Acceleration of the Rate of Reaction of Methanesulfonyle fluoride and Acetylcholinesterase by Substituted Ammonium Ions*

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(Received for publication, July 23, 1962)

Methanesulfonyle fluoride is an oxydiaphoric (acid-transferring) inhibitor of acetylcholinesterase. It reacts with the enzyme to produce a methanesulfonyle enzyme derivative. The group in the enzyme that is sulfonylated lies near the anionic site and is possibly the same group that is acetylated during the normal catalytic activity of the enzyme, i.e. the basic group of the esteratic site (1, 2).

Other anhydrides of methanesulfonic acid—in particular, those containing suitable quaternary ammonium functions—are also oxydiaphoric inhibitors and produce the same methanesulfonyle enzyme. The rate of reaction of the enzyme with these inhibitors is slowed by simple inhibitors such as tetramethylammonium ion (1, 2). In the case of methanesulfonyle fluoride, the situation is quite different, possibly in part because the small size of the leaving group—fluorine—may allow the reaction to proceed even if the anionic site is occupied by tetramethylammonium ion. The situation is far more complicated, however, because the experimental fact is that the rate of reaction is increased by a factor of 6. With tetraethylammonium ion, the rate of reaction is increased by a factor of more than 30.

In this paper we describe the effect of a number of substituted ammonium ions on the rate of this reaction.

EXPERIMENTAL PROCEDURE

The enzyme was obtained from the main electrical organ of Electrophorus electricus and purified by column chromatography. The preparation used in this work had a specific activity of 230 \( \mu \)moles of acetylcholine hydrolyzed per mg of protein per hour. The reaction medium was 2.7 \( \times \) 10\(^{-4}\) M acetylcholine in 0.1 M NaCl, 0.02 M MgCl\(_2\), and 0.02 M sodium phosphate, pH 7.0, at 25°. The acetylcholine concentration was measured colorimetrically by the hydroxamic acid method (3). Other anhydrides of methanesulfonic acid—in particular, those containing suitable quaternary ammonium functions—are also oxydiaphoric inhibitors and produce the same methanesulfonyle enzyme. The rate of reaction of the enzyme with these inhibitors is slowed by simple inhibitors such as tetramethylammonium ion (1, 2). In the case of methanesulfonyle fluoride, the situation is quite different, possibly in part because the small size of the leaving group—fluorine—may allow the reaction to proceed even if the anionic site is occupied by tetramethylammonium ion. The situation is far more complicated, however, because the experimental fact is that the rate of reaction is increased by a factor of 6. With tetraethylammonium ion, the rate of reaction is increased by a factor of more than 30.

In this paper we describe the effect of a number of substituted ammonium ions on the rate of this reaction.

RESULTS

The ability to accelerate the rate of reaction of methanesulfonyle fluoride with acetylcholinesterase is widespread among simple substituted ammonium ions; see Tables I and II. Both the normal and accelerated reactions follow second order kinetics. The pseudo first order equation is

\[
2.3 \log \left( \frac{E'}{E_0} \right) = -k(t)
\]

where the concentration of inhibitor \((I)\) is very much larger than the concentration of enzyme \((E)\). For the normal reaction \(k = 2.5\) liters per mole per second. Typical examples of acceleration are shown in Figs. 2 and 3.

The maximal acceleration yet attained is with tetraethylammonium ion, which increased the specific rate constant by a factor of more than 30. The question arises whether a sulfonyl enzyme is formed under these circumstances and, if so, whether it is the same as the sulfonyl enzyme formed without acceleration. This question was investigated by comparing the rates of reactivation (2) of enzyme samples inhibited in the normal and in the accelerated reactions. The reactivator was phenyl 3-pyridyl \(\text{anti-}\)ketoxime methiodide at 7.5 \(\times\) 10\(^{-3}\) M, pH 8.0, 25.0°. The accelerator was 3.5 \(\times\) 10\(^{-4}\) M tetraethylammonium ion, which produces an acceleration of 15-fold. The rates of reactivation were the same; half-times were 6.8 and 7.3 hours for enzyme inhibited by the normal reaction and 7.3 and 7.6 hours for enzyme inhibited in the accelerated reaction. We conclude, therefore, that the inhibited enzyme is the same in both cases; i.e. the same methanesulfonyle enzyme is formed.

