{[E]_{\text{tot}}} = \frac{k_E [S] \cdot \left(1 + \frac{a_{SA} [S]}{K_{SA}}\right) \left(1 + \frac{a_{SI} [S]}{K_{SI}}\right)}{\left(1 + \frac{[S]}{K_D}\right) \left(1 + \frac{[S]}{K_{SA}}\right) \left(1 + \frac{[S]}{K_{SI}}\right)} \quad (3)$$

In addition to a term representing the active site where hydrolysis occurs (with apparent substrate dissociation constant K_D), it includes a substrate inhibition site (with apparent dissociation constant K_{SI}), and a substrate activation site (with apparent dissociation constant K_{SA}) (18). Binding of substrate to the inhibition site decreases substrate turnover ($a_{SI} < 1$) while binding of substrate to the activation site increases substrate turnover ($a_{SA} > 1$). When substrates fully equilibrate with AChE, these apparent dissociation constants can represent actual thermodynamic binding (e.g., K_D is the Michaelis constant K_M). However, some substrates proceed through the catalytic pathway too fast to equilibrate with AChE, and in this case Eq. (3) simply offers an empirical fit that allows detection of substrate inhibition or activation without a mechanistic context (14). After assigning a_{SI} as zero and fixing k_E at the value measured with Eq. (1), data were fitted to Eq. (2) by nonlinear regression with SigmaPlot (vers.11.0) to obtain fitted values of K_D , K_{SI} , K_{SA} , and a_{SA} .

3. Results and Discussion

3.1. Stability of Ac-homoSCh, Ac-norSCh, and the hydrolysis product northiocholine (norSCh)

The AChE substrates Ac-*homo*SCh and Ac-*nor*SCh (Fig. 1) were synthesized as outlined in the Experimental methods. Non-enzymatic hydrolysis rate constants (k_{nonE}) for AcSCh and Ac-*homo*SCh were almost negligible at the AChE concentrations employed (k_{nonE} values were 16×10^{-6} and $8 \times 10^{-6} \text{ mM}^{-1}\text{min}^{-1}$, respectively). However, k_{nonE} for Ac-*nor*SCh was much larger ($700 \times 10^{-6} \text{ mM}^{-1}\text{min}^{-1}$), as expected from the electron withdrawing effect of the quaternary ammonium group on the stability of the thioester bond, and this k_{nonE} became quite significant at the highest substrate concentrations examined in this report. For example, at 50 mM Ac-*nor*SCh, the non-enzymatic hydrolysis rate v_{nonE} was about 0.5 $\Delta A_{412}/\text{min}$ in our assay. As a further complication, this rate at high concentrations of Ac-*nor*SCh was not linear but rapidly decreased: v_{nonE} decreased 26% over one minute with 50 mM Ac-*nor*SCh, in contrast to a decrease of < 3% over one minute with 5 mM Ac-*nor*SCh.

Mass spectrometry analysis of an aqueous dilution of Ac-*nor*SCh in assay buffer revealed that the hydrolysis product *nor*thiocholine (*nor*SCh: $(\text{CH}_3)_3\text{N}^+-\text{CH}_2-\text{SH}$) was not stable but proceeded along two additional reaction pathways. The first pathway generated trimethylamine $(\text{CH}_3)_3\text{N}$, $(\text{M}+1)^+ 60$) and was inferred to be a decomposition of *nor*SCh that produced trimethylamine, formaldehyde, and hydrogen sulfide. The second pathway produced 1-((3-mercaptopropanoyl)thio)-N,N,N-trimethylmethanaminium $(\text{CH}_3)_3\text{N}^+-\text{CH}_2-\text{S}-\text{CO}-(\text{CH}_2)_2-\text{SH}$, $(\text{M})^+ 194$), and this product can be generated by an irreversible nucleophilic displacement of trimethylamine from *nor*SCh by the enolate tautomer of Ac-*nor*SCh. With 50 mM Ac-*nor*SCh in assay buffer, the second pathway was predominant over the first, but addition of 2 mM DTNB to this mixture decreased the 194 $(\text{M})^+$ ion peak by more than a factor of two. These observations indicated that Ac-*nor*SCh and DTNB compete for reaction with *nor*SCh and that this competition will reduce the rate of formation of the chromophoric reporter group, the thiolate dianion of DTNB.

