

Kinetics and Mechanism of Hydrolysis of Acetylthiocholine by Butyrylcholine Esterase

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Kinetics and mechanism of hydrolysis of acetylthiocholine by the enzyme butyrylcholine esterase was studied. The spectrophotometric Ellman's method and potentiometric pH-stat method were used for continuous determination of the actual concentration of the products thiocholine and acetic acid in the reaction mixture. The validity of the Michaelis-Menten (Briggs-Haldane) equation in the whole course of the reaction under used conditions was proved. The corresponding kinetics parameters (V_m and K_M) were calculated from the obtained dependences of concentration of thiocholine or acetic acid vs. time and compared. From this comparison the deciding kinetic role of the step producing thiocholine was derived. The values of initial molar concentration of the enzyme and of the rate constants of the kinetic model were estimated.

Introduction

This work creates a part of the research of new suitable inhibitors of cholinesterases as potential drugs against the Alzheimer demence. The inhibition effect of every new drug has to be compared with the uninhibited reaction of the same type. This paper deals with the kinetics and mechanism of uninhibited enzymatic decomposition of acetylthiocholine (ATCH) by butyrylcholine esterase (BCHE) to thiocholine (TCH) and acetic acid (HA). The reaction course was measured in the initial stage and up to high conversion by two independent methods, i.e. by continuous measurement of the dependence of either concentration of thiocholine [TCH] vs. time (t) using the Ellman's method (Ellman, 1961) or [HA] vs. t by the potentiometric pH-stat method (Giacobini, 2000).

Materials and Methods

Theory

The formal reaction scheme of enzymatic hydrolysis of the substrate S (i.e. ATCH) by the enzyme E (i.e. BCHE) to product P (i.e. TCH) and HA can be expressed by the steps



The symbols = and \rightarrow declare the reversible (equilibrium) and irreversible (one way) reaction steps with the rate constants k_1 , k_{1r} (r denotes the reverse step), k_2 , k_3 and equilibrium constant $K_1 = k_{1r}/k_1$. A steady state for all reaction components including E (i.e. E and ES) exists during the whole reaction course on conditions that the initial concentration $[S]_0 \gg [E]_0$ and the initial concentrations of all other components are zero.

Experimental

Chemicals

Butyrylcholinesterase (BCHE): hydrolysate from the horse plasma, pressed in pellets ca. 6 g, kept in refrigerator at 5 °C. Two pellets were solved in 200 ml of demineralized water. This enzyme preparation was divided into the 10 ml portions which were kept frozen at ca. –6 °C. For the daily experiments a portion was melted, kept at 5 °C and used only that day.

Acetylthiocholine (ATCH) iodide: substrate from firm Sigma-Aldrich, Prague, CZ, kept at 5 °C. From this substrate a fresh water solution of

chosen volume and concentration was prepared for daily experiments.

5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB, Ellman's reagent): Sigma-Aldrich, Prague, CZ, kept at laboratory temperature. From this substance the analytical aqueous solution 2×10^{-3} M was prepared and kept at ca 5 °C.

Buffer: Sørensen's phosphate buffer pH 8.0, concentration 0.07 M, ionic strength $I = 0.186$ M (defined by NaCl 0.1 M) was used. The buffer was prepared from $\text{Na}_2\text{HPO}_4 \cdot 12 \text{ H}_2\text{O}$ and KH_2PO_4 (both p.a. quality, Lachema Brno, CZ). The reserve solution was kept in darkness at laboratory temperature.

Methods and apparatus

Ellman's method (Ellman, 1961) was realized by means of a diode-array spectrograph HP 8452A, Hewlett-Packard, USA. This method is based on the spectrophotometric determination of the yellow anion Y, produced by the reaction of thiocholine P from the reaction (2) with DTNB in the reaction mixture. Y has the maximum absorbance at 412 nm (A). The value of A is taken as proportional to the actual concentration of P, thus $[\text{P}] = A / (\epsilon \cdot d)$, where ϵ is the absorption coefficient of Y at 412 nm and d is the thickness of the cuvette. The value of $\epsilon = 14150 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used (Dodds and Rivory, 1999). A glass cuvette with the total volume 30 ml and optical path 2 cm provided with a glass propeller was used as the reactor. The cuvette was filled with chosen volumes of buffer pH 8 ($I = 0.14$), DTNB solution and enzyme preparation. This mixture was thermostated at 25 °C. The reaction was started by fast (< 1 s) homogenisation of the chosen volume of ATCH solution in the vigorously mixed reaction mixture. The initial concentrations of reactants are given in the description of single experiments in the chapter Results.