The next question is whether the phenomenon of acceleration arises from binding at the anionic site or at some other site, or sites, in the enzyme molecule. It arises from binding at the anionic site and if we assume that the sites are independent, the following scheme is appropriate,

\[
E + I \xrightarrow{k_3} E'
E + A \xrightarrow{K_A} E\cdot A
E\cdot A + I \xrightarrow{k_4} E'
\]

* This work was supported by the Division of Research Grants and Fellowships of the National Institutes of Health, Grant B-573 (C13), by United States Public Health Service and Research Career Award GM-R3-13, 012, and by National Science Foundation Grant 18926.
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Acceleration of rate of reaction of methanesulfonyl fluoride with acetylcholinesterase by ammonium ions

The second order rate constant for the normal reaction is 2.5 liters per mole per second at 25°C, pH 7.0. α is the maximal number of times faster that the reaction proceeds in the presence of the indicated substituted ammonium ion. $K_A$ is the dissociation constant for the accelerator which fits the data to the equation in the text. $K_I$ is the dissociation constant for the ion obtained from the inhibition of the hydrolysis of acetylcholine (4, 5).

<table>
<thead>
<tr>
<th>Ion</th>
<th>Formula</th>
<th>α</th>
<th>$K_A$</th>
<th>$K_I$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Dimethylammonium</td>
<td>(CH₃)₂NH⁺</td>
<td>9.5</td>
<td>$3.2 \times 10^{-3}$</td>
<td>$2.6 \times 10^{-2}$</td>
</tr>
<tr>
<td>2. Trimethylammonium</td>
<td>(CH₃)₃NH⁺</td>
<td>7.5</td>
<td>$4.2 \times 10^{-3}$</td>
<td>$4.8 \times 10^{-3}$</td>
</tr>
<tr>
<td>3. Tetramethylammonium</td>
<td>(CH₃)₄N⁺</td>
<td>6.0</td>
<td>$7 \times 10^{-4}$</td>
<td>$1.2 \times 10^{-3}$</td>
</tr>
<tr>
<td>4. Tetraethylammonium</td>
<td>(C₂H₅)₂N⁺</td>
<td>33.0</td>
<td>$5 \times 10^{-4}$</td>
<td>$2.5 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

where $I$ is methanesulfonyl fluoride, $A$ is the accelerator, and $E'$ is the methanesulfonyl enzyme. This scheme is also appropriate for a site other than the anionic site, but if more than one site is involved, the scheme will become more complicated, and we should have to know how the acceleration depends upon the number of sites occupied. The rate constant derived from the scheme is

$$k = \frac{k_0 \left(1 + \frac{\alpha(A)}{K_A}\right)}{1 + \frac{(A)}{K_A}}$$

where $\alpha = k_A/k_b$. In most instances the value of $k$ was near its maximum at the highest concentration of accelerator used, so that the approximate value of $\alpha$ was directly determined and only $K_A$ remained as an adjustable constant. The values of $K_A$ required to fit the data with the equation are given in Table I. In some cases (dimethylammonium ion), the points fit the calculated curve very well, but with tetraethylammonium ion (Fig. 1), the fit was not very good.

In the four cases in which the studies were extensive enough to assign values for $K_A$, they are approximately the same as the dissociation constants for these ions, $K_D$, obtained from the inhibition of acetylcholine hydrolysis. This suggests that the anionic site may be involved in acceleration, but the argument is weak because none of these ions has any structural feature that involves a high degree of specificity for the anionic site.

A number of compounds are listed in Table II with the effect obtained at the highest concentration used. Compound 10, 3-hydroxyphenyltrimethylammonium ion, is a very potent reversible competitive inhibitor of acetylcholine hydrolysis and has structural features suggesting a high degree of specificity for the anionic site. For example, the 2- and 4- derivatives, and also the unsubstituted ion, are much weaker inhibitors. Therefore, this ion can reasonably be assumed to act only at the anionic site at low concentrations. This ion inhibits both the normal and accelerated reactions (Fig. 2). Inhibition of the normal reaction at several concentrations of 3-hydroxyphenyltrimethylammonium ion yielded $K_I = 3 \times 10^{-7}$ M, the same as determined from the inhibition of acetylcholine hydrolysis.

Tetra-n-butylammonium ion is also an effective inhibitor of the reaction, but the slight inhibition observed with tetra-n-propylammonium ion is not certain because of our low precision. The slight acceleration observed with phenyltrimethylammonium ion is probably real. This compound decidedly inhibits the acceleration produced by tetraethylammonium ion (Fig. 3), which indicates that, although any effect by itself is at best marginal, it occupies the acceleration sites.

