CONCERNING THE RATE LIMITING STEP IN THE HYDROLYSIS OF SUBSTITUTED PHENYL ACETATES BY ACETYLCHOLINESTERASE

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Kinetic parameters of acetylcholinesterase catalyzed hydrolysis of substituted phenyl acetates under the conditions of $S \gg E$ and $S \cong E$, were analyzed in terms of the Hammett plot. In both cases, the slope of the line changes from negative for the electron withdrawing substituents to positive for the electron donating substituents. It is suggested that formation of a hydrogen bonded tetrahedral intermediate may be rate limiting in the hydrolysis of some substrates by acetylcholinesterase.

Introduction

The mechanism of acetylcholinesterase action has been studied by many workers and it is generally accepted that the following scheme applies:

$$E + S \underset{}{\overset{k_s}{\rightleftharpoons}} ES \overset{k_{23}}{\underset{P_1}{\rightleftharpoons}} EP_2 \xrightarrow{k_{34}} E + P_2 \qquad (1)$$

where ES represents a Michaelis-Menten Complex, EP_2, an acyl enzyme, P_1 an alcohol, and P_2 acetic acid.

While de-acylation of the enzyme is assumed to be rate limiting for substrates such as acetylcholine or phenyl acetate (1,2), the acylation step is believed to be rate determining for a number of more slowly hydrolyzed analogs of both acetylcholine and phenyl acetate. Thus, for instance, considerable differences in the hydrolytic rates are seen in the acetyl -choline, -thiocholine, and -selenocholine series (3), as well as with the substituted phenyl acetates (4). The apparent change in the hydrolytic mechanism of acetylcholinesterase with substrate has not been adequately explained, although it has been postulated (3,4) that the ability

of a substrate to form a hydrogen bond between it and the enzyme may be the determining factor.

Since it is often possible to interpret reactivity of substituted phenyl acetates in electronic terms utilizing the Hammett σ - ρ relation, the effect of para- and meta-substituents on the rates of acetylcholinesterase catalyzed hydrolyses of phenyl acetates was investigated.

Materials

Electric eel acetylcholinesterase, E.C. 3.1.1.7. (ECHP, sp. activity approx. 1500 u/mg) was obtained from Worthington Biochemical Co. The enzyme normality was determined by titrations with O-nitrophenyldimethyl-carbamate (5). Phenyl, p- and m-tolyl acetates were obtained commercially (Fisher Scientific Co.) and were redistilled. All other substrates were prepared according to standard methods by reacting the appropriate phenol with acetic anhydride. Melting points and spectra of the products corresponded to the literature values.

Results

In Table I are listed the substrates together with the corresponding values of k_{cat}, K_m, k_{cat}/K_m, and log (k_{cat}/K_m). The data indicate that the effect of the substituents is on both K_m and on the rate constant k_{cat}. The Hammett plot of $\log(k_{cat}/K_m)$ and $\log(k_{cat})$ vs σ is shown in Figure 1. It is seen that a reasonably good linear relationship obtains; however, a clear break in the direction of the slope is evident on going from the electron withdrawing to the electron donating substituents. A change in the sign of ρ can be interpreted, although it does not prove it, as a change in the rate determining step. Figure 1 shows that the value of ρ is negative for the electron withdrawing substituents, with the p-nitrophenyl acetate being the poorest substrate in the series. This situation is the exact opposite of what would be expected if the acylation step involving the nucleophilic serine were rate determining, as is generally assumed for

TABLE I

STEADY STATE KINETIC PARAMETERS OF ACHE CATALYZED HYDROLYSIS OF PHENYL ACETATES

PHENYL ACETATE	$10^{-3}xk_{cat}$ (sec^{-1})	$10^{3}xK_m$ (M)	$10^{-6}x(k_{cat}/K_m)$ (M^{-1} sec^{-1})	$\log\frac{k_{cat}}{K_m}$	$\log k_{cat}$	σ
P-nitro	1.284±.082	3.41±.31	.376	5.57	3.11	1.27
p-acetyl	1.441±.045	3.79±.27	.381	5.58	3.16	0.873
m-acetyl	9.221±.239	3.06±.19	3.01	6.48	3.96	0.306
m-methoxy	10.641±.191	1.16±.07	9.2	6.96	4.02	0.115
H	17.198±.399	2.14±.13	8.01	6.9	4.24	0.
m-methyl	14.473±.397	1.27±0.11	11.6	7.06	4.16	-0.069
p-methyl	12.505±.386	2.44±.19	5.13	6.71	4.08	-0.170
p-methoxy	10.004±.429	5.33±.45	1.89	6.29	4.0	-0.268

The rates of hydrolyses of phenyl acetates were followed titrimetrically (Titrigraph SBR 3c, Radiometer type TTT 1-a, Denmark) at pH 7.0, 26°, under stream of nitrogen gas. The reaction mixture consisted of 10 ml of the appropriate substrate solution in 0.1 M NaCl and 5% MeOH (v/v), to which 10 µl of enzyme solution of known normality was added. Whenever necessary, the initial rates were corrected for non-enzymic hydrolysis, and for dissociation of the substituted phenols. Results of a minimum of two independent assays at each substrate concentration were evaluated by the method of Wilkinson (7). Standard σ values for phenols are used (6).

