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INFLUENCE OF pH ON BUTYRYLCHOLINESTERASE REACTION WITH ORGANOPHOSPHORUS INHIBITORS *

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Summary

The non-covalent enzyme · inhibitor complex dissociation constants and the enzyme phosphorylation rate constants were measured as functions of pH in butyrylcholinesterase (acylcholine acylhydrolase, EC 3.1.1.8) reaction with organophosphorus inhibitors $(C_2H_5O)_2P(O)SX$, where $X = (CH_2)_3SC_2H_5$ and $(CH_2)_6S^+(CH_3)C_2H_5$. Two ionizing groups, a basic and an acidic one, were revealed in the overall reaction of the enzyme inhibition within the pH range between 5 and 10.5. In the enzyme phosphorylation step only the acidic group was found, while the basic group appeared in the non-covalent binding step of both the ionic and non-ionic compounds. The results strongly imply the participation of the basic functional group in the conformation transition which affects the ability of butyrylcholinesterase to bind hydrophobic reagents in the acidic pH region.

Introduction

Butyrylcholinesterase (acylcholine acylhydrolase, EC 3.1.1.8) reaction with organophosphorus inhibitors is considered to involve two separate steps, a reversible complex formation followed by phosphorylation of the enzyme active center [1,2]. However, all the experimental data available on the pH dependence of the kinetic properties of the enzyme in this reaction concern the second-order rate constants and thus characterize only the overall reaction. Therefore, the present study is concerned with the influence of pH on the separate steps of the butyrylcholinesterase reaction with both the non-ionic and ionic organophosphorus inhibitors $(C_2H_5O)_2P(O)S(CH_2)_3SC_2H_5$ and $[(C_2H_5O)_2P(O)S(CH_2)_6S^+(CH_3)C_2H_5]CH_3SO_4^-$. We have found that the enzyme loses its ability to bind either of these inhibitors in the acidic pH region. The

* A preliminary account of this work was presented at the 12th FEBS Meeting in Dresden.

results imply that the reversible inactivation of butyrylcholinesterase at low pH can be associated with conformational transition triggered off by the protonation of a basic functional group, possibly the imidazole residue in the active center of the enzyme.

Methods

Horse serum butyrylcholinesterase was purchased from the Mechnikov Institute of Sera and Vaccine, Moscow. The organophosphorus inhibitors were purified chromatographically [3] to free them from any contaminants of high anticholinesterase activity. The other reagents and solutions were similar to those used in our earlier experiments [4].

The reactions of butyrylcholinesterase with the inhibitors were carried out under the pseudo-first-order conditions in a vessel of pH-stat (pH-121, BAT-15, B-705, USSR) at 25°C. At least 50-fold excess of the inhibitors was used in the kinetic measurements. The reaction medium was 0.15 M KCl, the pH of this solution was adjusted to the necessary value with the pH-stat and was checked during the kinetic runs. At appropriate time intervals 0.02—0.2 ml of the reaction mixture was diluted into 5.0 ml of 0.15 M KCl and 4 mM butyrylcholine and the enzyme residual activity was measured at pH 7.5 and 25°C using the second pH-stat (TTT-1, SBR-2, ABU-1, 'Radiometer', Denmark). The pseudo-first-order inhibition rate constant k_I was calculated from ln v_t versus t plots:

$$\ln v_t = \ln v_0 - k_I t \tag{1}$$

where v_0 is the enzyme activity at zero time. The enzyme · inhibitor complex dissociation constant K_Q and the phosphorylation step rate constant k_2 were calculated from $1/k_I$ versus $1/[Q]$plot (Fig. 1) according to the appropriate linear transformation of the following equation:

