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ON THE MECHANISM OF THE ACCELERATION OF METHANESULFONYLATION OF ACETYLCHOLINESTERASE WITH CATIONIC ACCELERATORS

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Summary

1. In order to elucidate some features of the mechanism of the acceleration of methanesulfonylation of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) with cationic accelerators, the methanesulfonylation of this enzyme by high concentrations of methanesulfonylfluoride, in the absence and presence of accelerators decamethonium and tetraethylammonium, was studied.

2. The results showed that the accelerator accelerates the reaction by electrostatically improving the binding between acetylcholinesterase and methanesulfonylfluoride without effecting the rate of the decomposition of the enzyme · inhibitor complex into the methanesulfonylated enzyme and product.

Introduction

It is known that some cationic competitive inhibitors of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) accelerate the sulfonylation of this enzyme with methanesulfonylfluoride [1]. Our recent work [2] suggested that the acceleration was electrostatic in nature: the cationic accelerator bound to the anionic site of the enzyme electrostatically effects the methanesulfonylation at the esteratic site. Nothing, however, is known about the detailed mechanism of this acceleration.

The sulfonylation of acetylcholinesterase with methanesulfonylfluoride proceeds presumably [3,4] according to the following scheme:

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_{+2}} E' + P_1 \qquad (1)$$

where E is the enzyme, more precisely: the esteratic site of the enzyme [5,6], I the inhibitor methanesulfonylfluoride, EI the complex between the enzyme and the inhibitor, E′ the methanesulfonylated enzyme; P_1 the product, and k_{+1},

k_{-1}, k_{+2}, rate constants. Under conditions of our experiments on the methanesulfonylation of acetylcholinesterase till now, the phenomenological course of the reaction was [3,5]

$$E + I \xrightarrow{k} E' + P_1 \tag{2}$$

where k is the second-order rate constant for the overall reaction. This constant is presumably [3,7,4,2]

$$k = \frac{k_{+1}\, k_{+2}}{k_{-1}} = \frac{k_{+2}}{K_i} \tag{3}$$

or, in terms of the corresponding thermodynamic activation quantities,

$$k = \frac{RT}{Nh} \exp[-(\Delta G^*_{+1} + \Delta G^*_{+2} - \Delta G^*_{-1})/RT] = \frac{RT}{Nh} \exp(-\Delta G^*/RT) \tag{4}$$

where R, T, N and h have their usual meaning; ΔG^*_{+1} is the free energy of activation of the enzyme · inhibitor complex formation, ΔG^*_{-1} the free energy of activation of the enzyme-inhibitor complex dissociation, ΔG^*_{+2} the free energy of activation of the decomposition of the complex into the methanesulfonylated enzyme and product, and ΔG^* the free energy of activation of the overall reaction (Fig. 1).

