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COMPETITION BETWEEN SUBSTRATES FOR ACETYLCHOLINESTERASE AND CHOLINESTERASE

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Summary

The kinetics of competition of pairs of two substrates for bovine erythrocyte acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) and horse serum cholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8) was studied so that the hydrolysis of only one substrate was measured at a time. The substrates were acetylthiocholine, phenylacetate and benzoylcholine; the same compounds, and also acetylcholine, were used as competiting substrates i.e. inhibitors. The substrate inhibition constants (K_{ss}) and Michaelis constants for the reaction of a single substrate were also determined. It was concluded that the substrate inhibition site in the enzyme does not show up in the competition between two substrates.

Introduction

It was shown earlier that coumarin derivatives are reversible inhibitors of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) [1,2]. From the kinetics of competition between substrates (acetylthiocholine and acetylcholine) and these compounds it was concluded that the coumarin derivatives bind to the substrate inhibition site in the enzyme. This site is commonly defined by the substrate inhibition constant K_{ss}. It was also postulated that the substrate inhibition site is an allosteric site of acetylcholinesterase. In this paper the kinetics of competition of pairs of two substrates for the enzyme were studied to verify to what extent the substrate inhibition sites of acetylcholinesterase and cholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8) are involved.

Material and Methods

The enzymes were bovine erythrocyte acetylcholinesterase (Winthrop Ltd., New York, N.Y., U.S.A.) and horse serum cholinesterase (Sigma Chemical

Comp., St. Louis, Mo., U.S.A.). The substrates were acetylthiocholine iodide (AChS), phenylacetate (PhAc), and benzoylcholine chloride (BzCh). The same compounds and also acetylcholine perchlorate (ACh) were used as competing substrates, i.e. inhibitors. All experiments were done in 100 mM phosphate buffer pH 7.4 at 25°C. The activity towards acetylthiocholine was measured by the spectrophotometric method of Ellman et al. [3] and towards phenylacetate by the spectrophotometric method of Krupka [4]. The activity towards acetylcholine was measured by the pH-stat method of Jensen-Holm [5]. The activity of serum cholinesterase towards benzoylcholine was measured by the spectrophotometric method of Kalow et al. [6] and the pH-stat method of Jensen-Holm [5]. The procedure was as follows: the enzyme solution (0.3 ml) in buffer was added to a buffer solution (2.7 ml) containing the substrate and inhibitor. Only the hydrolysis of the substrate was measured, while the hydrolysis of the competing substrate (inhibitor) was not measured (cf. ref. 7). The concentrations of inhibitors were such that the degree of enzyme inhibition was more than 20% and less than 80%.

Results and Discussion

The kinetic constants for the reaction of acetylcholinesterase and cholinesterase with a single substrate are listed in Table I. The Michaelis constant for acetylcholine and acetylcholinesterase was calculated from a plot of $[S]/v_0$ vs. [S], where v_0 is the enzyme activity.

For the reaction of benzoylcholine and cholinesterase the above plot is nonlinear. The Hill equation was therefore applied, and the Michaelis constant (K_m) and the Hill coefficient (n_H) were obtained from the χ^2 analysis, which was performed to derive the best set of K_m and n_H values which fits the experimental results. The substrate inhibition constant, K_{ss}, for benzyolcholine and cholinesterase was calculated from a plot of $1/v_0$ vs. [S]; this plot is linear indicating that the enzyme inhibited by the substrate itself does not hydrolyse

TABLE I

MICHAELIS CONSTANTS (K_m) AND SUBSTRATE INHIBITION CONSTANTS (K_{ss}) FOR ACETYLCHOLINESTERASE AND CHOLINESTERASE

n_H is the Hill coefficient. β is defined in the text. The range of substrate concentration used in the experiments is given in brackets.

Substrate (mM)	K_m/mM	K_{ss}/mM
Acetylcholinesterase		
Acetylcholine (0.01—1.0)	0.15 ± 0.01	—
Acetylcholine (1.0—100)	—	9 (β = 0.2)
Acetylthiocholine [8]	0.11	14
Phenylacetate [9]	2.6	—
Cholinesterase		
Benzoylcholine (0.01—10)	0.4 (n_H = 0.7)	—
Benzoylcholine (10—100)	—	56 ± 5 (β = 0)
Acetylcholine [10—13]	1.2—3.2	—
Acetylthiocholine [8]	0.6 (n_H = 0.8)	—
Phenylacetate [9]	3.8	—

Fig. 1.