$$k_I = \frac{k_2[Q]}{K_Q + [Q]} \tag{2}$$

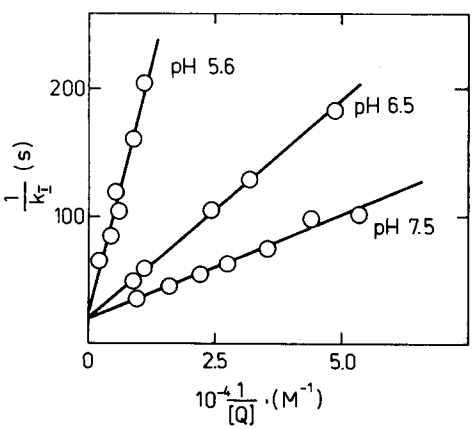


Fig. 1. Evaluation of k_2 and K_Q from the double-reciprocal plot for butyrylcholinesterase reaction with $(C_2H_5O)_2P(O)S(CH_2)_3SC_2H_5$ in the acidic and neutral pH regions.

where both constants K_Q and k_2 may be functions of pH and Q denotes inhibitor. The second-order rate constants of butyrylcholinesterase inhibition k_i were obtained under conditions $[Q] \ll K_Q$:

$$k_I = k_i[Q] \tag{3}$$

where $k_i = k_2/K_Q$. The constants k_i found under the pseudo-first-order conditions coincide within the error limits with that calculated as the ratio of k_2 and K_Q (see Tables I and II).

The pH dependences of the kinetic parameters were analyzed by the method of Wilkinson [5] on a Nairi-2 computer.

Results

The kinetic constants K_Q, k_2 and k_i for butyrylcholinesterase reaction with inhibitors $(C_2H_5O)_2P(O)S(CH_2)_3SC_2H_5$ and $[(C_2H_5O)_2P(O)S(CH_2)_6S^+(CH_3)\text{-}C_2H_5]CH_3SO_4^-$ at different pH values are listed in Tables I and II. The results indicate that the K_Q values for both inhibitors show a progressive increase in the acidic pH region while k_2 depends on pH only in the alkaline medium. This behavior of butyrylcholinesterase can be seen in more detail in Figs. 2 and 3 where the pK_Q — pH and log k_2 — pH profiles are presented. It appears that the shapes of the cruves obtained for the ionic and non-ionic inhibitors closely resemble each other. Hence the following common equations may be applied for both compounds used:

$$K_Q = K_Q^{lim}\left(1 + \frac{[H^+]}{K_2}\right) \tag{4}$$

TABLE I

KINETIC CONSTANTS FOR THE REACTION OF BUTYRYLCHOLINESTERASE WITH ORGANOPHOSPHORUS INHIBITOR $(C_2H_5O)_2P(O)S(CH_2)_3SC_2H_5$ AT DIFFERENT pH VALUES IN 0.15 M KCl AT 25°C

Variations quoted are standard errors.

pH	10^2k_2 (s^{-1})	10^3K_Q (M)	$10^{-2}k_i$ ($M^{-1} \cdot s^{-1}$)
5.0	5.0 ± 2.0	1.6 ± 0.5	0.389 ± 0.019
5.2	3.2 ± 0.6	1.0 ± 0.2	0.324 ± 0.014
5.3	4.5 ± 1.0	1.1 ± 0.3	0.501 ± 0.31
5.6	3.6 ± 0.9	0.56 ± 0.16	0.724 ± 0.73
6.0	5.7 ± 0.8	0.41 ± 0.07	1.41 ± 0.06
6.2	4.8 ± 0.3	0.24 ± 0.03	1.81 ± 0.09
6.5	3.6 ± 0.8	0.14 ± 0.05	3.08 ± 0.21
7.5	5.4 ± 0.9	0.10 ± 0.02	4.79 ± 0.18
8.5	4.5 ± 0.6	0.13 ± 0.02	4.78 ± 0.16
9.0	2.6 ± 0.7	0.09 ± 0.02	3.98 ± 0.14
9.3	3.6 ± 0.5	0.13 ± 0.03	2.95 ± 0.18
9.6	1.8 ± 0.4	0.14 ± 0.02	1.74 ± 0.16
9.8	1.6 ± 0.5	0.11 ± 0.02	1.96 ± 0.13
10.0	0.9 ± 0.3	0.09 ± 0.02	1.51 ± 0.10
10.3	0.9 ± 0.4	0.10 ± 0.03	0.790 ± 0.055
10.6	0.4 ± 0.2	0.12 ± 0.05	0.398 ± 0.028

