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Monitoring the reaction of carbachol with acetylcholinesterase by thioflavin T fluorescence and acetylthiocholine hydrolysis☆

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ABSTRACT

Acetylcholinesterase (AChE) contains a narrow and deep active site gorge with two sites of ligand binding, an acylation site (or A-site) at the base of the gorge and a peripheral site (or P-site) near the gorge entrance. The P-site contributes to catalytic efficiency by transiently binding substrates on their way to the acylation site, where a short-lived acyl enzyme intermediate is produced. Carbamates are very poor substrates that, like other AChE substrates, form an initial enzyme–substrate complex and proceed to an acylated enzyme intermediate which is then hydrolyzed. However, the hydrolysis of the carbamoylated enzyme is slow enough to resolve the acylation and deacylation steps on the catalytic pathway. Here we show that the reaction of carbachol (carbamoylcholine) with AChE can be monitored both with acetylthiocholine as a reporter substrate and with thioflavin T as a fluorescent reporter group. The fluorescence of thioflavin T is strongly enhanced when it binds to the P-site of AChE, and this fluorescence is partially quenched when a second ligand binds to the A-site to form a ternary complex. These fluorescence changes allow not only the monitoring of the course of the carbamoylation reaction but also the determination of carbachol affinities for the A- and P-sites.

1. Introduction

Acetylcholinesterase (AChE) hydrolyzes the neurotransmitter acetylcholine at one of the highest known enzymatic rates [1]. Partial inhibition of AChE activity in the brain, obtained with a number of inhibitors including carbamoyl esters, has been shown to have therapeutic benefits. AChE inhibitors that penetrate the blood–brain barrier elevate the levels of endogenous acetylcholine and are useful in the symptomatic treatment of Alzheimer's disease [2]. However, complete inactivation of AChE, which can occur with organophosphate chemical warfare agents [3], leads to toxic accumulation of acetylcholine and failure of cholinergic synaptic transmission, with consequent deterioration of neuromuscular junctions, flaccid muscle paralysis, and seizures in the central nervous system.

An essential feature of AChE activity at many synapses is the ability to hydrolyze acetylcholine within a millisecond of its release [4]. Insights into this high catalytic efficiency were obtained from ligand binding studies [5–7] and X-ray crystallography [8], which revealed a narrow active site gorge some 20 Å deep with two separate ligand binding sites. At the base of the gorge is the acylation or

Abbreviations: AChE, acetylcholinesterase; ATMA, 3-(acetamido)-*N*,*N*,*N*-trimethylanilinium; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); *Tc*AChE, *Torpedo californica* AChE; TMTFA, *m*-(*N*,*N*,*N*-trimethylammonio) trifluoroacetophenone.

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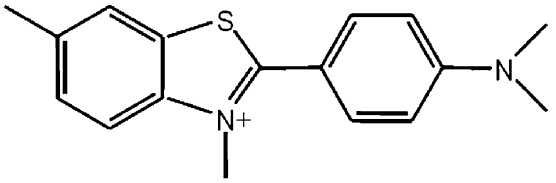

Fig. 1. Structure of thioflavin T.

A-site where residue W86[1] binds the trimethylammonium group of acetylcholine and H447, E334, and S203 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 [6] and thioflavin T [7].

Detailed information about the AChE catalytic mechanism can be obtained by characterizing intermediates formed on the catalytic pathway. A class of AChE inhibitors comprised of carbamoyl esters (here referred to as carbamates) is particularly useful in this regard. Carbamates are actually very poor substrates for AChE. Like other AChE substrates, they form an initial enzyme–substrate complex ES and proceed to an acylated enzyme intermediate which is then hydrolyzed. However, unlike carboxylic acid ester substrates, the hydrolysis of the carbamoylated enzyme is slow enough to resolve the acylation and deacylation steps. Despite this striking advantage offered by carbamates, few comprehensive analyses of their reactions with AChE have been undertaken. Here we report on the interaction of AChE with carbachol, a carbamate whose reaction mechanism with AChE is important because it is an isosteric analog of acetylcholine. We show that the reaction of carbachol with AChE can be monitored both with acetylthiocholine as a reporter substrate and with thioflavin T (Fig. 1) as a fluorescent reporter group. The fluorescence of thioflavin T is strongly enhanced when it binds to the P-site of AChE, and this fluorescence is partially quenched when a second ligand binds to the A-site to form a ternary complex [7]. In addition to monitoring the course of the carbamoylation reaction, we show that these fluorescence changes allow the determination of carbachol affinities for the A- and P-sites.