pH-stat method (Giacobini, 2000)

The pH-stat was constructed by the combination of the potentiostat Automatic Titrimeter OP-506, Automatic Burette OP-930 (both Radelkis, Budapest, Hungary) and usual PC. The method is based on the determination of the actual concentration of the acetic acid [HA] produced in the step (3). This is realized by the continuous titration of HA

with the analytical solution of NaOH keeping the pH of the reaction mixture at chosen constant (here pH 8.0) value, checked by the couple glass electrode – saturated AgCl electrode. From this measurement the dependence volume of analytical solution of NaOH (V) vs. t is obtained. The V value can be simply transformed to [HA] in the reaction mixture

$$[\text{HA}] = V \cdot [\text{NaOH}] / [(1 + 1.67) \cdot V_t] \quad (4)$$

where the number 1.67 respects the amount of NaOH spent for neutralisation of the dissociated part of the acid thiocholine (Brestkin *et al.*, 1974) and V_t is the total volume of the reaction mixture. In this method any buffer must not be principally used. Further, the reaction mixture must not be in the contact with the air because of present CO_2 which reacts at pH 8 immediately with the reaction mixture and decreases continuously its pH value. This complication was eliminated using the mixture of nitrogen and helium above the reaction mixture. The ionic strength was kept at the same value as in Ellman's method ($I = 0.14$ M) by means of water solution of 1 M NaCl.

The reaction was carried out in a glass closed vessel 100 ml thermostated at 25 °C with an electromagnetic stirrer. The vessel was filled at first with the chosen amounts of BCHE preparation, water and NaCl solution. This mixture was kept under inert atmosphere and its pH value was adjusted to 8.0 by means of the aqueous solution of NaOH under continuous control by galvanic couple mentioned above. The reaction was started by fast addition of the chosen volume of ATCH solution into the vigorously mixed reaction mixture. At the same time the automatic continuous addition of chosen analytical solution of NaOH and the on-line PC registration of its added volume V vs. t were started. The total initial volume of the reaction mixture was 36 ml, its increase during the addition of NaOH solution was ca 10% and was respected in the calculation of [HA] actual concentrations. The initial concentrations of reactants are given in the description of single experiments in the chapter Results.

The kinetics of hydrolysis of ATCH was studied by means of dependences $A \approx [\text{P}] \equiv [\text{TCH}]$ vs. t (Ellman's method) and $V \approx [\text{HA}]$ vs. t (pH-stat method) 1) without BCHE, 2) with BCHE at the a) start, b) whole course of the reaction at constant

[BCHE]₀, pH 8, ionic strength 0.14 M (NaCl) and temperature 25 °C.

Results

Hydrolysis of ATCH by BCHE at start of the reaction

The nonenzymatic ATCH decrease (0.11% w/w after 16 min of the reaction) can be fully neglected in the case of its parallel much faster enzymatic hydrolysis by BCHE.

Ellman's method

The mixture of phosphate buffer 0.05 M and DTNB 2×10^{-4} M was used as the comparing standard solution. The initial reaction mixture consisted of the phosphate buffer 0.05 M, [DTNB]₀ = 2×10^{-4} M and [BCHE]₀ = 0.1 ml of the enzyme preparation. The total volume of the reaction mixture was 12 ml. The series of dependences A vs. t was measured with various [ATCH]₀. From the linear initial parts of these dependences the saturation curve $v_o' = dA/dt$ vs. [ATCH]₀ was calculated – see Fig. 1, curve a. The monotoneous form of the curve without decrease of v_o' at high [ATCH]₀ values indicates no inhibition of the reaction by the substrate ATCH. Comparison of the experimental data with the Michaelis–Menten equation