TABLE II

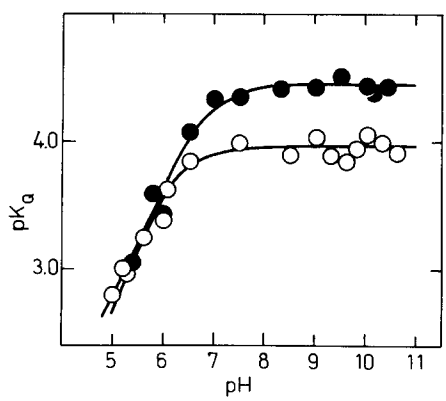
KINETIC CONSTANTS FOR THE REACTION OF BUTYRYLCHOLINESTERASE WITH ORGANOPHOSPHORUS INHIBITOR $[(C_2H_5O)_2P(O)S(CH_2)_6S^+(CH_3)C_2H_5]CH_3SO_4^-$ AT DIFFERENT pH VALUES IN 0.15 M KCl AT 25°C

pH	$10^2 k_2$ (s^{-1})	$10^4 K_Q$ (M)	$10^{-2} k_i$ ($M^{-1} \cdot s^{-1}$)
5.1	1.6 ± 0.7	25 ± 4	0.0645 ± 0.0041
5.4	2.0 ± 0.6	9 ± 2	0.214 ± 0.031
5.8	2.3 ± 0.6	2.5 ± 0.9	0.794 ± 0.045
6.0	2.5 ± 0.7	3.8 ± 1.1	0.603 ± 0.042
6.5	2.1 ± 0.5	0.83 ± 0.09	2.19 ± 0.20
7.0	1.9 ± 0.3	0.45 ± 0.05	4.24 ± 0.11
7.5	2.4 ± 0.6	0.43 ± 0.09	5.46 ± 0.23
8.3	1.4 ± 0.3	0.38 ± 0.09	4.67 ± 0.15
9.0	1.6 ± 0.3	0.36 ± 0.08	5.25 ± 0.18
9.5	0.8 ± 0.1	0.29 ± 0.09	2.46 ± 0.06
10.0	0.65 ± 0.08	0.36 ± 0.07	1.66 ± 0.06
10.1	0.38 ± 0.04	0.40 ± 0.07	1.18 ± 0.04
10.4	0.32 ± 0.05	0.36 ± 0.09	0.851 ± 0.031

$$k_2 = k_2^{\lim}\left(1 + \frac{K_1'}{[H^+]}\right)^{-1} \tag{5}$$

The above equilibrium and rate constants correspond to the reaction scheme:

$$\begin{array}{ccccc} & & E & \overset{aK_Q^{\lim}}{\rightleftharpoons} & EQ \\ & & \Downarrow K_1 & & \Downarrow K_1' \\ Q + & EH & \overset{K_Q^{\lim}}{\rightleftharpoons} & EHQ & \xrightarrow{k_2^{\lim}} \text{products}, \\ & & \Downarrow K_2 & & \\ & & EH_2 & & \\ & & \Downarrow & & \\ & & (EH_3) & & \end{array} \tag{6}$$



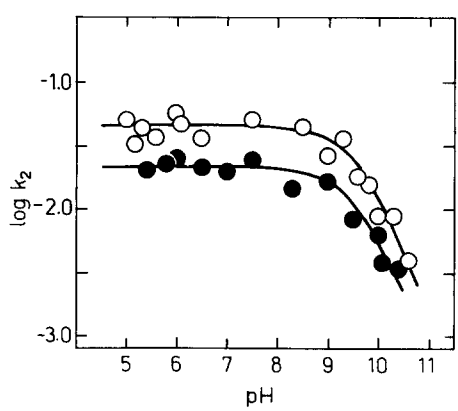


Fig. 2. Effect of pH on non-covalent binding step in butyrylcholinesterase reaction with $(C_2H_5O)_2P(O)S(CH_2)_3SC_2H_5$ (○) and $[(C_2H_5O)_2P(O)S(CH_2)_6S^+(CH_3)C_2H_5]CH_3SO_4^-$ (●).