the more slowly hydrolyzed substrates. On the other hand, the de-acylation step cannot be rate limiting for the phenyl acetates with the electron withdrawing substituents as it would lead to a paradoxical situation where

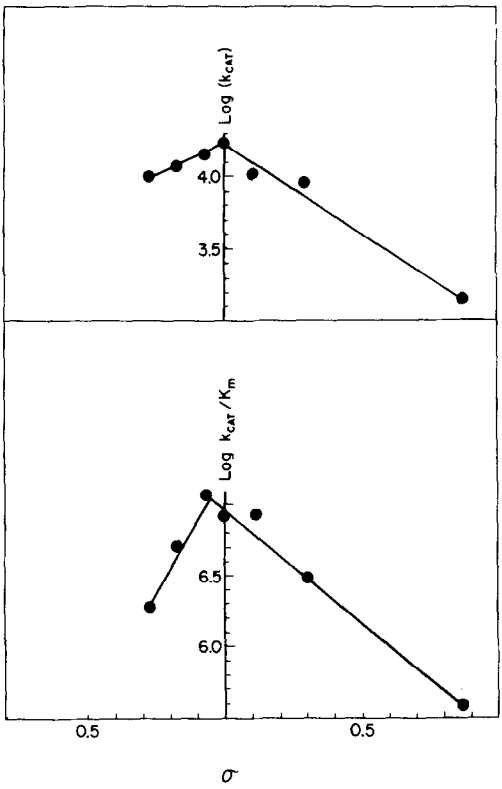

Figure 1.

Hammett plot of log(k_{cat}) and log(k_{cat}/K_m) obtained from the steady-state measurements. Standard σ values for phenols are used (6). P-nitrophenyl acetate is not included in the graph.

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apparently identical acetyl-enzymes de-acetylate at different rates. Indeed, stop-flow experiments conclusively eliminate this possibility. The positive slope obtained with the phenyl acetates bearing the electron donating substituents would indicate that, in this case, the rate limiting step could be acylation.

The results seem to indicate that a change in the rate of hydrolyses of the substituted phenyl acetates by acetylcholinesterase cannot be simply explained as a change in the rate determining step from de-acylation to acylation. It is suggested that the formation of a hydrogen bonded tetrahedral intermediate may be rate determining for substrates with strong

electron withdrawing substituents, and that acylation may become the rate determining step with substrates in which hydrogen bonding of the phenoxy oxygen is facilitated by the presence of the electron donating substituents in the para position on the phenyl ring. Supporting evidence for the above derives from the results in Table II and Figure 2, obtained under the conditions of approximately equal enzyme and substrate concentrations,

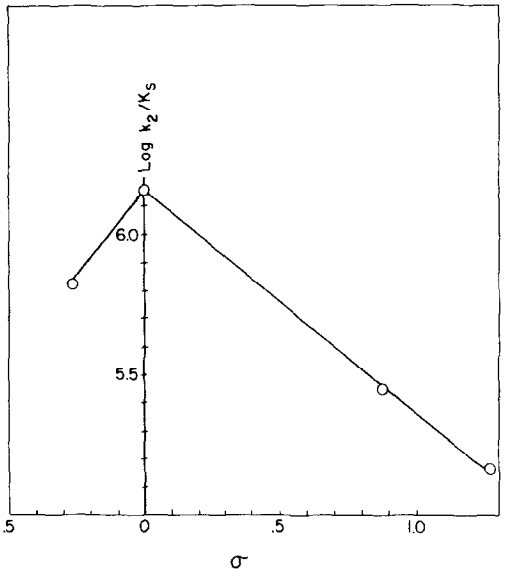

Figure 2.

Hammett plot of $\log(k_2/K_s)$ obtained under the conditions of E = S, at pH 5.1 and 26°. Standard σ values are used (6).

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i.e., under the conditions when essentially only the acylation step is observed. As in the steady state experiments, a negative ρ is obtained with the electron withdrawing substituents, and a change in the direction of the slope for the electron donating substituents is noted. Whereas, in this case the k_{23} and K_s constants cannot be dissociated, it can be said that the contribution of the k_{34} constant to the k_{obs} is rather negligible.

TABLE II

ACYLATION OF AChE BY SUBSTITUTED PHENYL ACETATES

PHENYL ACETATE	$10^6 x S_o$ (M)	$10^6 x E_o$ (M)	$10^6 x (k_2/K_s)$ (M^{-1} sec^{-1})	$\log k_2/K_s$
H	2.5	1.73	1.44±.4	6.16
p-methoxy	2.5	1.73	.668±.014	5.82
p-acetyl	2.5	1.73	.295±.025	5.47
p-nitro	2.5	1.73	.153±.006	5.18

The results were obtained by reacting approximately equal enzyme and substrate concentrations in a Durrum-Gibson stop-flow instrument. The final reaction mixture was 0.05 M in NaCl and sodium acetate buffer, pH 5.1 at 26°. The second order rate of reaction was observed by following the release of phenol at an appropriate wavelength. k_{obs} evaluated from the plot of $\log \Delta O.D._{t-t_o}$ vs t is equal, under these conditions, to k_2/K_s.