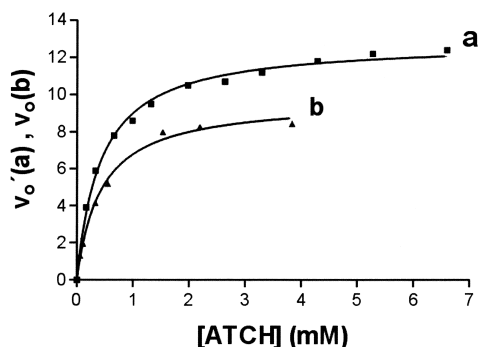


Fig. 1. Enzymatic hydrolysis of ATCH by BCHE measured at the start of the reaction by Ellman's method (dependence a: $v_o'(a) \equiv dA/dt$ [mAU·s⁻¹] $\equiv \epsilon \cdot d \cdot d[\text{TCH}]/dt$ vs. t), pH-stat method (dependence b: $v_o(b) \equiv d[\text{HA}]/dt$ [mM·s⁻¹] vs. dt). The points represent experimental data. The curves were obtained by comparison of these data with Equ. (5) by nonlinear regression with optimal parameters a) $K_M = 4.3 \times 10^{-4}$ M and $V_m = 4.2 \times 10^{-7}$ M·s⁻¹, b) $K_M = 4.3 \times 10^{-4}$ M, $V_m = 9.7 \times 10^{-7}$ M·s⁻¹.

$$v = -d[\text{S}]/dt = d[\text{P}]/dt = d[\text{HA}]/dt = v' / (\epsilon \cdot d) = V_m \cdot [\text{S}] / (K_M + [\text{S}]) \quad (5)$$

with respect to the value of $\epsilon = 14150 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and thickness of the cuvette $d = 2 \text{ cm}$, gave the values $K_M = 4.3 \times 10^{-4} \text{ M}$ and $V_m = 4.2 \times 10^{-7} \text{ M s}^{-1}$ (see Table II).

pH-stat method

was executed at the same temperature, pH, I and [BCHE]₀ (0.3 cm³ of the enzyme preparation in 36 cm³ of the total volume of the initial reaction mixture). The analytical solutions of NaOH 3.6 and 5.2 mM were used for the continuous titration of the arising HA. From the linear initial parts of the dependences V vs. t for various [ATCH]₀ the saturation curve $v_o = d[\text{HA}]/dt = -d[\text{ATCH}]/dt$ vs. [ATCH]₀ was calculated (see Fig. 1, curve b). Comparison of the experimental data with (5) by nonlinear regression gave the values $K_M = 4.3 \times 10^{-4} \text{ M}$ and $V_m = 9.7 \times 10^{-7} \text{ M s}^{-1}$ (see Table II).

Hydrolysis of ATCH by BCHE up to high conversion

The dependences $[\text{P}] \equiv [\text{TCH}]$ vs. t (Ellman's method) or $[\text{HA}]$ vs. t (pH-stat method) were measured up to long reaction times. The results were compared with the integrated form of the equation (5).

Ellman's method

Three experiments (1, 2, 3) were realized with [ATCH]₀ = 2.63, 3.95 and 1.32×10^{-5} M. The equation (5) can be after integration transformed by means of A to the form

$$t = K_M \cdot \ln[A_\infty / (A_\infty - A)] + k \cdot A / V_m \quad (6)$$

which can be further retransformed to

$$(1/A) \cdot \ln[A_\infty - A] = (V_m / K_M) \cdot t / A - k / K_M \quad (7)$$

where A_∞ is the absorbance of the final reaction mixture ($t \rightarrow \infty$, equilibrium) and $k = (\epsilon \cdot d)^{-1}$. The dependence of the left side of (7) vs. t/A gives a straight line. The values of K_M and V_m obtained from this linear regression (see Table Ia) were used as first estimates of the nonlinear regression of the

