Kinetics of Total Enzymatic Hydrolysis of Acetylcholine and Acetylthiocholine

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Kinetics and the mechanism of total *in vitro* hydrolyses (*i.e.* up to the exhaustion of substrate) of acetylcholine and acetylthiocholine by acetylcholinesterase and butyrylcholinesterase were studied *in vitro* in a batch reactor at 25 °C, pH 8 and ionic strength of 0.11 M. Every hydrolysis was monitored by 2–3 independent analytical methods. All studied types of enzymatic hydrolyses fulfilled the Michaelis–Menten reaction scheme with the irreversible second step. A table of obtained average values of rate constants and estimations of initial molar enzyme concentrations, and discussion of the results are presented.

Key words: Acetylcholine, Hydrolysis, Kinetics

Introduction

Acetylcholine (ACH) hydrolysis by acetylcholinesterase (ACHE) or butyrylcholinesterase (BCHE) plays an important role in impulse transmission at cholinergic synapses (Taylor, 1990). The surplus of these enzymes in brain cells is considered as one possible reason of Alzheimer disease. To remove this surplus various inhibitors of cholinesterases are used as drugs (Bar-On *et al.*, 2002). For *in vitro* studies of these hydrolyses acetylthiocholine (ATCH) is often used instead of ACH because of its similar qualitative kinetic behaviour and possibility of relative simple monitoring of the reaction course (Ellman *et al.*, 1961). We try already for a longer time to find new, more effective but nontoxic cholinesterase inhibitors. Their effectivity is tested at first on the mechanism of the reaction of substrate (ACH, ATCH) with enzyme (ACHE, BCHE) and inhibitor, preferably up to the final reaction state (*e.g.* equilibrium). These informations are obtained at least by two independent analytical methods, determining the time course of substrate concentration (ACH or ATCH) and/or two products [choline (CH) or thiocholine (TCH) and acetic acid (HA)]. The effect of the tested inhibitor follows from the comparison of the inhibited and uninhibited hydrolyses under the same conditions. We cannot trust fully on the uninhibited data from the literature (*e.g.* Bar-On *et al.*, 2002; Brestkin *et al.*, 1974; Reiner and Simeon-Rudolf, 2000), because of various reaction conditions, using only one analytical method (Ellman's method suitable only for ATCH hydrolysis or universal pH-stat method, both monitoring only products of hydrolysis, TCH or HA) and the fact that the majority of published kinetic measurements was done only for the initial reaction stadium (up to 5% conversion). Therefore, this paper deals with the kinetics of total uninhibited hydrolyses ACH + ACHE, ACH + BCHE, ATCH + ACHE and ATCH + BCHE, *i.e.* studies up to the total exhaustion of the substrate. The hydrolysis course was monitored by measuring the dependences of actual concentration of substrate and/or both products vs. reaction time (t) by four independent analytical methods: Ellman's spectroscopic (ELM), pH-stat (PHS), hydroxylamine (HXA) and HPLC method at the same temperature, pH value and ionic strength.

Experimental

Theory

The formal reaction scheme of enzymatic hydrolysis of the substrate S (*i.e.* ACH or ATCH) by the enzyme E (*i.e.* ACHE or BCHE) to products

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P (*i.e.* CH or TCH) and HA can be expressed by the steps

$$E + S \rightleftarrows ES, \quad (1)$$

$$ES \rightleftarrows EA + P, \quad (2)$$

$$EA + H_2O \rightarrow E + HA. \quad (3)$$

The symbols $\rightleftarrows$ and $\rightarrow$ denote the reversible (equilibrium) and irreversible (one way) reaction steps. If reaction (3) (due to the water excess) is very fast, it holds

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES, \quad (4a)$$

$$ES + H_2O \xrightarrow{k_2} E + P + HA. \quad (4b)$$

A steady state for the reaction components E and ES exists during the whole reaction course under condition that the initial molar concentration $[S]_0 \gg [E]_0$ and the initial concentrations of all other components are zero.