Fig. 3. Plot of log k_2 versus pH in butyrylcholinesterase reaction with $(C_2H_5O)_2P(O)S(CH_2)_3SC_2H_5$ (○) and $[(C_2H_5O)_2P(O)S(CH_2)_6S^+(CH_3)C_2H_5]CH_3SO_4^-$ (●).

TABLE III

INFLUENCE OF pH ON BUTYRYLCHOLINESTERASE REACTION WITH ORGANOPHOSPHORUS INHIBITORS $(C_2H_5O)_2P(O)SX$ IN 0.15 M KCl AT 25°C

X	Constants
$(CH_2)_3SC_2H_5$ *	$\log k_i^{lim}$ = 2.68 ± 0.09
	pK_1 = 9.5 ± 0.1
	pK_2 = 6.3 ± 0.2
	pK_Q^{lim} = 3.98 ± 0.08
	pK_2 = 6.2 ± 0.2
	$\log k_2^{lim}$ = −1.35 ± 0.06
	pK_1' = 9.6 ± 0.2
$(CH_2)_6S^+(CH_3)C_2H_5$	$\log k_i^{lim}$ = 2.77 ± 0.07
	pK_1 = 9.5 ± 0.1
	pK_2 = 6.7 ± 0.2
	pK_Q^{lim} = 4.46 ± 0.09
	pK_2 = 6.8 ± 0.2
	$\log k_2^{lim}$ = −1.69 ± 0.07
	pK_1' = 9.5 ± 0.2

* For the pH interval between 3 and 11 $\log k_i^{lim}$ = 2.75 ± 0.05, pK_1 = 9.47 ± 0.10 and pK_2 = 6.20 = 0.11 have been found [9].

and are listed in Table III. The solid lines in Figs. 2 and 3 were calculated from Eqns. 4 and 5 on the basis of these data.

The second-order rate constants decrease in both the alkaline and acidic pH regions and the dependences observed wihtin the pH interval used for kinetic measurements were described by the following equation:

$$k_i = k_i^{lim}\left(1 + \frac{K_1}{[H^+]} + \frac{[H^+]}{K_2}\right)^{-1}. \quad (7)$$

The constants obtained are listed in Table III. These results agree with the earlier data on butyrylcholinesterase reactions with organophosphorus inhibitors [6], methanesulfonyl fluoride [7] and substrates [8], where also two ionizing groups of the enzyme active center have been found. However, besides that a fourth pH form of the free enzyme with an apparent pK_a of 4.5 was established in log k_i versus pH plot for butyrylcholinestesrase reaction with cationic inhibitor $[(C_2H_5O)_2P(O)S(CH_2)_3S^+(CH_3)C_2H_5]CH_3SO_4^-$ [9]. This pH form is indicated in brackets in Eqn. 6. We have suggested that this ionizing group may belong to the anionic center of butyrylcholinesterase as it did not appear in reactions of non-ionic inhibitors with the enzyme [9]. The significant increase in the K_Q values in the acidic pH region did not allow exact separation of the binding and reaction constants at pH below 5. Therefore the protonation of the second basic group cannot be demonstrated on the level of the separate steps of the inhibition reaction.

Table III shows that the pK_2 values obtained for the cationic and non-ionic inhibitors differ approximately by 0.5 pK_a units. However, this discrepancy may be considered to remain within the limites of error, especially as the possible influence of the enzyme form EH_3 was not taken into account in calculating the pK_2 value for the cationic inhibitor. No other satisfactory explana-

tion has yet been given to this phenomenon.