Exper. no.	Linear regression according to a) (7) or b) (9)			Nonlinear regression according to a) (6) or b) (8)		
	$K_M \times 10^4$ M	$V_m \times 10^7$ M/s	R^2	$K_M \times 10^4$ M	$V_m \times 10^7$ M/s	R^2
a)						
1	0.75	2.3	0.9946	0.39	1.3	0.9967
2	2.2	6.2	0.9993	1.8	5.3	0.9999
3	0.84	2.6	0.9921	1.9	3.1	0.9896
b)						
4	1.6	6.4	0.9917	1.5	6.0	0.9983
5	1.9	6.2	0.9671	1.5	5.2	0.9910
6	2.9	9.4	0.9586	1.4	5.1	0.9925

Table I: Values of K_M , V_m and R^2 calculated from the experimental data a) obtained by Ellman's method (experiments 1, 2, 3) and b) obtained by pH-stat method (experiments 4, 5, 6). R is the correlation coefficient.

experimental dependence A vs. t according to (6). An example of the results for the experiment 2 see in the Fig. 2a. The values of K_M , V_m and the corresponding correlation coefficients R calculated by the described linear and nonlinear regression for all three experiments 1, 2, 3 are also given in the Table Ia.

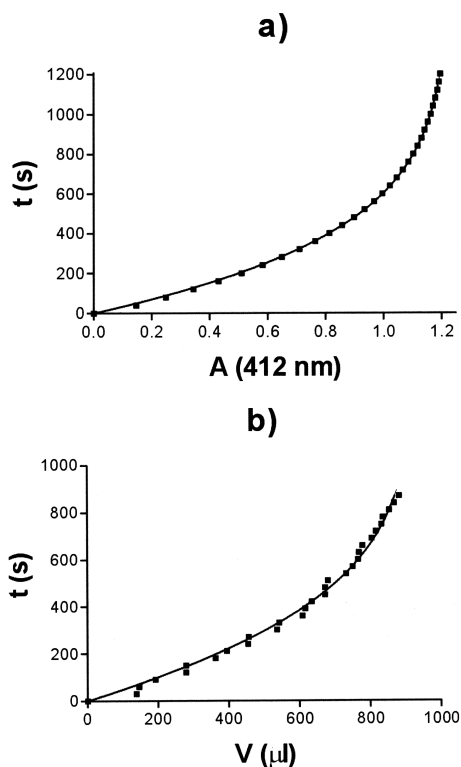


Fig. 2a,b. Hydrolysis of ATCH by BCHE up to high conversion of ATCH. a) Ellman's method: Graphical comparison of the data from the experiment 2 with relation (6), $R^2 = 0.9999$. b) pH-stat method: Graphical comparison of the data from the experiment 6 with relation (8), $R^2 = 0.9925$.

pH-stat method

The same initial conditions and concentrations were used as described in previous chapter "pH-stat method" with exception of the value of $[ATCH]_0$. Three experiments (4, 5, 6) were realized with $[ATCH]_0 = 1.24, 1.10$ and 0.549×10^{-4} M.

The Equ. (5) can be in this case transformed to the form

$$t = K_M \cdot \ln[V_\infty/(V_\infty - V)] + k' \cdot V/V_m \quad (8)$$

and after linearization

$$\left(\frac{1}{V}\right) \cdot \ln\left[\frac{V_\infty}{V_\infty - V}\right] = \left(\frac{V_m}{K_M}\right) \cdot \frac{t}{V} - k \cdot K_M \quad (9)$$

where V_∞ is the volume of the used analytical solution of NaOH in equilibrium. The constant k' has, after introducing the used analytical concentration $[NaOH]$, the total volume of the reaction mixture and the correction factor 0.67 (see equation (4)), the definition $k = 1.6633 \times 10^{-5} \cdot [NaOH]$. The dependence of the left side of (9) vs. t/V is linear for all three experiments 4, 5 and 6. The values of K_M and V_m obtained from this linear regression (see Table Ib) were used as first estimates of the nonlinear regression of the experimental dependences V vs. t according to (8). An example for the experiment 6 is given in the Fig. 2b. The values of K_M , V_m and R calculated from this comparison are also given in the Table Ib.

We take the results from nonlinear regression as more exact than from linear regression in both types of experiments.