2. Experimental methods

2.1. Materials

Recombinant human AChE was expressed as a secreted, disulfide-linked dimer in *Drosophila* S2 cells and purified as outlined previously [11]. Thioflavin T and carbachol (carbamoylcholine chloride) were from Sigma. Thioflavin T was recrystallized from water, and concentrations were assigned by absorbance at 412 nm with $\varepsilon_{412\,nm} = 36{,}000\,M^{-1}\,cm^{-1}$.

2.2. Steady-state measurements of AChE-catalyzed acetylthiocholine hydrolysis

Hydrolysis rates v for acetylthiocholine were measured by spectrophotometry in a coupled reaction in which thiocholine generated in the presence of DTNB was determined by formation of the thiolate dianion of DTNB at 412 nm ($\Delta\varepsilon_{412\,nm} = 14{,}150\,M^{-1}\,cm^{-1}$) [12]. Total AChE concentrations $[E]_{tot}$ were calculated assuming 450 units/nmol [7].[2]

2.3. Stopped-flow measurements of carbachol reaction with AChE

Stopped-flow methods were applied to measure the approach to the carbamoylation steady state with acetylthiocholine as a chromogenic reporter substrate or thioflavin T as a fluorescent reporter ligand. Absorbance was monitored on a Varian Cary 3 Bio UV–visible spectrophotometer at 412 nm and fluorescence, on a PerkinElmer LS-50B luminescence spectrometer, both thermostatted at 25 °C. Rigorous temperature control was essential for stable fluorescence signals. Fluorescence excitation was at 450 nm and emission at 490 nm, with excitation and emission slits of 10–20 nm. A thermostatted Hi-Tech SFA 20 stopped flow apparatus was used to rapidly mix equal volumes (300 μl) of AChE in one syringe and carbachol in the other. Measurements were recorded at fixed intervals as short as 33 ms (absorbance) or 20 ms (fluorescence). Acetylthiocholine as the reporter substrate and DTNB were added only to the carbachol syringe, but with a DTNB concentration after mixing of 10 mM to insure that its reaction with thiocholine was not rate limiting during the absorbance increase. The solution was buffered to pH 7.0 with a total of 30 mM phosphate and 70 mM Na^+ (added as Na_2PO_4 and NaOH). Thioflavin T as the reporter was added at equal concentrations to both syringes in 40 mM sodium phosphate, 0.04% Triton X-100 at pH 7.0. To maintain constant ionic strength when the range of carbachol concentrations extended to 60 mM, additional NaCl was included so that the sum of the carbachol and NaCl concentrations was 60 mM.

2.4. A two-site model of carbamoylation of AChE in the presence of a reporter substrate

With carbamate substrates, both the formation and hydrolysis of the acylated enzyme intermediate, or carbamoyl enzyme (EC), are slow enough to allow equilibrium assumptions. A general approach is given in Scheme 1 [13,14]. In this scheme, k_{12} is the overall carbamoylation rate constant and k_{21} is the overall decarbamoylation rate constant. P and COH are the products released

[1] Throughout this paper we number amino acid residues according to the human AChE sequence.

[2] One unit of AChE activity corresponds to 1 μmol of acetylthiocholine hydrolyzed/min under standard pH-stat assay conditions at pH 8 [7]. Our conventional spectrophotometric assay at 412 nm is conducted in pH 7 buffer. With wild type AChE and 0.5 mM acetylthiocholine, this assay results in 4.8 $\Delta A_{412\,nm}$/min with 1 nM AChE or about 76% of the pH-stat assay standard.