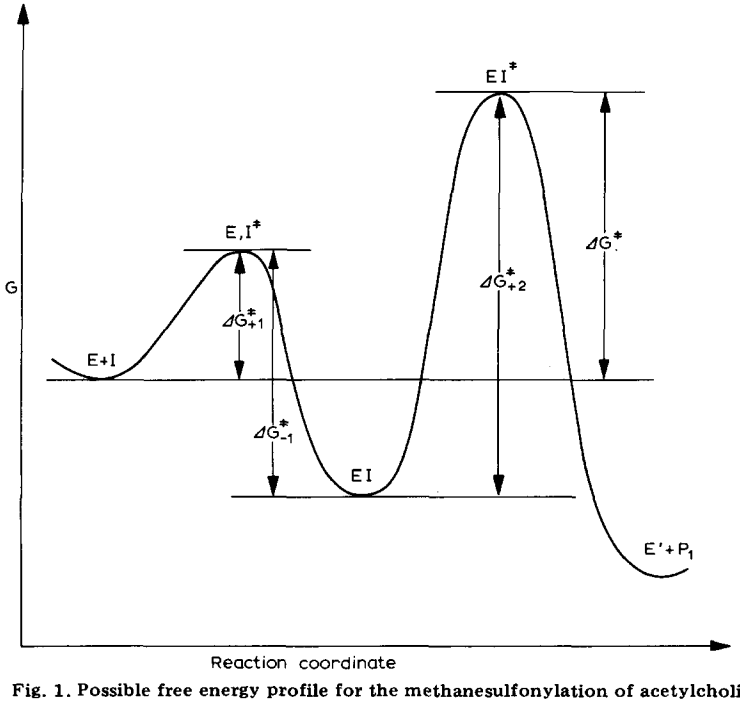

Fig. 1. Possible free energy profile for the methanesulfonylation of acetylcholinesterase. G, free energy; E, acetylcholinesterase; I, inhibitor (methanesulfonylfluoride); EI, enzyme · inhibitor complex; E′, methanesulfonylated enzyme; P_1, product; E, I*, activated complex for the formation of the enzyme · inhibitor complex; EI*, activated complex for the decomposition of the enzyme · inhibitor complex into the methanesulfonylated enzyme and product; ΔG^*_{+1}, free energy of activation of the enzyme · inhibitor complex formation; ΔG^*_{+2}, free energy of activation of the decomposition of the enzyme · inhibitor complex; ΔG^*_{-1}, free energy of activation of the enzyme · inhibitor complex dissociation; ΔG^*, free energy of activation of the overall reaction.

The second-order rate constant, k, was used in our work as the characteristic quantity for methanesulfonylation and, consequently, our findings up to the present are concerned with ΔG^*, the thermodynamic activation quantity of the overall reaction. The first question about the mechanism to be solved is: does the cationic accelerator increase the methanesulfonylation of acetylcholinesterase by increasing the binding of methanesulfonylfluoride to acetylcholinesterase or by increasing the decomposition of the enzyme-inhibitor complex into the methanesulfonylated enzyme and product? Or does it work in both ways?

The answer to these questions seems to be important since it would explain in some detail the acceleration of the methanesulfonylation of acetylcholinesterase with cationic accelerators and, possibly, it would throw some light on similar cases of acceleration with cations. In an attempt to get the answer we studied the methanesulfonylation of acetylcholinesterase with relatively high concentrations of methanesulfonylfluoride, in the presence and absence of accelerators.

Methods and Materials

The rate of the sulfonylation of acetylcholinesterase with methanesulfonylfluoride derived from Eqn. 1 is [4]

$$\ln \frac{[E]}{[E]_0} = -\frac{k_{+2}\, t}{1 + k_{+1}/[I]} = k_{app} t \tag{5}$$

and thus

$$\frac{1}{k_{app}} = \frac{1}{k_{+2}} + \frac{K_i}{k_{+2}} \cdot \frac{1}{[I]} \tag{6}$$

where [E] is the concentration of the uninhibited enzyme, $[E]_0$ the total concentration of the enzyme, [I] the concentration of methanesulfonylfluoride, t the time of methanesulfonylation, and k_{app} the apparent pseudo first-order rate constant for a given concentration of methanesulfonylfluoride.

In experimental conditions where the concentration of methanesulfonylfluoride is high enough (cf. ref. 3) the rate of methanesulfonylation follows Eqn. 6 and a plot of $1/k_{app}$ against $1/[I]$ gives a straight line with the intersection $1/k_{+2}$ and the tangens K_i/k_{+2}. Thus, if experiments can be carried out with relatively high concentrations of methanesulfonylfluoride, more precisely if the concentrations of methanesulfonylfluoride are not much lower than K_i, then it is possible to determine k_{+2} and K_i. The methanesulfonylation under these conditions, with and without added accelerators, would make it possible to determine the effect of an accelerator on individual steps of the methanesulfonylation.

We followed the inhibition of acetylcholinesterase with methanesulfonylfluoride in a relatively high concentration, with added accelerators decamethonium or tetraethylammonium and without them. The concentrations of methanesulfonylfluoride which turned out in a separate experiment to be still suitable for experimental work ranged up to about 20 mM; at these concentrations, the solubility of methanesulfonylfluoride was still adequate and the

half-times for methanesulfonylation were not too low.

