ARE IDENTICAL CATALYTIC GROUPS INVOLVED IN THE ACETYLATION AND DEACETYLATION STEPS OF ACETYLCHOLINESTERASE REACTIONS?

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Experiments have been carried out on acetylcholinesterase, using charged and neutral substrates and inhibitors, which lead to the following conclusions: Two ionizing groups, which function catalytically in the unprotonated form, are present at the active center; one of these groups (pK 5.6) functions only in the acetylation step in catalysis, while the other (pK 6.2) functions only in the deacetylation step, the reaction sequence being

$$E + S \underset{k\text{-}1}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + P \qquad (1)$$

where k_2 and k_3 are rate constants for acetylation and deacetylation, and E, ES and EA are the free enzyme, the enzyme-substrate complex and the acetyl enzyme, respectively. Following formation of ES and EA, the enzyme may undergo conformational changes which alter the relative positions of groups at the active center, so that different catalytic groups may participate in each of the two steps. Such an arrangement overcomes a fundamental difficulty in covalent catalysis, namely that structural features in a catalyst promoting formation of a compound with the substrate normally increase the chemical stability of the latter;and for this reason it is difficult to envisage efficient catalysis of both steps, formation and breakdown of the enzyme-substrate compound, unless the chemical environment of the covalent enzyme-substrate bond changes during the

course of the reaction. These ideas suggest that one of the two similar histidine residues found in chymotrypsin, trypsin and elastase (Walsh et al 1964; Smillie and Hartley, 1964) may function in acylation, while the other functions in deacylation. The new mechanism also resolves a difficulty encountered when acylation and deacylation of chymotrypsin are assumed to be the microscopic reverse of one another. The pH dependence of these two reactions has been found to be different, and this is not explained easily if the same catalytic groups are involved (Bender and Kezdy, 1964), but is understandable if different groups participate.

Acetylcholinesterase used in this study was a purified preparation from bovine erythrocytes and was supplied by Sigma Chemical Co. or Nutritional Biochemicals Corp. Initial rates of substrate hydrolysis were determined by automatic titration of acid released with 0.01 N NaOH. Reaction mixtures contained 0.10 M NaCl and 0.04 M $MgCl_2$. The temperature was maintained at 26°.

Ionization constants for groups in the free enzyme and in the Michaelis complex, K and K' respectively, were calculated from the following equations (Laidler, 1958):

$$Km\ (expt.)/V\ (expt.) = Km\ (1 + [H]/K)/V \qquad (2)$$

$$1/V\ (expt.) = (1 + [H]/K')/V \qquad (3)$$

Competitive and non-competitive inhibition constants, K_i and K_i' respectively, were determined by means of a procedure described before (Krupka, 1965).

As a preliminary experiment, neutral and charged substrates were shown to be hydrolyzed by the same active center in the experimental enzyme preparation. This was done in three ways. (a) The binding constant for 3-hydroxyphenyltrimethylammonium iodide, a potent and highly specific reversible inhibitor (Krupka, 1965), was approximately the same when the substrate was AcCh or phenyl acetate ($K_i = 9.0 \times 10^{-7}$ M). The reaction mixture in this experiment contained 1% methanol. Up to 2% methanol was shown to decrease the maximum velocity, V, to only a slight

extent (2 - 3%) and did not significantly affect the pH dependence of AcCh hydrolysis. (b) Inhibition of AcCh hydrolysis by tetramethylammonium chloride is markedly pH dependent (Bergman and Shimoni, 1952; Krupka, 1964). Virtually the same pH dependence was demonstrated in inhibition of phenyl acetate hydrolysis. (c) Initial rates of phenyl acetate hydrolysis were measured spectrophotometrically at 270 mμ, and inhibition of phenyl acetate hydrolysis by added AcCh was then determined. The reaction mixture consisted of 0.05 M sodium phosphate buffer, pH 7.5, and 2% methanol. Competitive and non-competitive inhibition constants were determined: $K_i = 7.9 \times 10^{-5}$ M and $K_i' = 3.5 \times 10^{-3}$ M. AcCh hydrolysis was then followed under identical conditions, except for omission of phenyl acetate, and it was shown that K_i and K_i' for AcCh as an inhibitor correspond to Km and K_s (the substrate inhibition constant) for AcCh as a substrate. This correspondence could occur only if AcCh and phenyl acetate compete for the same active center.

