Chemico-Biological Interactions 203 (2013) 38–43

Contents lists available at SciVerse ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

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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ARTICLE INFO

Article history:
Available online 6 October 2012

Keywords:
Acetylcholinesterase
Peripheral site
Active site gating
Acetylthiocholine analogs
Thioflavin T
Enzyme mechanism

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 M^{-1} 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.

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 S203[2] 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 to the A-site [11–13] and, with certain bound cationic

Abbreviations: A, absorbance; AChE, acetylcholinesterase; *Tc*AChE, AChE from *Torpedo californica*; AcSCh, acetylthiocholine; Ac-*homo*SCh, acetyl(*homo*)thiocholine; Ac-*nor*SCh, acetyl(*nor*)thiocholine; ATMA, 3-(acetamido)-*N*,*N*,*N*-trimethylanilinium; BSA, bovine serum albumin; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); *nor*SCh, *nor*thiocholine.

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

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http://dx.doi.org/10.1016/j.cbi.2012.09.017

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-homoSCh)

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 $MgSO_4$, 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. ^{1}H NMR ($CDCl_3$, 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*SCh was filtered through Celite and lyophilized. The residue was further purified by recrystallization with dichloromethane to afford a white solid: yield 13.5 g (80%). ^{1}H 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 $(M+)^+$.

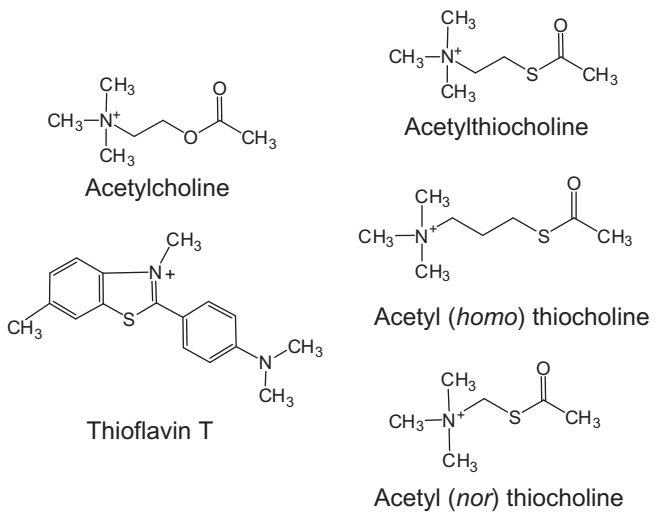

Fig. 1. AChE substrates, and thioflavin T, an inhibitor of AChE.

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. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.56 (s, 2H), 2.40 (s, 3H), 2.22 (s, 6H). MS *m/z* (ESI) 134.19 $(M + 1)^+$.

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 ^{1}H NMR. Yield 8.20 g (64%). ^{1}H 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 ε_{412nm} = 36,000 M^{-1} 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 ($\Delta\varepsilon_{412nm}$ = 14,150 M^{-1} - cm^{-1}) [14]. Total AChE concentrations (E_{tot}) were calculated assuming 450 units/nmol (which, under the assay conditions here, corresponds to 4.8 ΔA_{412}/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] << K_D, where K_D is defined in Eq. (2)), the most accurate way to measure the AChE-catalyzed second-order hydrolysis rate constant k_E is from Eq. (1) [16].

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