For such hydrolysis of S the classic Michaelis-Menten (Briggs-Haldane) equation

$$v = -d[S]/dt = d[P]/dt = d[HA]/dt = V_m[S]/(K_M + [S]) \quad (5)$$

holds, where v is the actual rate of the given enzyme reaction under given conditions (temperature, pH value, ionic strength etc.), and [S], [P] and [HA] are molar concentrations with the relation

$$[S] = [S]_0 - [P] = [S]_0 - [HA]. \quad (5a)$$

$V_m = k_2[E]_0$ is the maximum value of v at the saturation of E by S and $K_M = (k_{-1} + k_2)/k_1$ is the Michaelis constant. Equation (5), if valid in the whole course of the reaction, gives for every reaction time t after integration

$$t = (K_M/V_m) \ln([S]_0/[S]) + ([S]_0 - [S])/V_m. \quad (6)$$

Chemicals

Acetylthiocholine iodide p.a., acetylcholine chloride (min. 99%), substrates were kept at 0 °C. From these substrates a fresh aqueous solution of chosen volume and concentration was prepared for daily experiments.

Acetylcholinesterase: ACHE1, lyophilisate from electric eel; ACHE2, lyophilisate from bovine erythrocytes; all from Sigma, Prague, CZ, kept at −6 °C.

Butyrylcholinesterase: BCHE1, obtained from the Department of Toxicology, Purkyně Military Medicinal Academy Hradec Králové, CZ, hydrolysate from horse plasma, pressed in pellets of ca. 6 g; BCHE2, from Sigma, Prague, CZ, lyophilized powder from equin serum; both kept at −6 °C. A suitable amount of every enzyme preparation (EP) was dissolved in demineralized water. This EP solution was divided into suitable aliquots which were kept frozen at −6 °C. For the daily experiments a portion was melted, kept at 5 °C and used only that day.

5,5′-Dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent): Sigma, Prague, CZ, kept at 5 °C. From this substance the analytical aqueous solution (0.5 mM) was prepared and kept at 5 °C. Hydroxylamine hydrochloride p.a., potassium hydroxide p.a., hydrochloride acid conc., ferric nitrate $Fe(NO_3)_3 \cdot 9H_2O$ p.a., all Lachema, Brno, CZ.

Buffer: Sörensen's phosphate buffer, pH 8.0, ionic strength 0.275 M (defined by 0.5 M KCl) was used.

Methods and apparatus

Ellman's spectroscopic method (ELM) (Ellman *et al.,* 1961) was realized using a diode-array spectrograph HP 8453, Hewlett-Packard, USA. This method is based on the spectrometric determination of the yellow anion Y, produced by the reaction of TCH with DTNB directly in the reaction mixture. Y has the maximum absorbance at 412 nm (A). The value of A is taken as proportional to the actual molar concentration of TCH, thus $[TCH] = A/(\varepsilon d)$, where ε is the absorption coefficient of Y at 412 nm and d is the optical path of the cuvette. The value $\varepsilon = 14150\ M^{-1}\ cm^{-1}$ was used (Dodds and Rivory, 1999; Komers *et al.,* 2003).

A glass cuvette with the total volume of 30 cm^3 and $d = 2$ cm provided with a glass propeller was used as the reactor. The cuvette was filled with chosen volumes of buffer, pH 8, DTNB and substrate solutions. This mixture was thermostated at 25 °C. The reaction was started by fast (< 1 s) homogenization of the chosen volume of EP solution with the vigorously mixed reaction mixture. The final mixture had the ionic strength 0.11 M. The actual values of A were continuously measured vs. t and saved in a PC. The comparative water solution consisted of the same concentrations of buffer and DTNB to eliminate the weak absorption of the surplus of DTNB at 412 nm.

An automatic titrator 736 GP Titrinio, Metrohm Ltd., Herisau, Switzerland, combined with a PC was used as pH-stat. The PHS method (Hanin and Dudas, 2000) is based on the determination of the

actual concentration of the produced acetic acid, [HA]. This is realized by continuous titration of HA with the analytical solution of KOH keeping the pH value of the reaction mixture at chosen constant (here pH 8.0) value, checked by the couple glass electrode-Ag/AgCl electrode. From this measurement the dependence volume of the analytical solution of KOH vs. *t* is obtained and overcalculated to the dependence [HA] vs. *t*. In this method any buffer must not be principially used. Further, the reaction mixture must not be in contact with air because the present CO_2 reacts at pH 8 immediately with the reaction mixture and decreases continuously its pH value. This complication was eliminated using an argon atmosphere in and above the reaction mixture. The ionic strength was kept at the same value as in ELM (*i.e.* 0.11 M) by an aqueous solution of 0.5 M KCl. Monitoring of ATCH hydrolysis by pH-stat is problematic, because TCH, simultaneously rising with HA, behaves, analogous to HA, as a weak acid and reacts also with KOH. In Brestkin *et al.* (1974) there is a formula relating to the amount of KOH solution spent for neutralisation of the dissociated part of TCH, but we did not have a good experience in our experiments. Therefore the PHS method was not used in the hydrolyses of ATCH.

