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Determination of affinity and efficacy of acetylcholinesterase inhibitors using isothermal titration calorimetry☆

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ABSTRACT

Background: Acetylcholinesterase (AChE), an enzyme rapidly terminating nerve signals at synapses of cholinergic neurons is an important drug target in treatment of Alzheimer's disease and related memory loss conditions. Here we present comprehensive use of isothermal titration calorimetry (ITC) for investigation of AChE kinetics and AChE-inhibitor interactions.
Methods: Acetylcholinesterase (AChE, EC 3.1.1.7) from *Electrophorus electricus* was assayed for interactions with five well known AChE inhibitors, galanthamine, tacrine, donepezil, edrophonium and ambenonium. In ITC experiments the inhibitors were injected to the enzyme solution solely (for thermodynamic characterization of binding) or in presence of the substrate, acetylcholine (for determination of inhibitors potency).
Results: Detailed description of various experimental protocols is presented, allowing evaluation of inhibitors potency (in terms of IC_{50} and K_i) and thermodynamic parameters of the binding. The potency of tested inhibitors was in nano to micromolar range which corresponded to activities determined in conventional method. Binding of all inhibitors showed to be enthalpy driven and obtained K_a values demonstrated good correlation with the data from standard Ellman's assay.
Conclusions: Obtained results confirmed the usability of the ITC technique for comprehensive characterization of AChE-inhibitor interactions and AChE kinetics. The method reduced the complexity of reaction mixture and interference problems with the advantage of using natural substrates.
General significance: The work reports complete thermodynamic characteristics of the AChE — inhibitor complexes. Due to the universal character of ITC measurements, described protocols can be easily adapted to other enzymatic systems. This article is part of a Special Issue entitled Microcalorimetry in the BioSciences — Principles and Applications, edited by Fadi Bou-Abdallah.

1. Introduction

Isothermal titration calorimetry (ITC) has become a gold standard in characterization of molecular interactions due to universality, relatively simple protocols and accuracy of resultant data. Precise measurement of heat absorbed or generated during ligand-target binding event allows to study interactions in conditions resembling physiological with macromolecules in their native forms without any chemical modifications. Single experiment can provide a vast amount of data including unique information about the thermodynamics of studied ligand-target interaction [1–3]. Moreover as presented by Todd and Gomez [4] the ITC can be adapted to monitor progression of enzyme catalysed reactions. This enables the use of ITC also as a method for estimating the enzyme inhibitors activity and their mechanism of action.

Here we present tailored applications of ITC for study acetylcholinesterase (AChE) interaction with its substrates and inhibitors. Two substrates were used: acetylcholine (ACh), which is a neurotransmitter naturally occurring in the body and acetylthiocholine (ATCh) which is a derivative od ACh commonly used in the conventional AChE activity assay. Five well known drugs acting on AChE (i.e. galanthamine, tacrine, donepezil, edrophonium and ambenonium) were tested to validate the described protocols and further elucidate the AChE-inhibitor interaction.

The AChE enzyme plays an important role in functioning of cholinergic neuronal pathways. The enzyme terminates nerve impulse transmission at cholinergic synapses by rapid degradation of ACh, which is involved in both memory and learning. According to postulated cholinergic hypothesis impairment of the cholinergic pathways plays a central role in the development of neurodegenerative diseases such as Alzheimer's disease (AD), depression, schizophrenia, problems with the regulation of sleep and traumatic brain injury. The hypothesis also assumes a key role of the cholinergic system in deficiency of cognitive functions and dementia. A wide range of evidence shows that AChE inhibitors can improve cognitive function and interfere with the progression of AD. Nowadays, four out of five approved drugs for the symptomatic treatment of AD are AChE inhibitors [5]. Moreover,

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modulation of cholinergic function by AChE inhibitors is used as a pharmacological strategy for treatment of other disorders such as *Myasthenia Gravis* [6] or glaucoma [7]. Besides their therapeutic use compounds that effectively inhibit activity of the AChE are used also in agriculture as organophosphorus insecticides. The number of publications on the AChE inhibitors that have appeared in recent years demonstrates that development of new inhibitor structures, with high efficiency and selectivity ensuring low side-effects, acting desirably also on other elements of cholinergic signal transduction system is still an emerging issue. Therefore there is a great demand for robust and accurate techniques for the characterization of AChE-ligand interactions. ITC due to its versatility and label-free characteristic of the assay seems to be a technique of choice for comprehensive characterization of AChE inhibitors which, moreover, can be easily applied to investigate other enzymatic systems. The technique simultaneously provides data on affinity, stoichiometry of the enzyme-ligand binding and unique information on thermodynamics of the complex formation. The same instrumentation allows to monitor AChE enzymatic activity and therefore can provide data on inhibitors potency and their mechanism of action.

The possibility of using microcalorymetry in AChE activity measurements was suggested yet in 1980 by Rosenstein and Brown [8] which showed good correlation between signals seen in spectrophotometer and calorimeter. However, they assumed linear decrease of heat flow signal after the addition of the substrate to the enzyme solution which is true only for certain substrate/AChE concentrations and therefore may lead to unreliable results. Here we report and validate more accurate method. Detailed description of various experimental protocols will be presented, allowing accurate determination of acetylcholinesterase (AChE) kinetics, potency and affinity evaluation of AChE inhibitors, determination of their mechanism of action and the thermodynamic parameters of AChE-inhibitor binding. The advantages of the ITC assay will be discussed with the comparison to the conventional methods.