Table II: Average values of V_m and K_M determined by Ellman's and pH-stat method from the initial stage – Equ. (5) and from the whole course – Equ. (6) or (8) – of the enzymatic hydrolysis of ATCH by BCHE at 25 °C, pH 8.0, ionic strength 0.14 M and constant $[BCHE]_o$. Results correspond to data from (Breskin, 1974) measured at similar conditions: $V_m = 3.08 \times 10^{-7} \text{ M} \cdot \text{s}^{-1}$ and $K_M = 5.4 \times 10^{-4} \text{ M}$.

Dependence	Method	$V_m \times 10^7 \text{ M} \cdot \text{s}^{-1}$	$K_M \times 10^4 \text{ M}$
v_o vs. [ATCH] Equ. (5)	Ellman's	4.25	4.26
	pH-stat	9.73	4.35
[ATCH] vs. t Equ. (6) or (8)	Ellman's	3.27	1.37
	pH-stat	5.43	1.45

Discussion

Following conclusions can be made from the results given above:

1) The saturation curves measured by Ellman's and pH-stat methods are without any extreme, i. e. no inhibition by substrate exists in the enzymatic hydrolysis of ATCH by BCHE at pH 8.0, ionic strength 0.14 M and 25 °C.

2) The validity of the Michaelis-Menten (or Briggs-Haldane) reaction mechanism represented by the equation (5) is fulfilled for this reaction at given conditions not only at its initial stage but also in the whole extent of the reaction times.

3) The values of the average kinetic parameters V_m and K_M in (5), determined both for the initial stage of the reaction and for high conversions of ATCH concentration by Ellman's spectral or pH-stat potentiometric method, are practically identical, see Table II. They correspond also with the data from [Brestkin *et al.*, 1974] measured at similar conditions: $V_m = 3.08 \times 10^{-7} \text{ M s}^{-1}$, $K_M = 5.4 \times 10^{-4} \text{ M}$.

4) The step (2) with rate constant k_2 is rate determining in the reaction scheme described by the steps (1), (2) and (3). The Ellman's method determines namely the actual concentration of thiocholine [P], while the pH-stat method measures the concentration of acetic acid [HA]. If the values of

K_M and V_m obtained by both methods are practically identical, then $k_2 \ll k_3 \cdot [\text{H}_2\text{O}]$ and the faster step (3) does not make itself kinetically useful.

5) The values of single rate constants of the mentioned reaction scheme and the unknown molar concentration of BCHE can be estimated from the received experimental data.

The estimate was realized by means of the computation program GEPASI (Mendes 1993, 1997; Mendes *et al.*, 1998) allowing to solve the kinetics of the given reaction scheme numerically and to optimize the relevant kinetic constants with respect to the experimental dependences concentration vs. time of one or more reactants. The program requires in our case the stoichiometric equations of the scheme, initial composition of the reaction mixture, first estimation of the kinetic constants and the set (or sets) of experimental data. We used the reaction scheme represented by equations (1) and $\text{ES} \rightarrow (\text{H}_2\text{O}, k_2) \rightarrow \text{E} + \text{P} + \text{HA}$. The initial concentrations of the reactants were $[\text{S}]_o, [\text{ES}]_o \equiv [\text{P}]_o \equiv [\text{HA}]_o = 0$. The program allowed us to calculate not only the set of optimal kinetic constants k_1, k_{1r} and k_2 but also to estimate the optimal value of the not known initial molar concentration of the used enzyme $[\text{E}]_o$.

The data from experiment 2 (Ellman's method) were used because of their best fitting to theoretical Equ. (7) – see $R^2 = 0.9999$ in Table Ia. In this case $[\text{S}]_o = 3.95 \times 10^{-5} \text{ M}$ and the calculated optimal estimations of the initial concentration of enzyme and of rate constants are: $[\text{BCHE}]_o \equiv [\text{E}]_o = 4.74 \times 10^{-8} \text{ M}$, $k_1 = 79266 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{1r} = 7.436 \text{ s}^{-1}$ and $k_2 = 24.34 \text{ s}^{-1}$. The values of $K_M = (k_{1r} + k_2) / k_1 = 4.01 \times 10^{-5} \text{ M}$ and $V_m = k_2 \cdot [\text{E}]_o = 1.15 \times 10^{-6} \text{ M s}^{-1}$, calculated from these data, correspond well with the relevant values in the second line of Table Ia.

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