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The inhibition of butyrylcholinesterase by methanesulfonyl fluoride*

Methanesulfonyl fluoride is an irreversible inhibitor of cholinesterases[1]. Many other methanesulfonates inhibit acetylcholinesterase[2-5]. Some experiments[2,3] have indicated that the group in acetylcholinesterase that is sulfonylated is the same group which is acetylated during the normal enzymic activity. We investigated the reaction which had not yet been studied in detail, the reaction between methanesulfonyl fluoride and butyrylcholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8). Methanesulfonyl fluoride was chosen because of its simple structure and because of the acceleration connected with the sulfonylation of acetylcholinesterase by methanesulfonyl fluoride[6]. In investigating the reaction much interest was directed to the binding site on butyrylcholinesterase.

The rate of inhibition was followed by incubating 10 μl of the enzyme stock solution with 100 μl of the incubation solution of methanesulfonyl fluoride in final concentrations of 5–50 mM and by withdrawing, at time intervals of 1–10 min, samples for the assay[7] of enzyme activity. The enzyme–inhibitor solutions were diluted 100 times when added to assay solutions. The temperature was 25°. The equations and the analysis applied were previously[2] described for the inhibition of acetylcholinesterase by methanesulfonates.

In order to get information about the nature of binding by means of the energy of activation, experiments were also performed at 15°.

The reactivation of the inhibited butyrylcholinesterase was checked by first inhibiting the enzyme to about 85% and diluting the solution 50 times with reactivation solution containing buffer alone and buffer with reactivators (*cf.* ref. 3), and then, at time intervals of 2, 4, 6, 24 and 48 h, by withdrawing samples and analysing them on the activity of butyrylcholinesterase.

The site of sulfonylation was investigated by means of the inhibitors (+)-tubocurarine and eserine. Experiments with (+)-tubocurarine were carried out in the same way as inhibition experiments with methanesulfonyl fluoride alone except that, besides methanesulfonyl fluoride, (+)-tubocurarine was added to incubation solutions. In experiments with eserine, first this inhibitor was added to the enzyme for half an hour and then methanesulfonyl fluoride. The solution was dialysed for 48 h against tap water and finally assayed on the enzyme activity.

The acceleration of the reaction between butyrylcholinesterase and methanesulfonyl fluoride by tetraethylammonium, found previously by KITZ AND WILSON[6] for acetylcholinesterase, was tested by using incubation solutions with added tetraethylammonium.

The buffer used in all experiments was 0.1 M phosphate buffer (pH at 25° 7.0) with 0.01% gelatin. Butyrylcholinesterase preparation was prepared from horse plasma[8]; cakes of the 5th stage were used. The specific activity was about 2 units/mg of protein. The enzyme stock solution contained 100 mg of preparation per 1 ml buffer. The concentration of active centers in the stock solution, as estimated from the catalytic center activity[9] and the activity of the stock solution, was about 2 μM.

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The enzymic hydrolysis of acetylcholine followed the Michaelis–Menten equation over the substrate concentration range of 0.5–5 mM. The relation between the amount of the enzyme and the rate of hydrolysis of acetylcholine was slightly nonlinear; the assay readings were corrected accordingly. Methanesulfonyl fluoride stock solution was 1 M solution of methanesulfonyl fluoride, reagent grade, in spectro-quality acetone. Methanesulfonyl fluoride incubation solutions were prepared by mixing aliquots of the stock solution with the buffer. To avoid possible errors due to acetone, its concentration in incubation solutions was kept constant (5%). Incubation solutions were always prepared 1 min before each experiment. Incubation solutions with added (+)-tubocurarine or tetraethylammonium were prepared in the same way, with buffer containing (+)-tubocurarine or tetraethylammonium.

The time dependence of the inhibition[2] of butyrylcholinesterase by methanesulfonyl fluoride clearly showed that the inhibition is practically irreversible and progressive with time in accordance with equation $\ln(\varepsilon)/E_0 = -k_3't/(1 + K_i/[I])$. There is no evidence of possible multiple reversible butyrylcholinesterases as MAIN[10] has shown by means of the inhibition by organophosphates of a preparation of serum cholinesterase. From the dependence of k_{app}, the pseudo-first-order rate constant, on the concentration of methanesulfonyl fluoride it can be concluded that no or at most a weak reversible complex between butyrylcholinesterase and methanesulfonyl fluoride might be formed (see ref. 2). In the corresponding diagrams, the extrapolation of the lines towards the origin is small (but see ref. 2) so that this conclusion is rather safe. k_3', the second-order rate constant, for the inhibition at 25° for the condition $K_i \gg [I]$ is 0.12 l·mole^{-1}·sec^{-1}. k_3' for the inhibition at 15° is 0.047 l·mole^{-1}·sec^{-1}.

