NEW SUBSTRATES OF ACETYLCHOLINESTERASE

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1. Introduction

Acetylcholinesterase (AChE) has been the subject of intensive studies in the last 4 decades [1,2]. Occupying a central role in transmission of nervous signals and being an efficient hydrolase model enzyme, AChE attracted much interest, and a vast number of compounds were studied as substrates and inhibitors of the enzyme, for both theoretical and applied reasons.

Our aim was to examine the ability of new classes of compounds to serve as AChE substrates. A variety of compounds were tested as possible substrates of the *Electrophorus* enzyme and several highly active substrates were found:

(1) Phenothiazine carbonyl chloride was found to be a potent covalent inhibitor of acetylcholinesterase and could be used to titrate the enzyme active site;
(2) Enol acetates (vinyl acetate and its derivatives) are good substrates of the enzyme, yielding acetate and aldehyde upon hydrolysis;
(3) Alkylidenediacetates are hydrolyzed by acetylcholinesterase, yielding also acetate and aldehyde;
(4) Tropolone acetate is an excellent substrate of the enzyme;
(5) Several acetanilide derivatives are susceptible to acetylcholinesterase hydrolysis, although at a considerably slower rate than analogous esters.

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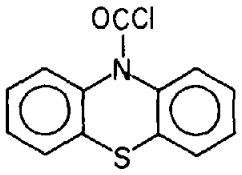
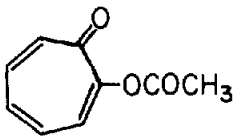
2. Materials and methods

Acetylcholinesterase from electric eel and acetylcholine chloride were purchased from Sigma. Phenothiazine-10-carbonyl chloride (PTCC, (a) in fig.1) was the product of Aldrich. Commercially available acetate esters were of high purity grade. Other acetates were prepared according to reported procedures. For example, 1-butenylacetate was prepared from



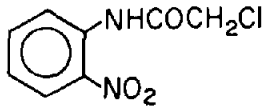
phenothiazine-10-carbonyl chloride

a



tropolone acetate

VI



o-nitro chloroacetanilide

VII

Fig.1.

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n-butanal and acetic anhydride [3], and tropolone acetate (VI, fig.1) from tropolone and acetic anhydride [4].

Hydrolysis rates of esters were followed pH-statically. In general the substrate was dissolved in acetonitrile and an aliquot was added to the pH stat vessel. The final concentration of acetonitrile was 1% (v/v). In this concentration acetonitrile did not affect the reaction rate. The reaction mixture contained 0.1 N NaCl and 0.02 M $MgCl_2$ and the enzymic hydrolysis was run at pH 8.0, 25°C, using 0.05 N NaOH as a titrant. Hydrolysis of *o*-nitro-chloroacetanilide (VII, fig.1) was followed spectrophotometrically at 400 nm. From the initial uptake of alkali in the pH stat, or from the initial absorbance change with time, the initial rate of hydrolysis was calculated. Lineweaver-Burk double reciprocal plot, $1/v$ vs $1/[S]$, generally yielded straight lines from which the kinetic parameters K_m and V_{max} were calculated by least squares analysis. The ratio $V_{max}/[AChE]$ gave k_{cat}, the turnover number of the enzyme with the substrate.

The active site concentration of AChE was determined by titrating it with phenothiazine carbonyl chloride (see section 3).

3. Results

3.1. *Inhibition of AChE with phenothiazine carbonyl chloride*

A μM stock solution of PTCC was prepared in acetonitrile. Aliquots (10 μl) of PTCC solution were added to AChE solution and several minutes after each addition aliquots of the enzyme solution were drawn for assay. It was found that enzymic activity was decreased in direct proportion to the added quantity of PTCC. The concentration of inhibitor required to abolish enzyme activity equalled the enzyme active site concentration. From the V_{max} of acetylcholine and the known concentration of the enzyme active site, a k_{cat}-value of 9.8×10^5 min^{-1} was found, which is in very good agreement with values derived by using other active site titrants [2].

3.2. *Enol acetates (vinyl acetate and butenylacetate)*

These were found to be good substrates of AChE (table 1). The k_{cat} of vinyl acetate is as high as that of acetylcholine, but the K_m-value is much higher. The butenyl derivative shows high affinity (low K_m), but the reactivity is slightly lower.