To minimize the distorting effect of high concentrations of Ac-*nor*SCh on its nonenzymatic and enzymatic hydrolysis rates measured with DTNB, we took three steps. First, stock Ac-*nor*SCh was weighed individually (~ 2 mg) for each assay above 20 μM Ac-*nor*SCh and assayed within three minutes after initial dissolution in water and 10-fold dilution into assay buffer. Although this tedious precaution decreased the precision of each assay and contributed to scatter of the data over a range of substrate concentrations, it substantially improved the reproducibility of hydrolysis rates at substrate concentrations > 5 mM. Second, initial rate measurements at concentrations of Ac-*nor*SCh > 5 mM were limited to the first minute after mixing. Third, the DTNB concentration in assays was increased from the conventional 0.33 mM to 2 mM. This change alone increased $\Delta A_{412}/\text{min}$ 2-fold at 50 mM Ac-*nor*SCh, as might be expected if Ac-*nor*SCh and DTNB compete for reaction with the *nor*SCh hydrolysis product. These steps appeared to eliminate the distorting effects of high concentrations of Ac-*nor*SCh, as v_{nonE} was observed to increase linearly with Ac-*nor*SCh concentration up to 50 mM.

3.2. Ac-homoSCh and Ac-norSCh are hydrolyzed much more slowly than AcSCh by AChE

AChE-catalyzed hydrolysis rates for all three substrates were fitted to Eq. (3) (Fig. 2), and the fitted parameters for Ac-*homo*SCh and Ac-*nor*SCh are compared to those for AcSCh in Table 1. Second order hydrolysis rate constants k_E were similar for Ac-*homo*SCh and Ac-*nor*SCh and about 60-fold smaller than the k_E for AcSCh. Maximal normalized hydrolysis rates $v/[E]_{\text{tot}}$ for Ac-*homo*SCh and Ac-*nor*SCh were about 5- and 8-fold smaller, respectively, than that for AcSCh. The data indicated that none of the three substrates followed classical Michaelis-Menten kinetics. All showed a substrate inhibition component, although its magnitude with Ac-*nor*SCh was uncertain because of the technical problems noted above in assaying this substrate at concentrations greater than 5 mM. Ac-*homo*SCh also exhibited substrate activation. Values of k_E and K_D for AcSCh were about 30% larger and 30% smaller, respectively, than previously reported (14). These differences largely resulted from the substitution of 0.01% BSA for 0.02% Triton X-100 to stabilize AChE activity. Triton X-100 has been observed to inhibit the activity of insect and eel AChEs (19, 20), and we found that 0.01% BSA was better than 0.02% Triton X-100 at stabilizing dilute AChE activity over periods of several hours. The value of the substrate activation parameter a_{SA} (3.1) obtained for Ac-*homo*SCh is comparable to that previously observed for the cationic acetanilide ATMA and for AcSCh with the human W86A mutant (14). AcSCh exhibits only barely detectable substrate activation with wild type human AChE, probably because the acetylation step is not rate limiting (14).

3.3. Do the low values of k_E for Ac-homoSCh and Ac-norSCh relative to k_E for AcSCh reveal that their entry to the A-site is partially limited by gating at the P-site?

Here we return to the question of whether the P-site does more than simply trap the substrate but in fact selectively gates entry to the A-site to provide specificity for AcSCh (and acetylcholine) relative to the close structural analogs Ac-homoSCh and Ac-norSCh. To define gating in enzyme kinetics terms, a model of the AChE catalytic pathway is required. This pathway for AcSCh is known to include acetyl enzyme intermediates in addition to the initial enzyme-substrate complexes (12, 13). Under second-order substrate hydrolysis conditions, however, ternary complexes involving two S molecules bound to E as well as all downstream acetylated intermediates become negligible because [S] approaches zero. In this case, Scheme 1 offers an appropriate reaction model (16).

This scheme involves two enzyme substrate intermediates, ES_P at the P-site and ES at the A-site, in addition to free enzyme E. An additional ligand specific to the P-site (I_P) is also included, and in the ternary complex ESI_P the acylation rate constant k_2 can be increased or decreased by the factor a_I . In the context of Scheme 1, the second-order rate constant k_E in the absence of I is given by Eq. (4).