TABLE I

Summary of Data on pH Dependence of Hydrolysis with Neutral and Cationic Substrates

Substrate	Relative Maximum Velocity	Rate Limiting Step	pK	pK'
phenyl acetate[1]	1.13	EA ⟶ E+A	5.64	6.35
AcCh	1.00	EA ⟶ E+A	6.20	6.26
isoamyl acetate[2]	0.20	ES ⟶ EA	5.65	5.65
methylaminoethyl acetate	0.098	ES ⟶ EA	6.1	5.4
dipropylmethylamino-ethyl acetate	0.054	ES ⟶ EA	6.0	5.5
4-trimethylamino-butyl acetate	0.019	ES ⟶ EA	6.1	5.2

[1]Reaction medium contained 1% methanol.

[2]Reaction medium contained 2% methanol.

Table I summarizes experiments on the pH dependence of hydrolysis of a number of neutral and charged acetyl ester substrates. The rate limiting step in hydrolysis of the best substrates, acetylcholine and phenyl acetate, is probably deacetylation, but with the more slowly hydrolyzed esters the rate limiting step is acetylation (Krupka, 1964). The values of pK, which reflect ionizations in the free enzyme only, and which are not influenced by ionizations in any E-S complex (Krupka and Laidler, 1960), are 5.65 with neutral substrates and 6.0 - 6.2 with cationic substrates. Two different groups must therefore ionize in the free enzyme. That which affects the hydrolysis of only cationic substrates (pK 6.0 - 6.2) cannot be an essential catalytic group; when protonated, it appears to repel cations from the active center. The other group, pK 5.6, is revealed in experiments with neutral substrates and must be essential for either formation or reaction of ES.

The values of pK' are obviously related, not to substrate charge, but to the rate limiting reaction step. When acetylation ($ES \longrightarrow EA$) is rate limiting, pK' is 5.2 - 5.65, indicating that the essential group in the free enzyme of pK 5.6 must function in acetylation. With deacetylation ($EA \longrightarrow E+A$) rate limiting, pK' is 6.2, showing that an ionizing group with this pK' value functions in deacetylation.

Let us now consider whether the non-essential ionizing group in E (pK 6.2), which plays no essential role in ES, is the same group that is essential in EA. We know that this group, in the free enzyme, repels cationic substrates from the active center. It should then be possible to show that it repels cationic inhibitors from E and EA. This has been done by measuring K_i for tetramethylammonium chloride, and K_i' for trimethylammonium chloride, as functions of pH. K_i and K_i' are measures of binding to E and EA, respectively (Krupka, 1964). Figures 1 and 2 show plots of $-\log K_i$ and $-\log K_i'$ against pH. Inflections, given by the point where $-\log K_i$ is 0.3 pK units below the maximum, appear at approximately

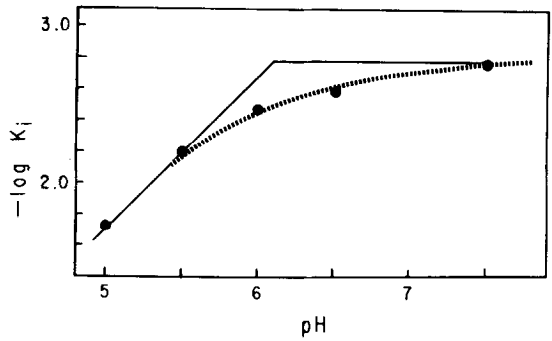

Fig. 1. Plot of pK_i against pH for competitive inhibition of AcCh hydrolysis by tetramethylammonium chloride. The pK of the ionizing group which affects binding, corresponding to the pH at which pK_i is 0.3 units below its maximum value, and shown by the point of intersection of the guide lines of slopes 1 and 0, is 6.10.