The enzyme was incubated with a high excess of methanesulfonylfluoride for various times, with and without added decamethonium or tetraethylammonium; the remaining enzyme activity was then determined after extensive dilution. The apparent first-order rate constant was calculated from the time dependence of methanesulfonylation, according to Eqn. 5. Six different concentrations of methanesulfonylfluoride were always used in order to determine six first-order rate constants. Then the reciprocals of the first-order rate constants were plotted against the reciprocals of methanesulfonylfluoride concentrations, in accordance with Eqn. 6.

A typical run was as follows (cf. refs. 5 and 8). 100 μl of incubation solution, with the concentration of methanesulfonylfluoride ranging from 2.5 to 20 mM, was added to 10 μl of enzyme stock solution. After various times of incubation, from 10 to 60 s, 10 μl of the mixture was withdrawn by means of an Eppendorf pipette 3130 and introduced into 15.0 ml of the reaction mixture for the determination of acetylcholinesterase activity. Then, the enzyme activity was determined according to the method of Ellman et al. [9], using spectrophotometer Opton PM 4 with the corresponding device for automatic changing of tubes and a recorder. In experiments with accelerators, their concentrations were near saturation (cf. refs. 8 and 10); the concentration of decamethonium was 0.1 mM and that of tetraethylammonium 5 mM. Since the methanesulfonylation in the presence of decamethonium or tetraethylammonium is about 30 times faster than without the accelerator, the concentrations of methanesulfonylfluoride in those experiments were lowered correspondingly.

The enzyme used was acetylcholinesterase, Worthington, ECH 36E653, 1000 units/mg. The stock solution of the enzyme contained 1 mg of the preparation in 1 ml of the buffer solution according to Britten and Robinson [11]; NaCl was added to the buffer solution up to a total ionic strength of 0.2. Methanesulfonylfluoride was of Eastman Organic Chemicals. The stock solutions of this inhibitor were prepared in spectroquality acetone and the concentrations of methanesulfonylfluoride ranged from 0.5 to 4 M. In order to avoid a source of errors by acetone, the concentration of this substance in incubation solutions was kept constant. In experiments with accelerators, the methanesulfonylfluoride stock solutions were 0.075—0.2 M. Incubation solutions were always prepared immediately before each experiment. The buffer solution for the incubation was the buffer solution of Britten and Robinson [11] with added NaCl. The buffer solution of Britten and Robinson [11] had a pH of 8.3 which is approximately the optimum for the methanesulfonylation of acetylcholinesterase (cf. ref. 5). All experiments were carried out in a thermostat at 25°C.

Results

Our experimental results are summarized in Figs. 2—4.

From these figures and by means of Eqn. 6 the values of K_i and k_{+2} for the methanesulfonylation under various experimental conditions were obtained. The corresponding thermodynamic quantities, ΔG°_{EI}, the standard free energy change of the enzyme · inhibitor complex formation, and ΔG^{*}_{+2}, the free energy of activation of the decomposition of the complex, were calculated by means