The reaction was carried out in a closed and thermostated glass vessel (100 ml) with an electromagnetic stirrer. The vessel was filled at first with the chosen amounts of ACH and KCl aqueous solutions. This mixture was kept under argon atmosphere for 10 min and then automatically adjusted to pH 8.0 by the aqueous solution of 0.01 M KOH under continuous PC control by galvanic couple mentioned above. The reaction was started by fast addition of the chosen volume of EP solution into the vigorously mixed reaction mixture. At the same time the automatic continuous addition of 0.01 M KOH and the on-line PC registration of its added volume vs. *t* were started. The increase of the initial volume of the reaction mixture by the added KOH solution was respected in the calculation of the dependence [HA] vs. *t*.

The original HXA method, developed from Hestrin (1949) and described by Štěpánková *et al.* (2005), determines, in contrast to ELM and PHS methods, the actual concentration of the substrate. The principle is: From the actual reaction mixture [including substrate, products, buffer (pH 8), EP solution and water] samples are taken in the chosen time intervals. Every sample is immediately and vigorously mixed with an aqueous solution of hydroxylamine and KOH. The rapid change of pH stops the enzyme hydrolysis and hydroxylamine reacts in strongly alkaline medium with the substrate (ACH or ATCH) forming acethydroxamic acid and methanol. Acidification of this mixture with HCl and addition of Fe^{3+} ions results in a red-brown complex, ACH- or ATCH-acethydroxamic acid product. The absorbance *A* (540 nm) of this final solution is measured in the glass cuvette with optical path of 2 cm by the diode-array spectrograph HP 8453 with PC Chemstation and recalculated to actual concentration [ACH] or [ATCH].

The greatest advantage of the original HPLC method is the possibility of simultaneous determination of actual ACH, CH and HA (or ATCH, TCH and HA) concentrations in one measurement. All methods described above measure either the concentration of substrate (HXA) or one product (ELM or PHS). The principle is: The sample taken from the actual reaction mixture is most quickly acidified with hydrochloridic acid. This pH change stops immediately the enzyme activity and therefore also the hydrolysis. The acidified sample is then directly used for the isocratic HPLC analysis with refractometric detection. Using suitable calibration curves the dependences [ACH], [CH] and [HA] (or [ATCH], [TCH] and [HA]) vs. *t* are obtained. More detailed description of the measuring procedure is given by Štěpánková *et al.* (2005).

Every used EP solution was tested on its hydrolyzing power, *i.e.* its enzyme preparation (catalytic) activity (EPA). The catalytic activity of 1 unit (U) has such amount of the given EP, which converts 1 μmol of the given substrate in 1 min at the given reaction conditions. The daily determined EPA value of the given EP solution was used to calculate its suitable volume which had to be added into the initial reaction mixture to reach the chosen initial EP activity.

Measuring procedures

All experiments were done at 25 °C, pH 8, ionic strength 0.11 M and EPA 3.5 U in the initial reaction mixture. At ACHE2 the value of EPA could be only 0.5 U because of small solubility of this solid EP in water. The addition of EP solution started the reaction. The initial concentrations of substrate, $[S]_0$, are presented in the description of

single experiments in chapter Results and Discussion.

Ellman's method: 10 ml phosphate buffer, 5 ml 0.5 mM DTNB, 1 ml 1 mM ATCH and *x* ml water were thermostated at 25 °C. Then *y* ml of EP solution were added to reach EPA 3.5 U (0.5 U by ACHE2) in 25 ml of the initial reaction mixture. HXA and HPLC methods need no DTNB, the PHS method also no buffer and 0.5 M KCl was used to ensure the ionic strength 0.11 M. The total volumes of the ELM, PHS and HXA reaction mixtures were 25 ml, of the HPLC method 75 ml. Samples taken from actual reaction mixtures were processed as follows. HXA method: 1 ml sample was mixed with 2 ml of the mixture 2 M hydroxylamine hydrochloride/3.5 M KOH [1:1 (volumetric)]. After 2 min 1 ml of the mixture HCl conc./water (1:2) and 1 ml 0.37 M Fe^{3+} were added. The red-brown colour was measured at 540 nm. HPLC method: 5 ml sample were acidified with 0.2 ml 1 M HCl and analyzed by HPLC.