$$k_E = \frac{k_S k_1 k_2}{k_{-S} k_{-1} + k_2 (k_{-S} + k_1)} \quad (4)$$

As emphasized by Quinn (21), the rate constant k_E monitors conversion of free enzyme and free substrate to a transition state(s) in the acylation stage of catalysis, and it incorporates all initial reversible steps in the pathway up to the first irreversible step. The value of k_E can be limited by any of the three forward steps in Scheme 1. Under equilibrium conditions (where the bond-breaking step k_2 is much smaller than simple binding steps like k_{-1}), $k_E = k_2/K_M$, where $K_M = K_S K_I$ ($K_S = k_{-S}/k_S$ and $K_I = k_{-1}/k_1$), and k_2 is rate limiting. The binding steps that comprise K_M cannot be determined individually. Because of the strength of their ester or amide bonds, many carbamate and acetanilide substrates of AChE fall into this category (16). At the other extreme, the forward steps may be so much faster than the reverse steps that they become irreversible ($k_2 \gg k_{-1}$ and $k_1 \gg k_{-S}$), and then $k_E = k_S$, the substrate association rate constant. This is thought to be the case with AcSCh (13), as its k_E of $10^8 \text{ M}^{-1}\text{s}^{-1}$ (Table 1) is close to the expected diffusion-controlled value for k_S . In this case also, the binding steps that comprise K_M cannot be determined individually. In Scheme 1, k_1 is the rate constant at which S moves from the P-site to the A-site, and we interpret k_1 as a gating rate constant for S entry to the A-site. For k_1 to be rate limiting, k_{-S} must be $\gg k_1$ but $k_2 \gg k_{-1}$. This gives $k_E = k_1/K_S$.

Some information is available about rate constants that comprise K_M . Competitive binding experiments with fasciculin 2 and AcSCh (12) or thioflavin T and Ac-homoSCh or Ac-norSCh³ indicate that K_S for all three substrates is around 1 mM. Assuming that all have similar k_S values that reach or exceed the near diffusion-controlled limit noted above for AcSCh, these substrates would have k_{-S} values $\sim 10^5 \text{ s}^{-1}$ for their dissociation from the AChE P-site. Based on the arguments above and the k_E for AcSCh in Table 1, this value implies that the gating rate constant k_1 for AcSCh must be $\sim 10^5 \text{ s}^{-1}$. By the same arguments, the 60-fold lower values of k_E for Ac-homoSCh or Ac-norSCh in Table 1 rule out k_S being rate-limiting for these substrates and leave k_1 or k_2 as the possible rate-limiting steps for k_E . In this case, $k_{-S} \gg k_1$ and k_E in Eq. 4 reduces to the expression in Eq. (5).

³V. Beri and T. L. Rosenberry, manuscript in preparation.

$$k_E = \frac{k_1 k_2}{K_S (k_{-1} + k_2)} \quad (5)$$

Two additional experiments argue that k_2 is itself not rate-limiting and that k_1 contributes to rate limitation of k_E . The first measured solvent deuterium oxide (D_2O) isotope effects, which can identify enzyme-catalyzed steps that are rate-limited by proton transfer (21). With AChE, k_{cat} is a measure of the turnover rate at high $[S]$ and is primarily determined by rate constants like k_2 that involve proton transfer. For acetylthiocholine and phenyl acetate, k_{cat} was slowed by factors of 2.03 ± 0.05 and 2.45 ± 0.08 , respectively, when H_2O was replaced by D_2O (22), values typical of enzyme-catalyzed acyl transfer reactions. In contrast, k_E was slowed by factors of only 1.21 ± 0.02 and 1.48 ± 0.05 , respectively, indicating that k_E was limited primarily by steps before k_2 . The D_2O isotope effects on k_E for Ac-*homo*SCh or Ac-*nor*SCh were 1.59 ± 0.05 and 1.81 ± 0.03 , respectively ³, a range that indicates only partial rate determination by a proton transfer step.

The second experiment examined inhibition of AChE hydrolysis at low $[S]$ by the P-site inhibitor thioflavin T (Fig. 3). Particular attention was given to the parameter α (see Eq. (2)), the ratio of k_{E+I} to k_E $[I]=0$ at saturating concentrations of inhibitor. Previous studies have found that k_E values for slowly hydrolyzed substrates which equilibrate with AChE, like carbachol and ATMA, are unaffected by bound inhibitor ($\alpha = 1$) (16), while rapidly hydrolyzed substrates that do not equilibrate are strongly inhibited ($\alpha = 0.002 \pm 0.003$ for AcSCh (16); ($\alpha = 0.039 \pm 0.002$ for phenyl acetate ³). Values of α for Ac-*homo*SCh and Ac-*nor*SCh of (0.16 and 0.26, Fig. 3) are the first we have found to fall in a mid-range that suggests partial rate determination by both k_1 and k_2 .