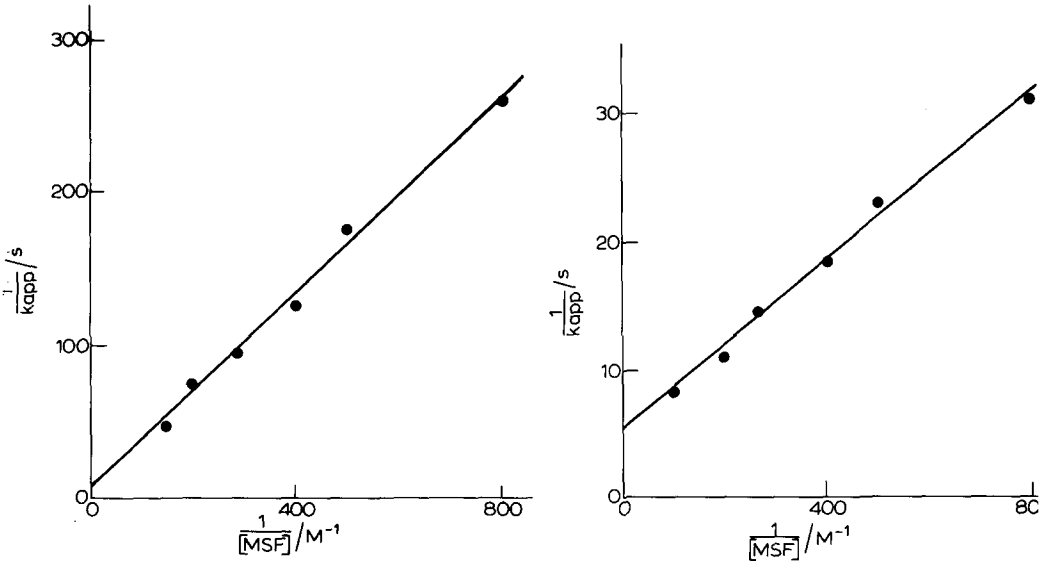

Fig. 2. Dependence of the first-order rate constants for the methanesulfonylation of acetylcholinesterase, k_{app}, on the concentration of methanesulfonylfluoride (MSF) for the reaction in the absence of accelerator. The straight line was obtained by the method of least squares. Three such diagrams were made out of three analogous experiments.

Fig. 3. Dependence of the first-order rate constants for the methanesulfonylation of acetylcholinesterase, k_{app}, on the concentration of methanesulfonylfluoride (MSF) for the reaction with added accelerator decamethonium (0.1 mM). The straight line was obtained by the method of least squares. Three such diagrams were made out of three analogous experiments.

of the usual thermodynamic equations. These kinetic and thermodynamic values, together with analogous ones from our earlier work [8,10], are presented in Table I.

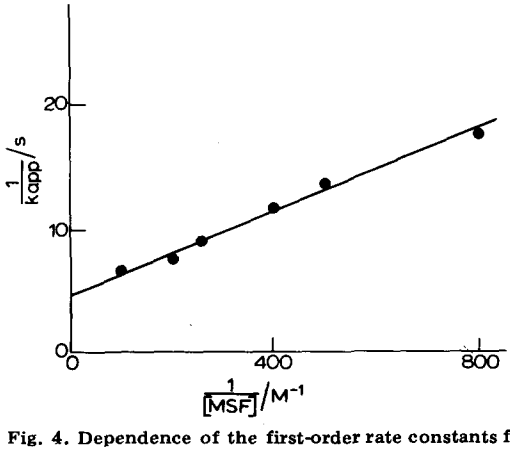

Fig. 4. Dependence of the first-order rate constants for the methanesulfonylation of acetylcholinesterase k_{app}, on the concentration of methanesulfonylfluoride (MSF) for the reaction with added accelerato tetraethylammonium (5 mM). The straight line was obtained by the method of least squares. Three suc diagrams were made out of three analogous experiments.

TABLE I

KINETIC AND THERMODYNAMIC DATA FOR THE METHANESULFONYLATION OF ACETYLCHOLINESTERASE UNDER VARIOUS EXPERIMENTAL CONDITIONS

Data apply to the reaction $E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_{+2}} E' + P_1$ · E, acetylcholinesterase; I, inhibitor (methanesulfonylfluoride); EI, enzyme · inhibitor complex; E', methanesulfonylated enzyme; P_1, product; k_{+1}, k_{-1}, k_{+2}, rate constants; MSF, methanesulfonylfluoride; C-10, decamethonium; TEA, tetraetylammonium; k, rate constant for the overall reaction; $\Delta G^{\ddagger}$, free energy of activation of the overall reaction; K_i, enzyme · inhibitor complex dissociation constant; ΔG°_{EI}, standard free energy change of the enzyme · inhibitor complex formation; k_{+2}, rate constant for the decomposition of the enzyme · inhibitor complex into the methanesulfonylated enzyme and the product; $\Delta G^{\ddagger}_{+2}$, free energy of activation of the decomposition of enzyme · inhibitor complex. Each value in the table is the average from three determinations. The values from different determinations never differed more than 10%.

Experiment	k ($M^{-1} \cdot s^{-1}$)	$\Delta G^{\ddagger}$ (kcal/mol)	K_i (M)	ΔG°_{EI} (kcal/mol)	k_{+2} (s^{-1})	$\Delta G^{\ddagger}_{+2}$ (kcal/mol)
MSF	3.1 *	16.8 *	0.058	−1.68	0.185	18.4
MSF + C-10	88 **	14.7 **	0.0060	−3.03	0.180	18.5
MSF + TEA	85 *	14.7 *	0.00315	−3.41	0.200	18.4

* From ref. 8.