Calculations

From the original measurements the experimental dependences of the actual concentration of substrate [S] and/or products [P] and/or [HA] vs. reaction time *t* were calculated. These dependences were tested for validity of equations (4) by three independent mathematical procedures: The nonlinear regression program from the PC editor PRISM 2.01 compared (fitted) the experimental data with the integrated kinetic equation (6). The PC program GEPASI solving the kinetic of all the biochemical problems (Mendes, 1993, 1997; Mendes and Kell, 1998; http://gepasi...) was used for fitting the same data with the differential kinetic equation (5) or with the system of differential kinetic equations describing the reaction in equation (4). If experimental data fulfilled the Michaelis-Menten equation (5) the first two calculations gave the optimal values of K_M and V_m. From the positive solving of equations (4) the optimal values of k_i of all rate constants and estimation of absolute initial enzyme molar concentration $[E]_0$ were obtained.

Results and Discussion

At 25 °C, pH 8, ionic strength 0.11 M and EPA 3.5 U (0.5 U at ACHE2) following hydrolyses were studied (with the initial substrate concentrations $[S]_0$): ACH + ACHE1 (4 mM), ACH + ACHE2 (2.5 mM) , ACH + BCHE1 , ACH + BCHE2 (4 mM), ATCH + ACHE1, ATCH + ACHE2, ATCH + BCHE1 and ATCH + BCHE2

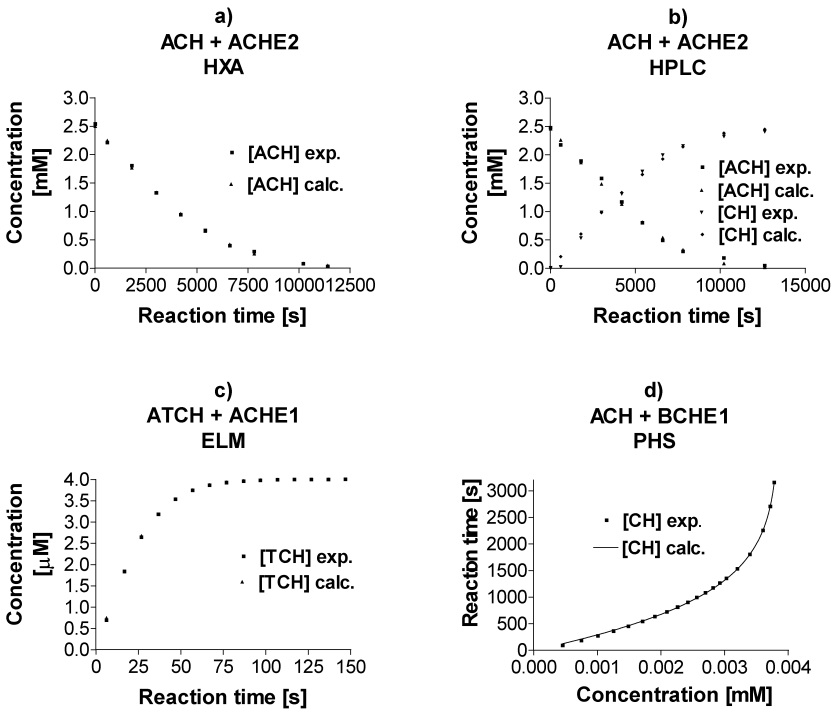

Fig. 1. Hydrolysis of a) ACH + ACHE2, HXA method: initial concentration $[ACH]_0 = 2.5$ mM, evaluated by GEPASI, optimal $k_1 = 25860$ (M s)$^{-1}$, $k_{-1} = 19.37$ s^{-1}, $k_2 = 12.21$ s^{-1}, $[E]_0 = 54$ nM, standard deviation sd = 33.9 μM; b) ACH + ACHE2, HPLC method: $[ACH]_0 = 2.5$ mM, evaluated by GEPASI, optimal $k_1 = 43820$ (M s)$^{-1}$, $k_{-1} = 21.48$ s^{-1}, $k_2 = 9.532$ s^{-1}, $[E]_0 = 47.63$ nM, sd = 97.7 μM; c) ACH + ACHE1, ELM method: $[ATCH]_0 = 0.04$ mM, evaluated by GEPASI, optimal $K_M = 35.11$ μM, $V_m = 2.363$ μM s^{-1}, sd = 17.7 nM; d) ACH + BCHE1, PHS method: $[ACH]_0 = 3.838$ mM, evaluated by PRISM, optimal $K_M = 7.96$ mM, $V_m = 11.47$ μM s^{-1}, correlation coefficient $R^2 = 0.9995$.

