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DISSOCIATION CONSTANTS OF THE ESTERATIC CENTER OF BUTYRYLCHOLINESTERASE

M. R. PAVLIČ
Institute of Pathophysiology, University of Ljubljana, Ljubljana (Yugoslavia)

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SUMMARY

1. The pH dependence of the rate of sulfonylation of butyrylcholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8) by methanesulfonyl fluoride was studied in an attempt to obtain some new information about dissociating groups in the esteratic center of this enzyme and to determine the corresponding dissociation constants.

2. Results obtained by following the rates of sulfonylation at different pH values point to a reaction mechanism in which two dissociating groups in the esteratic center are involved, with $pK_1 = 6.2$ and $pK_2 = 10.1$. If both groups are protonated the resulting esteratic center is inactive; if only the first group is dissociated the esteratic center reacts with $k_a'' = 0.30\ l \cdot mole^{-1} \cdot s^{-1}$, and if both groups are dissociated the esteratic center reacts with $k_a''' = k_a''/2$.

INTRODUCTION

The sulfonylation of cholinesterases by methanesulfonyl fluoride is practically a simple irreversible pseudo first-order reaction[1,2]. Sulfonylation takes place at the esteratic center of the enzyme while the anionic center remains free[2,3]. The sulfonylation of cholinesterases by methanesulfonyl fluoride is, therefore, a suitable tool for the study of the esteratic centers of cholinesterases.

In the present work the sulfonylation of butyrylcholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8) by methanesulfonyl fluoride was studied at different pH values in order to obtain some information about dissociating groups of the esteratic center of this enzyme and to determine the corresponding dissociation constants.

METHODS AND MATERIALS

The basic reaction was

$$E + I \overset{k_a}{\rightarrow} E' + P \qquad (1)$$

where E represents the esteratic center of the enzyme butyrylcholinesterase; I, the

inhibitor methanesulfonyl fluoride; E', the sulfonylated esteratic center; P, the product, and k_a, the second-order rate constant at a given pH.

The experimental procedure was as follows (*cf.* ref. 3): First, the apparent pseudo first-order rate constant at a given concentration of methanesulfonyl fluoride and a given pH was determined from the time dependence of the inhibition. Three different concentrations of methanesulfonyl fluoride were always used (1–10 mM). The enzyme activity was measured photometrically (Hestrin[4]) by the decrease in acetylcholine concentration. k_a was then determined from the dependence of the apparent constant on the concentration of methanesulfonyl fluoride. Experiments were repeated at pH values from 5.3 to 11.

Experiments were carried out in a thermostat at 25 °C. The universal buffer solution of Britten and Robinson[5] was used, but since this solution has different ionic strengths at different pH values, NaCl was added to all solutions, so that a total ionic strength of 0.2 was maintained.

The stability of butyrylcholinesterase at a given pH was checked by preincubating the enzyme for 15 min at a given pH value, diluting the solution 100 times with a buffer solution, pH 7.0, and determining the enzyme activity. No decrease in enzyme activity was ever noticed in the pH range from 5.3 to 11. The stability of methanesulfonyl fluoride at different pH values has been established before[3].

The butyrylcholinesterase used was Code CHE 9 KD, 6 units/mg, Worthington. The enzyme stock solution contained 200 mg of the preparation in 1 ml buffer solution with 0.1% gelatin, pH 7.0. The enzymic hydrolysis of acetylcholine obeyed the Michaelis–Menten equation over the tested concentration range from 0.2–4 mM.

The methanesulfonyl fluoride stock solution was a 1 M solution of methanesulfonyl fluoride, reagent grade, from Eastman Organic Chemicals, in spectroquality acetone. The incubation solutions were prepared by mixing various amounts of the stock solution with the buffer solution. The incubation solutions were prepared immediately before each experiment.

RESULTS AND DISCUSSION

Our experimental results are summarized in Fig. 1.