2. Materials and methods

2.1. Materials

Acetylcholinesterase from *Electrophorus electricus* (AChE, EC 3.1.1.7), acetylcholine chloride (ACh), acetylthiocholine iodide (ATCh), galanthamine hydrobromide (from *Lycoris* sp.), tacrine hydrochloride (9-amino-1,2,3,4-tetrahydroacridine hydrochloride), donepezil hydrochloride monohydrate and bovine serum albumin lyophilized powder (BSA) were purchased from Sigma Aldrich (St. Luis, MO, USA). Edrophonium chloride was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA) and ambenonium chloride from Tocris Bioscience (Bristol, United Kingdom). Purified water from Direct-Q UV system (Millipore, Milford, MA, USA) was used to prepare buffers and standard solutions.

To prepare buffer solutions 2-amino-2- (hydroxymethyl)propane-1,3-diol (TRIS) Sigma Aldrich (St. Luis, MO, USA) of analysis quality was used. The buffer solutions were filtered through a 0.22 μm membrane filter before their use. For calorimetric analysis all the solutions were degassed on Degassing Station from TA Instruments (Lindon, UT, USA) immediately before placing in the instrument.

Stock solutions of tested inhibitors (galanthamine, tacrine, donepezil, edrophonium and ambenonium) were prepared (2–10 mM) and further diluted in deionized water or in the relevant buffer.

2.2. Calorimetric measurements of AChE kinetics

Calorimetric measurements were performed using Nano ITC Isothermal Titration Calorimeter (TA Instrumantes, Lindon, UT, USA) characterized by 950 μL of sample cell volume with a 250 μL dosing syringe. During all experiments the reference cell was filled with degassed water. Before starting the measurement, the system was equilibrated at desired temperature. The stirring rate provided by the injector paddle rotation was set to 250 rpm and it was kept constant.

The lyophilized AChE sample was reconstituted in 50 mM TRIS–HCl pH 7.5 buffer to achieve an activity concentration of ~1330 U•mL^{-1}. The protein was further diluted to 0.005 U•mL^{-1} (0.08 nM of binging sites) in 50 mM TRIS–HCl pH 8.0 with the 0.1% addition of BSA as an enzyme stabilizing factor. The ACh solution was prepared in the same buffer.

Molar enthalpy of acetylcholine hydrolysis was determined by injecting 10 μL of 20 mM substrate into the ITC instrument sample cell filed with solution containing 0.2 U of AChE activity (3.4 nM of binging sites). The heat generated after complete conversion of injected substrate was integrated and corresponding molar enthalpy of substrate enzymatic hydrolysis was calculated in terms of kJ•mol^{-1} of substrate. To evaluate the contribution of proton exchange between the system and the buffer to the estimated enthalpy of reaction the same measurement was repeated in three buffer systems with flowing enthalpies of protonation: 3.6 kJ kJ•mol^{-1} for phosphate buffer, 20.4 kJ•mol^{-1} for HEPES and 47.45 kJ kJ•mol^{-1} for TRIS buffer.

The AChE saturation curve for enzyme kinetics analysis was constructed in an experiment with multiple, cumulative injections of substrate, as described by Todd and Gomez (2001). In brief the method links the instrument registered heat flow with the rate of enzymatic reaction taking place in the sample cell. The 5 mM acetylcholine solution was delivered into the sample cell (filled with AChE solution) as 25 automatic, cumulative injections, each of 10 μL with the interval of 120 s. The signal of a blank titration (inhibitor solution to buffer solely) was subtracted from resulting heat flow signal of substrate hydrolysis. Thermal power generated by the enzymatic reaction after each addition of the substrate was defined, converted to respective reaction rates (μmol•min^{-1}) and plotted against respective substrate concentrations. Substrate concentration after each injection was corrected by the amount of acetylcholine digested in the period between injections. Kinetic parameters K_M and V_{max} were found by fitting the Michaelis–Menten equation to the obtained saturation curve using non-linear regression function of Prism 5 software (GraphPad Software, Inc., CA, USA).

The calorimetric measurements of AChE kinetics were conducted at 25 °C.

2.3. Calorimetric determination of AChE inhibitors' potency

Two approaches were used, allowing to determine both IC_{50} and K_i value of an inhibitor.

For the IC_{50} value determination the instrument cell was filed with 0.2 U•mL^{-1} of AChE (3.4 nM of binging sites) in 50 mM TRIS–HCl pH 8.0 with the 0.1% addition of BSA. Tested compounds were delivered to the cell together with 20 mM ACh in a form of single 10 μL injection. Five inhibitor concentrations were assayed as an addition to the AChE solution. The concentrations resulting in 20 to 80% reduction of ACh hydrolysis rate were used. This was observed as a respective decrease in maximal level of heat flow generated during the enzymatic reaction. The maxima of the signals were then plotted against used inhibitor concentrations allowing for interpolation of the IC_{50} concentration.

K_i value was defined using same procedure as for calorimetric determination of enzyme kinetic parameters (2.3). However, in this case the saturation curves were measured in the presence of inhibitor. As a proof of concept two different concentrations of edrophonium were analysed. Double reciprocal plots of $1/V_{max}$ versus 1/[ACh] (Lineweaver–Burk plot) measured in presence of inhibitor were constructed to assess the inhibitor mechanism of action [9]. The respective K_i value was calculated using Prism 5 software (GraphPad Software, Inc., CA, USA). Alternatively slopes of these reciprocal plots can be plotted against tested inhibitor concentrations where the x-intersect of obtained line equals to $-K_i$ value [9].