On hydrolysis of these compounds aldehyde was formed, which was detected by coupling the reaction to lactate dehydrogenase and NADH system:

$$CH_2 = CHOCOCH_3 + H_2O \rightarrow$$

$$H^+ + CH_3COO^- + CH_3CHO \qquad (1)$$

3.3. *Alkylidene diacetates, $RCH(OCOCH_3)_2$*

These are also susceptible to AChE hydrolysis. The k_{cat}-values of propylidene and benzylidene diacetates were ~60% that of acetylcholine and K_m was in the mM range (table 1). In this case the products were also acetate and aldehyde:

$$CH_3CH_2CH(OCOCH_3)_2 + H_2O \rightarrow$$

$$2\,H^+ + 2\,CH_3COO^- + CH_3CH_2CHO \qquad (2)$$

Table 1
Kinetic parameters of acetylcholinesterase catalyzed hydrolysis of different substrates[a]

Substrates	k_{cat} (min^{-1})	K_m (mM)
I Acetylcholine	9.8×10^5	0.4
II Vinyl acetate, CH_2=CHOCOCH$_3$	10.5×10^5	67.0
III 1-Butenyl acetate, CH_3CH_2CH=CHOCOCH$_3$	5.7×10^5	1.1
IV Propylidene diacetate, $CH_3CH_2CH(OCOCH_3)_2$	6.0×10^5	1.7
V Benzylidene diacetate, $C_6H_5CH(OCOCH_3)_2$	6.6×10^5	1.5
VI Tropolone acetate	10.2×10^5	0.55
VII *o*-Nitro-chloroacetanilide	1.6×10^4	2.5
VIII *o*-Nitro-dichloracetanilide	1.2×10^4	0.6

[a] pH 8.0, 25°C, in 0.1 N NaCl, 0.02 M $MgCl_2$ and 1% acetonitrile

3.4. *Tropolone acetate (VI, fig.1)*

Since aryl acetates are known to be good substrates of AChE [5,6] and as we found that enol acetates were also good substrates of AChE, we decided to examine tropolone acetate, VI (fig.1), which possesses both vinyl and aromatic properties [7]. This compound proved to be an excellent substrate of AChE, with kinetic parameters similar to those of the natural substrate (table 1).

3.5. *Aromatic amides*

While many acetate esters are good substrates of AChE, amides are hardly affected by the enzyme. The amide analogue of acetylcholine was found to have a k_{cat} ~4500-fold smaller than that of acetylcholine [8]. As aromatic groups are generally better leaving groups than their aliphatic analogues, it was of interest to test the ability of aromatic amides to serve as substrates of AChE. To accomplish this end, *o*-nitrochloroacetanilide and *o*-nitro-dichloroacetanilide were prepared. When AChE was added to solutions of these compounds the amide spectrum was changed to that of nitroaniline. The k_{cat} values were $>10^4$ min^{-1} (table 1), much slower than that of aromatic esters [5,6], but ~2 orders of magnitude faster than the amide analogue of acetylcholine ($k_{cat} = 2.2 \times 10^2$ min^{-1}) [8].

4. Discussion

Table 1 lists new types of good substrates of AChE, some of which have the same k_{cat} as acetylcholine. Inspection of these substrates indicates that they are 'activated' esters and therefore are prone to undergo fast reaction with the enzyme to form acyl intermediate.

The quaternary ammonium group of acetylcholine causes the increased sensitivity of this substrate to nucleophilic attack. The specific rate constant of hydroxide catalyzed hydrolysis of it is 25-fold greater than that of the uncharged ester ethyl acetate [9]. Vinyl acetate is highly activated and undergoes hydroxide catalyzed hydrolysis 100-fold faster than its saturated analogue ethyl acetate [10]. The high reactivity of alkylidene diacetates (IV,V) is probably due to the presence of 2 electron-withdrawing ester groups attached to the same carbon atom.

A minimal mechanism for AChE action is a 2 step reaction:

$$\mathrm{ROCOCH_3} + \mathrm{EOH} \underset{}{\overset{K_s}{\rightleftharpoons}} \mathrm{ROCOCH_3 \cdot EOH} \xrightarrow{k_2} \mathrm{EOCOCH_3} \xrightarrow[\mathrm{H_2O}]{k_3} \mathrm{EOH} + \mathrm{H^+} + \mathrm{CH_3COO^-} \qquad (3)$$

in which EOH is the AChE serine enzyme, $EOCOCH_3$ is the transient acetyl enzyme, k_2 is acylation rate and k_3 is deacylation rate constant.

With all acetyl substrates a common acetyl enzyme intermediate is formed which undergoes hydrolysis with the same rate constant k_3. However, the reactivity of the substrate affects k_2 and k_{cat} which equals $k_2k_3/(k_2 + k_3)$ [11,12]. In the cases in which $k_2 > k_3$, k_3 is rate limiting and $k_{cat} \simeq k_3$, the upper limit value. This is expected to occur with highly activated ester substrates.

Data reported in literature indicate that AChE has a broad specificity and catalyzes the hydrolysis of cationic as well as neutral esters [2,9,13]. The low K_m values and high k_{cat} values found for the substrates investigated in this study show that many compounds can be very good substrates of the enzyme. However, the uniqueness of acetylcholine in nervous processes lies in its specific properties and interaction at the neuromuscular junction rather than in its sensitivity to hydrolysis by the esterase.

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