It is seen in Fig. 1 that the pH dependence of the sulfonylation of butyrylcholinesterase by methanesulfonyl fluoride is bell shaped. A bell-shaped curve for pH

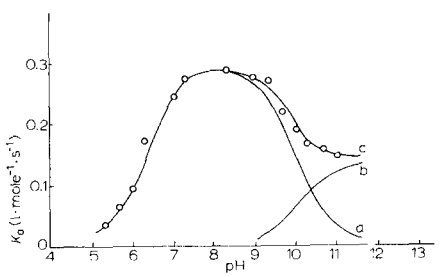


Fig. 1. The pH dependence of the second-order rate constant for the inhibition of butyrylcholinesterase by methanesulfonyl fluoride at 25 °C. k_a is the second order rate constant for the inhibition at a given pH. Each point in the figure is the average of three determinations. Curves a, b and c are theoretical curves (see text).

dependence of an enzymic reaction can be interpreted in many ways (*cf.* refs 6–8). In our case, the interpretation in terms of dissociation constants of some groups seems to be the most plausible since it has been shown already (*cf.* ref. 9) that in reactions with cholinesterases, including butyrylcholinesterase, pH-dependent dissociating groups in their esteratic centers are involved.

Let us assume, in accordance with other findings on cholinesterases (*cf.* refs 9, 10), that the esteratic center of the enzyme exists in the following three dissociating forms:

$$EH^{2+} \overset{K_1}{\rightleftharpoons} E\,H \overset{K_2}{\rightleftharpoons} E^- \tag{2}$$

In this scheme K_1 and K_2 refer to the first and the second stage of the dissociation of the esteratic center.

If only the EH form is active (*cf.* refs 3, 11) then the activity of the esteratic center must be proportional to the amount of this form:

$$k_a = \frac{k_a''}{1 + \frac{K_2}{(H^+)} + \frac{(H^+)}{K_1}} \tag{3}$$

Here, k_a'' is the second-order rate constant for the sulfonylation of the second form of the esteratic center (EH). The denominator is the second Michaelis pH function. For $(H^+) \gg K_2$ or for $(H^+) \ll K_1$, respectively,

$$k_a = k_a'' - \frac{1}{K_1} k_a(H^+) \tag{4}$$

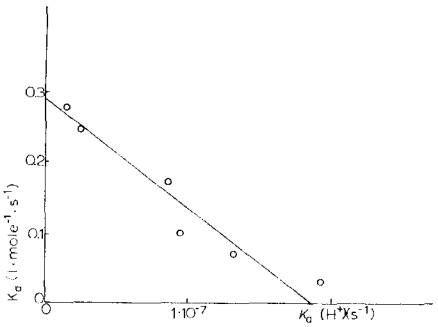
$$k_a = k_a'' - K_2 \frac{k_a}{(H^+)} \tag{5}$$

A plot of k_a against $k_a\,(H^+)$, or k_a against $k_a/(H^+)$, gives a line with K_1 as the reciprocal of the tangent of the line, or K_2 as the tangent of the line, respectively. In both cases, the intersection of the line with the y axis gives k_a''.

Our experimental data were plotted according to Eqns 4 and 5, as shown in Figs 2 and 3, respectively. The data in the low pH range (Fig. 2) are in accordance with Eqn 4 but the data in the high pH range (Fig. 3) are not in accordance with Eqn 5. Thus, our experimental results at high pH values do not agree with our assumptions.

Judging from the relatively high activities of the enzyme at high pH values (Figs 1 and 3) it seems possible that our assumption of only one active form of the esteratic center may be wrong. Not only the second form, EH, but also the third form, E^-, seems to be active. The contribution of this form to the activity measured at the lower pH values is negligible, so the line in Fig. 2 fitting the experimental points again represents Eqn 4 but the dotted experimental curve in Fig. 3 then corresponds to the activity of the second form *plus* the activity of the third form. From this figure, K_2 can be obtained as the tangent to the experimental curve at $x = 0$; k_a'' is the intersection of this tangent* with the y axis and k_a''', the second order rate constant for the sulfonylation of the third form of the esteratic center, is the extrapolated

* It should be mentioned here that, at first, for determining K_2 the line fitting best for the first four experimental points in Fig. 3. was used[12]; the value thus obtaiend was $pK_2 = 10.3$.