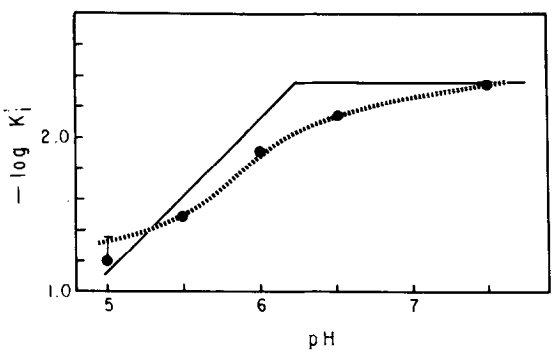

Fig. 2. pK_i' vs. pH for non-competitive inhibition of AcCh hydrolysis by trimethylammonium chloride. Inflection point at pH 6.24 (see Fig. 1).

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pH 6.2 in both cases. This supports the idea that the group which is non-functional in E and ES is essential in EA.

This idea was confirmed by another experiment, involving tetraethylammonium bromide (TEA). This inhibitor was known to become bound to EA without blocking deacetylation (Krupka, 1965). If, as the above evidence suggests, an inhibitory proton and a substituted ammonium ion compete for the active center, the following equilibrium of EA species should prevail

$$\dot{E}\dot{A}H \rightleftharpoons EA \rightleftharpoons EA\dot{T} \qquad (4)$$

where $\overset{+}{E}AH$ is unreactive, but where EA, and in the case of TEA, $\overset{+}{E}AI$ as well, undergo deacetylation. The inhibitor, I, should therefore increase the maximum velocity of AcCh hydrolysis at low pH, since hydrolysis of this substrate is rate-limited by deacetylation. The data in Figure 3 bear out this prediction and confirm the hypothesis. It may also be shown that the degree to which TEA increases V can be quantitatively predicted from the model shown in equation 4. By contrast, when TEA is replaced by tetrapropylammonium, which blocks deacetylation (EAI is unreactive; Krupka, 1965), V is lowered at all pH values.

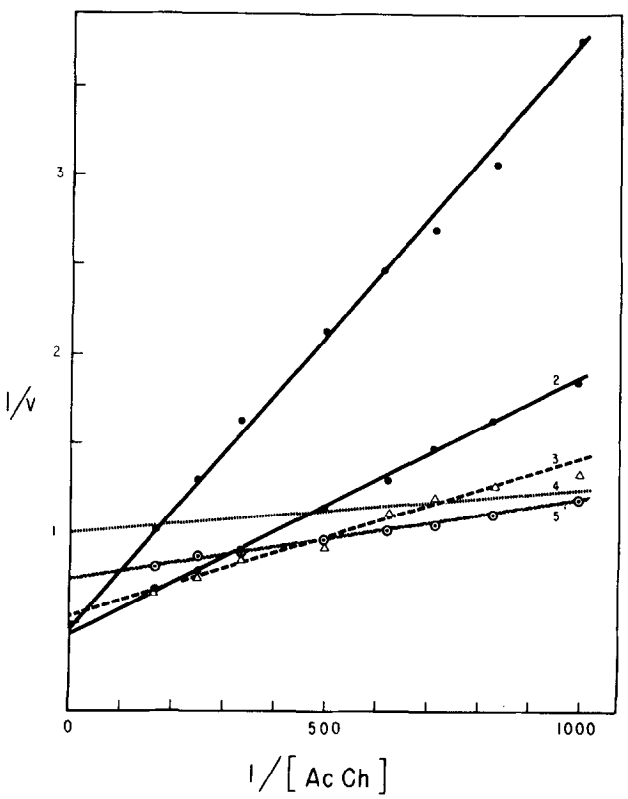

Fig. 3. Effects of TEA on AcCh hydrolysis, pH 5.0. Units of AcCh concentration are molar, and V in the absence of TEA is arbitrarily set at unity (line 4). Each line corresponds to rates in the presence of fixed concentrations of TEA: (1) 4.78×10^{-2}M; (2) 1.90×10^{-2}M; (3) 9.56×10^{-3}M; (4) no inhibitor (5) 4.75×10^{-3}M.

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