Partial rate determination implies that k_1 and k_2 would have comparable values in the expression for k_E in Eq. (4). For both k_1 and k_2 to be partially rate-limiting steps in k_E , k_{-1} and k_2 in Eq. (5) must be approximately equal. Rearranging Eq. (5) and assuming from above that K_S is about 1 mM, this approximate equality indicates that k_1 can be only a little larger than k_E . From the values of k_E for Ac-*homo*SCh and Ac-*nor*SCh in Table 1, the corresponding k_1 these values would be $10^3 - 10^4 \text{ s}^{-1}$. Such k_1 values are one to two orders of magnitude lower than the minimum k_1 calculated above for AcSCh. More precise estimates require application of non-equilibrium treatments to several converging kinetic and thermodynamic analyses of the interaction of these substrates with AChE, and such studies are in progress. It is unclear what feature of the P-site would control the magnitude of k_1 and dictate preferential gating specificity for AcSCh relative to these two close analogs. But it seems clear that we can add substrate gating specificity to the list of functions played by the P-site in AChE.

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Abbreviations

A	absorbance
AChE	acetylcholinesterase
TcAChE	AChE from <i>Torpedo californica</i>
AcSCh	acetylthiocholine

Ac-homoSCh	acetyl(<i>homo</i>)thiocholine
Ac-norSCh	acetyl(<i>nor</i>)thiocholine
ATMA	3-(acetamido)- <i>N,N,N</i> -trimethylanilinium
BSA	bovine serum albumin
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
norSCh	(<i>nor</i>)thiocholine

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Fig. 1.
AChE substrates, and thioflavin T, an inhibitor of AChE.

Fig. 2.

(A) Hydrolysis rates for the three indicated substrates of AChE were normalized by the total AChE concentration ($v/[E]_{\text{tot}}$) and plotted against the substrate concentration. Plots were fitted to Eq. 3 as outlined in Table 1 to obtain kinetic parameters. (B) Data for Ac-homoSCh and Ac-norSCh in panel A are shown in expanded form. (C) and (D) Experimental points and fitted curves in panel B were transformed to the Eadie-Hofstee format (23) in panel C to clarify the observation of substrate activation for Ac-homoSCh, where points to the right of the maximal $v/[E]_{\text{tot}}$ are concave upward. Intercepts on the x-axes correspond to values of k_E , which were known with great precision (Table 1).

Fig. 3.

Inhibition of the enzymatic hydrolysis of (A) *Ac-homoSCh* and (B) *Ac-norSCh* by the P-site inhibitor thioflavin T. Initial experimental traces were analyzed with Eq. (1) to obtain values of k_{E+I} and $k_E [I]=0$. The initial $[S]_0$ values were $10 \mu\text{M}$, and $[E]_{\text{tot}}$ was 7.0 nM . The ratios $k_E [I]=0/k_{E+I}$ were analyzed with Eq. (2) to obtain K_I for thioflavin T and α . For *Ac-homoSCh*, $K_I = 1.33 \pm 0.05 \mu\text{M}$ and $\alpha = 0.16 \pm 0.01$ (averages of 5 independent experiments). For *Ac-norSCh*, $K_I = 1.33 \pm 0.08 \mu\text{M}$ and $\alpha = 0.26 \pm 0.02$ (averages of 2 independent experiments).

Scheme 1.

Table 1

Analysis of substrate hydrolysis according to the three-site model in Eq. (3).

Substrate	k_E mM ⁻¹ s ⁻¹	a_{SA}	K_{SA} mM	K_{SI} mM	K_D mM
AcSCh	(102 ± 4) × 10 ³	__ <i>b</i>	__ <i>b</i>	16 ± 1	0.057 ± 0.005
Ac- <i>homo</i> SCh	1650 ± 70	3.1 ± 0.2	5.1 ± 1.1	107 ± 17	0.33 ± 0.03
Ac- <i>no</i> SCh	1540 ± 60	__ <i>b</i>	__ <i>b</i>	65 ± 20	0.47 ± 0.03

Values of k_E were obtained by fitting second order hydrolysis rates with Eq. (1) (the number n of independent k_E measurements for each substrate was 4 – 7). Substrate hydrolysis profiles like those in Fig. 2 were then fitted to eq. 3 with k_E fixed to obtain the three equilibrium constants K_{SA} , K_{SI} and K_D and the relative acceleration constant a_{SA} (n was 3 for Ac-*homo*SCh and 1 for AcSCh and Ac-*no*SCh).

b With AcSCh and Ac-*no*SCh, no substrate activation phase was apparent and K_{SA}^{-1} was fixed at zero for fitting, removing a_{SA} as a variable. For these two substrates, standard errors were calculated from the fitting procedure.