** From ref. 10.

Discussion

It follows immediately from Fig. 2 that a complex of enzyme and methanesulfonylfluoride is formed during the methanesulfonylation and that the reaction, indeed, proceeds according to Eqn. 1. If it were not so and if the reaction proceeded according to Eqn. 2 the straight line in the diagram would go through the origin of the coordinate system, since in this case k_{app} is necessarily $k_{app} = k\,[I]$. The complex formed, however, is weak. Its dissociation constant, K_i, is 0.058 M and the corresponding standard free energy change of the complex formation, ΔG°_{EI}, is −1.67 kcal/mol (Table I, cf. refs. 2 and 4). Another property of this complex can be deduced at this stage: K_i for this complex is a true dissociation constant and not a Michaelis-Menten kind of constant, since, on the one hand, k_{+2} is low and, on the other hand, k_{+1} may safely be assumed as being high (cf. ref. 12).

An important deduction can be made directly from Figs. 2—4 and Table I: the presence of both typical accelerators decamethonium and tetraethylammonium practically does not change k_{+2} but it does profoundly change K_i. In other words the accelerator accelerates the methanesulfonylation of acetylcholinesterase by improving the binding between acetylcholinesterase and methanesulfonylfluoride without effecting the rate of the decomposition of the enzyme-inhibitor complex into the methanesulfonylated enzyme and product.

It should be noted here that the values for k in the presence of decamethonium and tetraethylammonium which can be obtained from the described experiments (cf. Table I) by means of the equation $k = k_{+2}/K_i$ are lower than the corresponding values reported earlier (cf. Table I and refs. 8 and 10). This is in agreement with the above finding that the acceleration is due to an

improved binding since in this case the acceleration must be lower at higher concentration of methanesulfonylfluoride.

In terms of thermodynamics, the accelerator can improve the binding in two different ways: by increasing the free energy level of the reactants acetylcholinesterase and methanesulfonylfluoride or by decreasing the free energy level of the complex between acetylcholinesterase and methanesulfonylfluoride. The two possible ways are illustrated in Fig. 5. There can be, of course, a combination of the ways.

Using the thermodynamic data from Table I and assuming for the free energy of activation of the complex formation a value of 5 kcal/mol (cf. refs. 1 and 2), a quantitative free energy profile for the methanesulfonylation of acetylcholinesterase has been constructed and is shown in Fig. 6 (full line). Under the assumption that the accelerator increases the free energy level of the reactants, probably of the enzyme, the quantitative free energy profile for the methanesulfonylation in the presence of the accelerator decamethonium or tetraethylammonium is as shown by the dotted line in Fig. 6.

Summarizing, the mechanism of the acceleration of methanesulfonylation of acetylcholinesterase by cationic accelerators consists in an electrostatically improved binding between acetylcholinesterase and methanesulfonylfluoride. However, the molecular events of this mechanism remain to be elucidated.