(4 mM for HXA and HPLC, 40 μM for ELM). ACH hydrolyses were monitored by PHS, HXA and HPLC, ATCH hydrolyses by ELM, HXA and HPLC method. Every experiment was tested for the validity of equations (4) and (5). The regression coefficient (R^2 in PRISM) or standard deviation (sd in GEPASI) and the graphical comparison of experimental and theoretical curves served as validity criteria. Examples of results are given in Fig. 1. For every combination S + E and every used analytical method average values (and their deviations) of optimal kinetic parameters (K_M, V_m, k_i), statistic parameters (R^2, sd), initial molar concentrations of substrate $[S]_0$ and enzyme $[E]_0$ were calculated. Because the average values of K_M and V_m obtained from the corresponding values of k_i and $[E]_0$ were comparable with the same parameters calculated directly from (5) and (6) for all presented combinations S + E, the Michealis-Menten equation (5) and the more general reaction scheme (4) are simultaneously valid for the studied hydrolyses. Table I contains the summary of average values of optimal k_i and $[E]_0$ calculated for every combination of S + E from all experiments performed by all used analytical methods. Based on these informations, following conclusions can be expressed about the studied total (*i.e.* up to [S] = 0) *in vitro* enzymatic hydrolyses:

1. The course of all tested enzymatic hydrolyses can be described by the Michaelis-Menten (Briggs-Haldane) form (5) of the reaction scheme (4). Both products, *i.e.* CH or TCH and HA, rise simultaneously. The reaction step (3) is therefore much faster than the kineticly deciding step (2).

2. The mean kinetic parameters K_M and V_m, determined for the same hydrolysis by the same analytical method and computed by three independent procedures, correspond well.

3. Table I shows that the average k_i values determined for the same hydrolysis performed by the same enzyme obtained from various sources (ACHE1 or 2, BCHE1 or 2) differ substantially. These kinetic differencies are probably caused by the structural differencies of both enzyme types.

4. Great average deviations of some k_i values in Table I signify, that these kinetic parameters measured by two or three independent analytical methods for the same combination S + E differ also substantially.

5. Because all types of experiments were reproduced many (meanly 12) times and mostly in various and distant dates, the quantitative differencies described in 4. cannot be explained by experimental errors. We suppose that they are caused mainly by variations in the composition of the reaction mixtures used for single analytical methods: ELM method requires a surplus of DTNB, PHS method must not contain any buffer but includes KCl and argon in addition, only HXA and HPLC methods (requiring removing of samples) use nearly identical reaction milieu. Also the initial concentration of substrate could affect the qualitative and/or quantitative parameters of the reaction mechanism (*e.g.* by substrate inhibition): Our ELM method needed $[S]_0 = 0.04$ mM because of the great value of absorption coefficient of the yellow product Y, while the HXA, PHS and HPLC methods required (according to their sensitivity) $[S]_0$ in order of 2.5 to 4 mM.

Videlicet, the course of the studied hydrolyses depends, besides common conditions (temperature, pH, ionic strenght, initial concentration of S and EP), sometimes also on special conditions (*e.g.* type or absence of buffer, presence of special reagent in the reaction mixture, history of the used enzyme).

Table I. Mean optimal values and deviations of rate constants k_i and molar initial enzyme concentration $[E]_0$ according to (4) calculated for every combination S + E from all experiments and used methods. Detailed description in text.

Combination	k_1 [10^{-4} (M s)$^{-1}$]	k_{-1} [s^{-1}]	k_2 [s^{-1}]	$[E]_0$ [10^7 M]
ACH + ACHE1	6.69 ± 0.81	87.7 ± 3.74	44.6 ± 20.0	0.983 ± 0.406
ACH + ACHE2	3.85 ± 0.94	56.7 ± 38.2	17.3 ± 7.6	0.766 ± 0.326
ACH + BCHE1	25.1 ± 21.6	488 ± 290	2256 ± 2208	1.61 ± 0.12
ACH + BCHE2	3.90 ± 0.72	144 ± 32	42.0 ± 5.3	1.63 ± 0.24
ATCH + ACHE1	26.0 ± 17.1	130 ± 105	33.9 ± 15.2	0.930 ± 0.405
ATCH + ACHE2	20.4 ± 10.8	52.0 ± 36.1	12.8 ± 6.8	0.528 ± 0.256
ATCH + BCHE1	35.4 ± 9.32	95.6 ± 87.2	38.7 ± 3.3	1.71 ± 0.24
ATCH + BCHE2	33.8 ± 15.9	234 ± 156	60.3 ± 35.3	1.15 ± 0.37

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