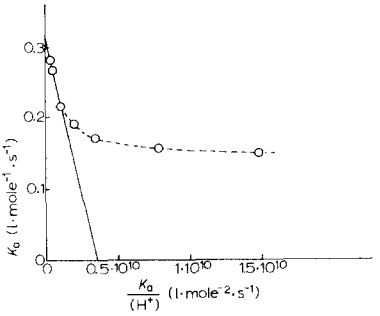


Fig. 2. The dependence of the rate of the inhibition of butyrylcholinesterase by methanesulfonyl fluoride at 25 °C on the H^+ concentration in the range from $5 \cdot 10^{-8}$ to $5 \cdot 10^{-6}$ M. k_a is the second order rate constant for the inhibition at a given pH. The data for the low pH values from Fig. 1 are plotted according to the equation $k_a = k_a'' - k_a (H^+)/K_1$ (Eqn 4). From this diagram the following values have been derived (see text): $k_a'' = 0.29$ l·mole^{-1}·s^{-1}; $K_1 = 6.5 \cdot 10^{-7}$ M.

Fig. 3. The dependence of the rate of the inhibition of butyrylcholinesterase by methanesulfonyl fluoride at 25 °C on the H^+ concentration in the range from $1 \cdot 10^{-9}$ to $1 \cdot 10^{-11}$ M. k_a is the second-order rate constant for the inhibition at a given pH. The data for high pH values from Fig. 1 are plotted as k_a against $k_a/(H^+)$. From this diagram the following values have been derived (see text): $k_a'' = 0.31$ l·mole^{-1}·s^{-1}; $k_a''' = 0.15$ l·mole^{-1}·s^{-1}; $K_2 = 8.5 \cdot 10^{-11}$ M.

value of k_a at very high x values. The constants thus obtained are: $K_1 = 6.5 \cdot 10^{-7}$ M, $pK_1 = 6.2$; $K_2 = 8.5 \cdot 10^{-11}$ M, $pK_2 = 10.1$; $k_a'' = 0.30$ l·mole^{-1}·s^{-1} (average), and $k_a''' = 0.15$ l·mole^{-1}·s^{-1}. If these values for K_1, K_2 and k_a'' are introduced in Eqn 3, Curve a in Fig. 1. is obtained which represents the activity of the esteratic center with only its second form being active. The activity of the esteratic center with only the third form being active is

$$k_a = \frac{k_a'''}{1 + \frac{(H^+)}{K_2} + \frac{(H^+)^2}{K_1 K_2}} \qquad (6)$$

Here, the denominator is the third Michaelis pH function. If the corresponding values are introduced in Eqn 6 Curve b in Fig. 1 is obtained. The activity of the esteratic center with its second and third form being active is the sum of Curves a and b and is shown in Fig. 1 as Curve c. Our experimental points fit the resultant curve very well*.

Thus, our experimental data agree well with the following assumptions and derivations: In the sulfonylation of butyrylcholinesterase by methanesulfonyl fluoride, the esteratic center of the enzyme exists in three dissociating forms (Eqn 2) of which the second and the third are active; their activities expressed as the corresponding second-order rate constants are $k_a'' = 0.30$ l·mole^{-1}·s^{-1} and $k_a''' = 0.15$ l·mole^{-1}·s^{-1}, respectively. The constants for the first and the second stage of the dissociation are $K_1 = 6.5 \cdot 10^{-7}$ M and $K_2 = 8.5 \cdot 10^{-11}$ M, respectively.

Since the constants K_1 and K_2 are widely separated they may be referred to two dissociating groups. In any case, the groups in question must be on the enzyme and

* Note that the rates obtained in the present work are higher than those previously determined[2]. This fact is primarily due to the two different enzyme preparations as it was checked repeatedly.

not on the inhibitor as methanesulfonyl fluoride does not dissociate. Furthermore, the groups must be in the esteratic center, as only there does the reaction take place. It is unlikely that some groups outside the esteratic center would influence the reaction in such a characteristic manner (see Fig. 1).