Discussion

The results reported herein agree best with the findings of Bergman, et al (4), who suggested the following scheme for the acetylcholinesterase-catalyzed hydrolyses of phenyl acetates:

$$EH + S \underset{}{\overset{k_s}{\rightleftharpoons}} EH...S \overset{k_{23}}{\underset{P_1}{\searrow}} EP_2 \xrightarrow{k_{34}} E + P_2 \qquad (2)$$

These authors assumed that a hydrogen bond formation must take place between a substrate and an electrophilic group in the enzyme. They concluded that formation of the EH...S complex or subsequent acylation may be rate limiting but that the deacylation step cannot. It is more realistic not to equate the EH...S with the Michaelis-Menten complex as is implied by scheme 2, and it is proposed that the following scheme describes the results more satisfactorily:

$$EH + S \underset{}{\overset{K_S}{\rightleftharpoons}} EHS \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} EH...S \xrightarrow{k_{34}} EP_2 \xrightarrow{k_{45}} E + P_2 \qquad (3)$$
$$\searrow P_1$$

Scheme 3 also describes satisfactorily the results obtained by Hillman and Nautner (3) with acetylcholine and its analogs, acetylthiocholine and acetylselenocholine. These authors found that the rates of hydrolysis by acetylcholinesterase decrease in order: acetylcholine > acetylthiocholine > acetylselenocholine, presumably because the ability of the acyloxy oxygen atom of acetylcholine to form hydrogen bonds exceeds that of sulfur and selenium atoms. The authors suggested that the decreased rate with the latter two acetylcholine analogs could be due to the rate determining formation or breakdown of the tetrahedral intermediate (EH...S in scheme 3).

Recapitulation

Evidence is presented which suggests that in the acetylcholinesterase-catalyzed hydrolysis of phenyl acetates, and probably also in the hydrolysis of acetylcholine and its analogs, participation of hydrogen bonded intermediate may be important and may become rate determining with substrates in which the acyloxy oxygen is electron-deficient. When the formation of hydrogen bonded intermediate ceases to be rate limiting, as for instance in hydrolysis of phenyl acetates with electron donating substituents, a change in the mechanism from apparent electrophilic to apparent nucleophilic is noted. It is reasonable to assume that, in this instance, the acylation of the active serene is being observed. Qualitatively, the preceding interpretation is also supported by the fact that, in phenyl acetates with electron donating substituents, hydrogen bond formation between the phenolic oxygen and the enzyme will be relatively efficient as compared to decreased efficiency of nucleophilic attack on the acyloxy group, whereas exactly the reverse will be true for the phenyl acetates with electron withdrawing groups. Finally, although the two

effects are treated separately, it is likely that the action of the two groups[1] in the enzyme must be concerted, explaining why the unsubstituted phenyl acetate itself is a better substrate than any one substituted phenyl acetate. It is of interest that if deacylation step k_{45} becomes rate determining in the hydrolysis of phenyl acetate and acetylcholine, then it must be said that these two substrates are probably rather unique in this respect, although few other substrates, e.g., acetylthiocholine, might also qualify. It should be added that the k_{cat} values for the substrates for which deacylation is rate limiting should be, within experimental error, identical.

In summary, scheme 1 does not adequately describe acetylcholinesterase-catalyzed hydrolysis of phenyl acetates and is probably an oversimplification; any rigorous treatment of the kinetics of acetylcholinesterase catalysis should consider a probable contribution of EH...S intermediate to the overall mechanism of this enzyme.

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REFERENCES

1. Krupka, R.M. Biochem. Biophys. Research Comm. 19: 531, (1965).

2. Wilson, I.B. Ann. N.Y. Acad. Sci. 144: 664, (1967).

[1] It is assumed that two groups, one nucleophilic and one electrophilic (4), participate in the active site. A more exhaustive discussion of this point can be found in the paper of Hillman and Mautner (3).

3. Hillman, G.R. and H.G. Mautner. Biochem. 9: 2633, (1970).

4. Bergman, F., S. Rimon, and R. Segal. Biochem. J. 68: 493, (1958).

5. Bender, M.L., Begue, Canton, M.L., Blakeley, R.L., Brubacher, L.J., Feder, J., Gunter, C.R., Kezdy, F.J., Killheffer, J.V., Marshall, T.H., Miller, C.G., Roeske, R.G., and Stoops, J.K. J. Amer. Chem. Soc. 88: 5890, (1966).

6. Hammett, L.P. Physical Organic Chemistry, p. 188, McGraw-Hill, New York, (1940).

7. Wilkinson, G.N. Biochem. J. 80: 324, (1961).