Experiments at different temperatures made it possible to calculate the energy of activation for the sulfonylation of butyrylcholinesterase by methanesulfonyl fluoride. It was calculated by means of the equation $A = [RT_2T_1/(T_2 - T_1)] \times \ln k_2/k_1$ with $k_2 = k_3'$ at 298°K and $k_1 = k_3'$ at 288°K. The value obtained is $A = 16$ kcal·mole^{-1}. This considerable energy of activation speaks for a chemical reaction between butyrylcholinesterase and methanesulfonyl fluoride. This is in agreement with implications made by various authors[1-5] for the reaction between acetylcholinesterase and methanesulfonates.

The reactivators: buffer, 1 M hydroxylamine, 0.1 M choline and 0.02 M pyridine-2-aldoxime methiodide did not increase the activity of the 85% inhibited enzyme, even after 48 h of incubation. Thus, the inhibition of buryrylcholinesterase by methanesulfonyl fluoride is in fact practically irreversible.

(+)-Tubocurarine did not have any effect on the inhibition of butyrylcholinesterase by methanesulfonyl fluoride. The curves obtained from experiments where (+)-tubocurarine in final concentrations of 0.01, 0.1 and 1 mM was added to incubation solutions of methanesulfonyl fluoride were almost identical with the curves where no (+)-tubocurarine was added. This indicates that methanesulfonyl fluoride, regardless of whether or not it binds to the active surface of butyrylcholinesterase, does not bind to the anionic site of the enzyme. The question of an expected steric hindering of the binding of methanesulfonyl fluoride to the esteratic site remains moot at this stage. The preincubation of butyrylcholinesterase with 1 and 2 μM eserine, respectively, completely protected the enzyme from the inhibition by methanesulfonyl fluoride. The activity of the preincubated enzyme after dialysis was the same as the activity of the control (enzyme alone), whereas the activity of the un-

protected butyrylcholinesterase was 10% of the control when 10 mM methanesulfonyl fluoride was used. The protective effect of eserine shows that the active surface of butyrylcholinesterase must be involved in the inhibition. This finding and the failure of (+)-tubocurarine to inhibit the sulfonylation strongly suggest that it is the esteratic site of butyrylcholinesterase which reacts with methanesulfonyl fluoride.

Tetraethylammonium, which was shown by KITZ AND WILSON[6] to accelerate the reaction of acetylcholinesterase and methanesulfonyl fluoride up to about 3300%, added to incubation solutions in concentrations from 1 to 50 mM, accelerated the inhibition of butyrylcholinesterase by methanesulfonyl fluoride up to a factor of around 3. Although the concentration of tetraethylammonium which caused a 3-fold acceleration (50 mM) is very high, the acceleration is probably not due to the increased ionic strength; control experiments with the same concentrations of KCl and NH_4Cl did not accelerate the sulfonylation.

Comparing the inhibition of butyrylcholinesterase by methanesulfonyl fluoride with that of acetylcholinesterase[2,4,6] two important differences are noticed: firstly, the inhibition of butyrylcholinesterase is about 20 times slower and, secondly, the acceleration of the inhibition by tetraethylammonium is around 10 times lower in the case of butyrylcholinesterase. Since it is very probably the esteratic site of both cholinesterases which is sulfonylated, these differences point to dissimilarities not only in the active surface in general but also in the esteratic site of both enzymes. Whether or not this has something to do with the minor importance of an acidic group in the esteratic site of butyrylcholinesterase as suggested by the studies of AUGUSTINSSON AND ISACHSEN[11] and other similar questions require further investigations.

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1 D. K. MYERS AND A. KEMP, *Nature*, 173 (1954) 33.
2 R. KITZ AND I. B. WILSON, *J. Biol. Chem.*, 237 (1962) 3245.
3 J. ALEXANDER, I. B. WILSON AND R. KITZ, *J. Biol. Chem.*, 238 (1963) 741.
4 D. E. FAHRNEY AND A. M. GOLD, *J. Am. Chem. Soc.*, 85 (1963) 997.
5 J. F. RYAN, S. GINSBURG AND R. J. KITZ, *J. Biochem. Pharmacol.*, 18 (1969) 269.
6 R. KITZ AND I. B. WILSON, *J. Biol. Chem.*, 238 (1963) 745.
7 S. HESTRIN, *J. Biol. Chem.*, 180 (1949) 249.
8 F. STRELITZ, *Biochem. J.*, 38 (1944) 86.
9 W. K. BERRY, *Biochem. J.*, 49 (1951) 614.
10 A. R. MAIN, *J. Biol. Chem.*, 244 (1969) 829.
11 K. B. AUGUSTINSSON AND T. ISACHSEN, *Acta Chem. Scand.*, 11 (1957) 750.

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