Differently from the basic group the dissociation constant of the acidic functional group is independent of the structure of the inhibitors used. Moreover, the data in Table III show that this dissociation constant is not shifted upon binding of inhibitor as $K_1 = K_1'$. The equality of K_1 and K_1' implies that $a = 1$ in the reaction scheme given by Eqn. 6.

Discussion

It is generally accepted that the bond-breaking steps in reactions of cholinesterases are assisted by an imidazole group [10]. The present data are not at variance with this idea although this basic group did not appear in log k_2 versus pH plot. The latter fact may be connected with the absence of the appropriate equilibrium between the enzyme pH forms of EHQ and EH_2Q as the form EH_2 is not able to bind inhibitors (see Eqn. 6). Furthermore, a basic group with $pK_a = 6.4$, which is probably imidazole, has been found in log k_{cat} versus pH plots for butyrylcholinesterase-catalyzed hydrolysis of thiocholine esters [11] where enzyme deacylation can be considered to be the rate limiting step (our unpublished data). Therefore, it is reasonable to suppose that the same basic group participates also in the enzyme acylation and phosphorylation steps.

Besides that, the protonation of the same basic group affects the ability of butyrylcholinesterase to bind both the ionic and non-ionic compounds. In the latter case the loss of binding ability cannot be explained by neutralization of the effective charge of the anionic binding site as has usually been done in the case of cationic reagents [12]. From the above it follows that butyrylcholinesterase loses its hydrophobic binding center in the acidic pH region that may occur as a result of a conformational transition. In this connection two possibilities may be noted.

Firstly, the loss of the binding center can be associated with the disruption of a hydrogen bond between some acidic group and the crucial basic group, triggered off by the protonation of the last residue.

Secondly, the protonated basic group may form an ion pair with some anionic group, which is accompanied by the change in the structure of the enzyme active center.

The second possibility is quite similar to Bergmann's hypothesis [13] according to which the protonation of the catalytically important imidazole residue neutralizes also the effective negative charge of the anionic center and thus governs the binding of alkylammonium ions and other cationic reagents in

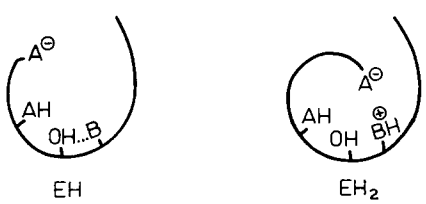


Fig. 4. Schematic drawing of the active center of butyrylcholinesterase pH forms EH (left) and EH_2 (right). Here AH and B denote the acidic and basic (imidazole) groups, OH is the nucleophilic serine hydroxyl and A^- belongs to the anionic site.

the active center of cholinesterases. The present data suggest that this ion pair formation between the imidazolium ion and the anionic group may be accompanied by conformation transition as illustrated in Fig. 4. The enzyme form EH_2 is unable to bind either ionic or non-ionic reagents as the active center is closed by the polypeptide chain containing the anionic residue A^-.

It should be noted that ion pair formation between BH^+ and A^- strongly stabilizes the enzyme form EH_2, while the location of the lone imidazolium ion in the hydrophobic 'cleft' of the active center is thermodynamically unfavourable, just as the transfer of a cationic group from water into any hydrophobic phase needs approximately $3.2 \cdot 10^4$ J/mol on the free energy scale [14]. An extra effect that stabilizes the location of ionic substituents in hydrophobic surroundings in the active center of cholinesterases has recently been established in reactions of these enzymes with cationic inhibitors [4,15] and substrates [16]. This stabilizing effect has been explained by ion-pair formation between the anionic center and cationic substituent [4]. Thus, the situation seems to be very similar to the one suggested by the second hypothesis given above and therefore, we consider it to be the more reasonable version.

The results for butyrylcholinesterase phosphorylation step are consistent with the dependence of k_2 on the acidic group AH (Fig. 4). This group is believed to be the phenolic hydroxyl of tyrosine [17]. The role of this group in catalysis is not clear yet. The possible participation of an acidic group in acetylcholinesterase reactions has been discussed by several authors [18,19].

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