Thus, the dissociation constants of the two groups in the esteratic center of butyrylcholinesterase are, according to our results, $pK_1 = 6.2$, and $pK_2 = 10.1$. Different authors have obtained different values for those constants (*cf.* refs 13–16). But their experimental conditions were complicated by a possible influence of the anionic center and, in some cases, by the possibility of the enzyme–substrate complex being responsible for the observed constants. For the reasons given above, our values for the dissociation constants seem to be more reliable. Far from being conclusive, our results speak for histidine and tyrosine in the esteratic center of butyrylcholinesterase (*cf.* refs 17 and 18).

It should be noted here that our analogous experiments with acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) showed[3] that the pH dependence of the sulfonylation of acetylcholinesterase by methanesulfonyl fluoride did follow the equation based on the assumption that there was only one active dissociation form of the esteratic center. However, in those experiments the pH dependence of sulfonylation at very high pH values displayed a similar, but much less expressed, behaviour as it does in the case of butyrylcholinesterase. The slight discrepancies between theory and experiment were interpreted as being due to relatively great errors at low reaction rates; this may or may not be true. The above interpretation of the discrepancies, applied to acetylcholinesterase, gives a value of $pK_2 = 10.2$ (previously, 10.3) for the acidic group and, for the activity of the third form of the esteratic center, a value of about one tenth or less of the activity of the second form. However, the reaction mechanism of the sulfonylation of acetylcholinesterase by methanesulfonyl fluoride seems to be different from that of butyrylcholinesterase; the dissociation state of the acidic group in the esteratic center being relatively much more important in acetylcholinesterase than in butyrylcholinesterase.

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REFERENCES

1 R. Kitz and I. B. Wilson, *J. Biol. Chem.*, 237 (1962) 3245.
2 M. Pavlič, *Biochim. Biophys. Acta*, 198 (1970) 389.
3 M. Pavlič, *Period. Biol.*, 73 (1971) 69.
4 S. Hestrin, *J. Biol. Chem.*, 180 (1949) 249.
5 H. T. S. Britten and R. A. Robinson, in H. M. Rauen, *Biochemisches Taschenbuch*, Springer Verlag, Berlin, 1956, pp. 649, 651, 654.
6 J. G. Kirkwood, *Disc. Faraday Soc.*, 20 (1955) 78.
7 W. P. Jencks, *Annu. Rev. Biochem.*, 32 (1963) 639.
8 J. L. Webb, *Enzyme and Metabolic Inhibitors*, Vol. 1, Academic Press, New York and London, 1963, p. 649.
9 E. Usdin, in A. G. Karczmar, *Anticholinesterase agents*, Pergamon Press, Oxford, 1970, p. 46.
10 I. B. Wilson and F. Bergmann, *J. Biol. Chem.*, 185 (1950) 479.
11 E. Reiner and W. N. Aldridge, *Biochem. J.*, 105 (1967) 171.

12 M. Pavlič, *Proc. 1st Eur. Biophys. Congr., Baden near Vienna, 1971*, Vol. 1, Verlag der Wiener Medizinischen Akademie, 1971, p. 165.
13 F. Bergmann, R. Segal, A. Shimoni and M. Wurzel, *Biochem. J.*, 63 (1956) 684.
14 L. A. Mounter, H. C. Alexander, K. D. Tuck and Lien Tien H. Dien, *J. Biol. Chem.*, 226 (1957) 867.
15 W. Kallow, *Can. J. Physiol. Pharmacol.*, 142 (1964) 161.
16 E. I. C. Wang, *Biochim. Biophys. Acta*, 198 (1970) 236.
17 I. B. Wilson and F. Bergmann, *J. Biol. Chem.*, 186 (1950) 683.
18 F. Bergmann, S. Rimon and R. Segal, *Biochem. J.*, 68 (1958) 493.