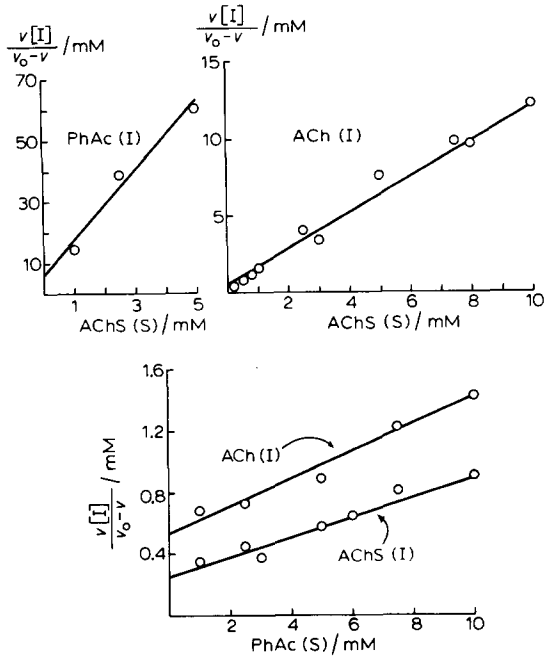

Fig. 2.

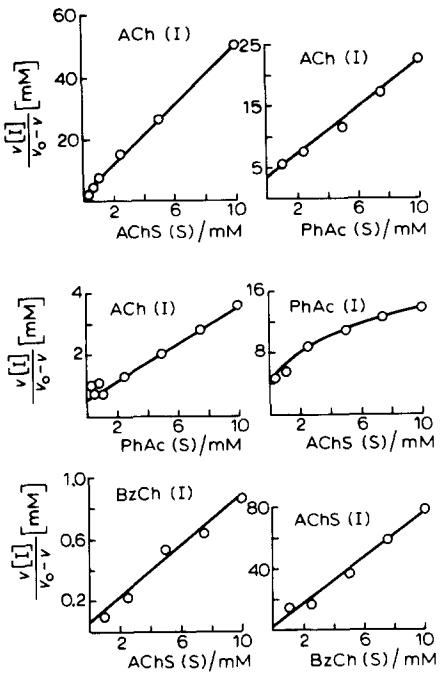

Fig. 1. Inhibition of acetylcholinesterase. Substrates (S) and inhibitors (I) are indicated in the figure. The symbols on the ordinate are defined in the text.

Fig. 2. Inhibition of cholinesterase. Substrates (S) and inhibitors (I) are indicated in the figure. The symbols on the ordinate are defined in the text.

at a measurable rate, i.e. $\beta = 0$ (cf. ref. 1, 8 and 14). The above plot for the reaction of acetylcholine and acetylcholinesterase is non-linear indicating that β is different from zero. The K_{ss} and β values given in Table I were obtained from the experimental data in a way described earlier [8] by the least-squares estimation.

The competition between two substrates for their reaction with the enzyme was studied so that the hydrolysis of only one substrate was measured at a time. For instance, when acetylthiocholine was the substrate and acetylcholine the inhibitor, the hydrolysis of acetylthiocholine was measured by the Ellman spectrophotometric method [3]; by this method only the formation of thiocholine, and not choline is determined. All results are presented in Figs. 1 and 2. The substrate concentration is plotted on the abscissa. The ratio v [I]/($v_0 - v$) is plotted on the ordinate; v_0 is the enzyme activity when no inhibitor is present, and v is the activity at the same substrate concentration but with the inhibitor present. [I] is the inhibitor concentration; the range of the inhibitor concentrations used in the experiments is given in Table II. At each substrate concentration at least two inhibitor concentrations were tested, and each experiment was repeated 2 or 3 times. Consequently, each point in the figures is a mean of at least four independent experiments. It was possible to calculate mean values because the ratio $v[I]/(v_0 - v)$ was constant at any given substrate concentration irrespective of the concentration of the inhibitor. This is illus-

TABLE II

INTERCEPTS (± STANDARD ERRORS) ON THE ABSCISSA $K(S)$ AND ORDINATE $K(I)$

Calculated from results presented in Figs. 1 and 2. The range of inhibitor concentration used in the experiments is given in brackets.