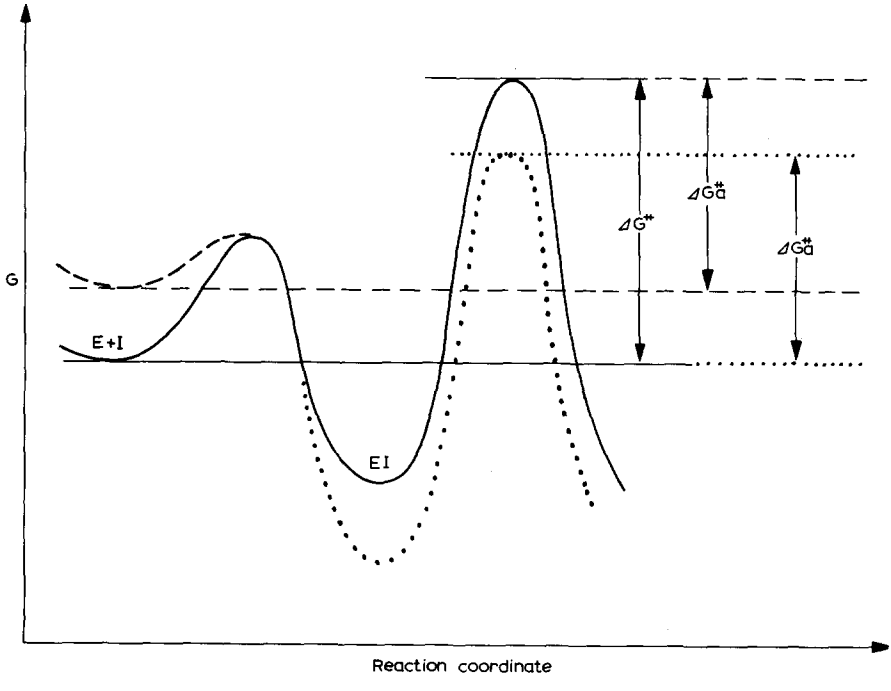

Fig. 5. Possible free energy profile for the methanesulfonylation of acetylcholinesterase in the absence (full line) and presence (interrupted and dotted lines) of an accelerator. G, free energy; E, acetylcholinesterase; I, inhibitor (methanesulfonylfluoride); EI, enzyme · inhibitor complex: ΔG^*, free energy of activation of the overall reaction in the absence of accelerator; ΔG_a^*, free energy of activation of the overall reaction in the presence of accelerator. For details see text.

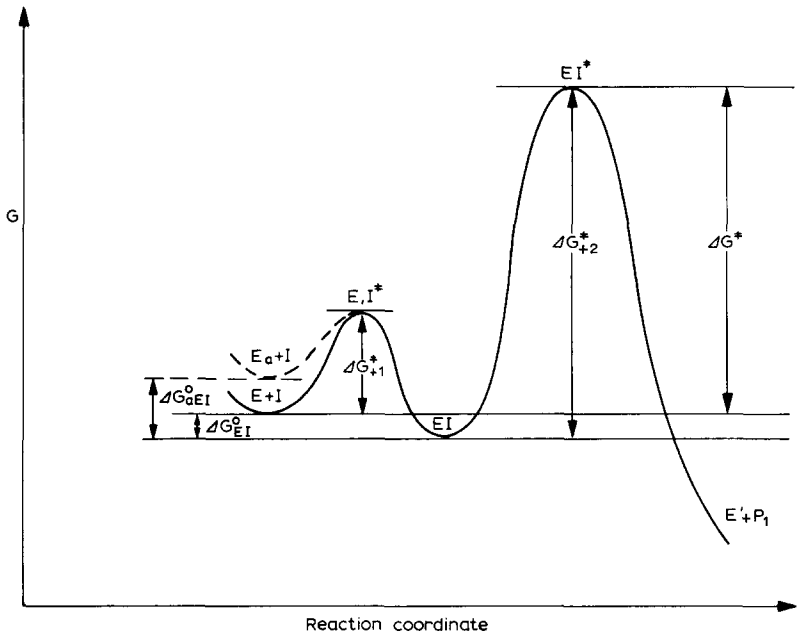

Fig. 6. Quantitative free energy profile for the methanesulfonylation of acetylcholinesterase in the absence (full line) and presence (interrupted line) of the accelerator decamethonium or tetraethylammonium. G, free energy; E, acetylcholinesterase; I, inhibitor (methanesulfonylfluoride); EI, enzyme · inhibitor complex; E', methanesulfonylated enzyme; P_1, product; E_a, enzyme raised by the accelerator to a higher free energy level; E, I*, activated complex for the formation of the enzyme · inhibitor complex; EI*, activated complex for the decomposition of the enzyme · inhibitor complex into methanesulfonylated enzyme and product; ΔG^*_{+1}, free energy of activation of the enzyme · inhibitor complex formation; ΔG^*_{+2}, free energy of activation of the decomposition of the enzyme · inhibitor complex; ΔG^*, free energy of activation of the overall reaction; ΔG°_{EI}, standard free energy change of the enzyme · inhibitor complex formation in the absence of accelerator; $\Delta G^\circ_{a,EI}$, standard free energy change of the enzyme · inhibitor complex formation in the presence of accelerator. For explanation see text.

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