Substrate	$K(S)$/mM	Inhibitor (mM)	$K(I)$/mM
Acetylcholinesterase			
Acetylthiocholine	0.17 ± 0.17	Acetylcholine (0.5—10)	0.22 ± 0.22
Acetylthiocholine	0.22 ± 0.14	Phenylacetate (2.6—15)	4.9 ± 6.1
Phenylacetate	3.8 ± 0.8	Acetylthiocholine (0.2—5)	0.25 ± 0.03
Phenylacetate	6.2 ± 1.2	Acetylcholine (0.5—5)	0.54 ± 0.05
Cholinesterase			
Acetylthiocholine	0.53 ± 0.17	Acetylcholine (5—20)	2.6 ± 0.77
Acetylthiocholine	≈1.0	Phenylacetate (1—10)	≈4.0
Acetylthiocholine	0.37 ± 0.55	Benzoylcholine (0.05—1)	0.03 ± 0.04
Phenylacetate	2.5 ± 0.4	Acetylthiocholine (0.2—4)	0.69 ± 0.08
Phenylacetate	1.6 ± 0.4	Acetylcholine (5—15)	3.1 ± 0.7
Benzoylcholine	0.17 ± 0.45	Acetylthiocholine (10—50)	1.3 ± 3.3

trated in Table III for the inhibition of acetylcholinesterase by acetylcholine in the presence of acetylthiocholine as substrate.

In the kinetic equations derived earlier [1,2,9], the mechanism of competition between substrates and inhibitors can be identified from the numerical values of the intercepts on the abscissa and ordinate, if the results are plotted as in Figs. 1 and 2. When the intercept of the line or curve with the abscissa, $K(S)$, equals the Michaelis constant, substrate and inhibitor compete in the catalytic site. When $K(S)$ equals K_{ss}, the competition takes place in the substrate inhibition site. The intercept with the ordinate, $K(I)$, corresponds to the dissociation constant between enzyme and inhibitor. In Table II are listed the intercepts calculated for the reactions presented in Figs. 1 and 2. In all reactions but one the plot of $v[I]/(v_0 - v)$ vs. $[S]$ is linear. The exception is the hydrolysis of acetylthiocholine by cholinesterase with phenylacetate as inhibitor (Fig. 2); the intercepts in that case were assessed from the initial slope of the curve. For both enzymes, the calculated $K(S)$ and $K(I)$ constants are all of the same order of magnitude as the corresponding Michaelis constants for the same compound when used as a single substrate. This is true irrespective of whether the enzyme is or is not inhibited by the single substrate (i.e. K_{ss} does or does not reveal itself within the studied concentration range). It also applies irrespective of whether the reaction of the single substrate follows the Michaelis or Hill equation.

The standard errors of the calculated constants are large (cf. Table II). Therefore, the differences which exist between K_m on one side, and $K(S)$ and $K(I)$ on the other side cannot be taken as meaningful in terms of reaction mechanisms. However, the values of $K(S)$ and $K(I)$ for acetylcholine, acetylthiocholine and benzoylcholine are all on the average two orders of magnitude lower than the corresponding substrate inhibition constants K_{ss}. This implies that $K(S)$ and $K(I)$ do not correspond to K_{ss}. It also implies that the competition between two substrates is basically a competition in the catalytic site of the enzyme. The substrate inhibition site does not show up in the kinetics of competition

TABLE III

COMPETITION BETWEEN ACETYLTHIOCHOLINE (AS SUBSTRATE) AND ACETYLCHOLINE (AS INHIBITOR) FOR ACETYLCHOLINESTERASE

[I] is the concentration of acetylcholine; v and v_0 is the rate of actylthiocholine hydrolysis in presence and absence of acetylcholine.

Acetylthio-choline (mM)	Acetyl-choline (mM)	$\frac{v\,[I]}{v_0 - v}$ (mM)
0.10	0.05	0.30
0.10	0.10	0.31
0.10	0.50	0.30
0.25	0.25	0.40
0.25	0.50	0.45
0.50	0.50	0.78
0.50	1.0	0.80
0.75	0.75	1.0
0.75	1.5	0.96
1.0	0.50	1.6
1.0	1.0	1.6
1.0	2.5	1.8
1.0	5.0	1.4
2.5	2.5	4.3
2.5	5.0	3.8
3.0	1.0	3.6
3.0	5.0	3.5
3.0	10	3.1
5.0	1.0	7.7
5.0	5.0	7.5
5.0	7.5	7.9
5.0	10	6.5
7.5	7.5	9.3
7.5	10	10
8.0	1.0	9.0
8.0	5.0	10
8.0	10	9.6
10	5.0	15
10	10	12

between substrates. This is different from the competition between the same substrates and coumarin derivatives where the substrate inhibition site was kinetically prevalent [1,2].

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