**DISCUSSION**

It is important to know whether the anionic site is the accelerator site, but it is difficult to tell. The concentrations required to produce acceleration are such that the anionic sites will be populated, even if they are not involved in acceleration. If there is a separate acceleration site, binding at the anionic site possibly will sterically inhibit the sulfonation. Even if this were so, we should still realize a net acceleration if the value of $k_A$ is at least several times greater than $k_b$. In this circumstance, however, we should have to account for the fact that the reactions with other anhydrides of methanesulfonic acid are not accelerated, but inhibited (1). A possible explanation might be that in these cases the anhydride itself occupies the accelerator sites but is not an accelerator. It is simpler to suppose temporarily that because of the small size of the leaving group the sulfonation reaction with methanesulfonyl fluoride is not inhibited by some of the ions bound at the anionic site. At first sight this might appear to be sterically impossible if the site of sulfonation is the esteratic site, especially in the case of tetraethylammonium ion, but there are conformations of this ion that would allow a close approach of the fluorine atom. Since we are not certain that the esteratic site is sulfonylated, but only that the
**Table II**

**Effect of substituted ammonium ions on rate of reaction of methanesulfonyl fluoride with acetylcholinesterase**

The concentration given is the highest used. $k_3$ is the second order rate constant for the normal reaction, and $k$ is the rate constant obtained in the presence of the ion. $K_I$ is the dissociation constant of the ion obtained from the inhibition of acetylcholine hydrolysis (4, 5).

<table>
<thead>
<tr>
<th>Ion</th>
<th>Formula</th>
<th>Concentration</th>
<th>$K_I$</th>
<th>$k/k_3$*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5. Triethylammonium</td>
<td>$(C_3H_7)_3NH^+$</td>
<td>$4 \times 10^{-3}$</td>
<td>$8 \times 10^{-4}$†</td>
<td>15.5</td>
</tr>
<tr>
<td>6. 1-Methylpyridinium</td>
<td>$N\text{-CH}_2^+$</td>
<td>$3.5 \times 10^{-3}$</td>
<td>$1.1 \times 10^{-4}$</td>
<td>2.1</td>
</tr>
<tr>
<td>7. Phenyltrimethylammonium</td>
<td>$N\text{(CH}_3)_3^+$</td>
<td>$7.5 \times 10^{-4}$</td>
<td>$5.3 \times 10^{-5}$</td>
<td>1.25</td>
</tr>
<tr>
<td>8. Tetra-n-propylammonium</td>
<td>$(C_4H_9)_4N^+$</td>
<td>$5 \times 10^{-3}$</td>
<td>$1 \times 10^{-4}$</td>
<td>0.90</td>
</tr>
<tr>
<td>9. Tetra-n-butyrammonium</td>
<td>$(C_6H_{15})_4N^+$</td>
<td>$3 \times 10^{-3}$</td>
<td>$2 \times 10^{-4}$</td>
<td>0.20</td>
</tr>
<tr>
<td>10. 3-Hydroxyphenyltrimethylammonium</td>
<td>$N\text{(CH}_3)_3^+$</td>
<td>$3.5 \times 10^{-9}$</td>
<td>$3 \times 10^{-7}$</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* If $k/k_3 > 1$, acceleration occurs; if $k/k_3 < 1$, inhibition occurs.
† Estimated.

**Fig. 2.** The effects of a reversible inhibitor and an accelerator on the rate of irreversible inhibition produced by reaction of methanesulfonyl fluoride with acetylcholinesterase. Curve 1, methanesulfonyl fluoride, $1.3 \times 10^{-4}$ M, $+ 3$-hydroxyphenyltrimethylammonium iodide, $1.2 \times 10^{-8}$ M; Curve 2, methanesulfonyl fluoride; Curve 3, methanesulfonyl fluoride $+ 3$-hydroxyphenyltrimethylammonium iodide $+$ tetraethylammonium iodide, $1.8 \times 10^{-4}$ M; Curve 4, methanesulfonyl fluoride $+$$+$ tetraethylammonium iodide.
site of sulfonylation is near the anionic site, this question may not arise.

Evidently there are many possibilities, and a reasonably certain conclusion cannot yet be made.

Even the significance of the phenomenon is uncertain, although it is tempting to speculate that it may explain why quaternary esters as substrates have higher maximal velocities than nonquaternary esters, but the accelerations obtained with the ammonium ions do not correlate with the rate at which the enzyme hydrolyzes esters containing these functions. There also remains the question whether this phenomenon is of any physiological significance.

Finally, what is the nature of the phenomenon itself? In some ways it brings to mind the unmasking of functional groups by denaturing agents and suggests that perhaps some considerable change in conformation occurs. All of these questions require further studies.

SUMMARY

Methanesulfonyl fluoride reacts with acetylcholinesterase to produce a methanesulfonyl enzyme. The sulfonation occurs near the anionic site, possibly at the esteratic site.

A number of substituted ammonium ions, notably tetraethylammonium ion, very greatly increase the rate of this reaction. The highest increase yet observed is 3300%. The resulting inhibited enzyme (methanesulfonyl enzyme) can be reactivated at the same rate as enzyme inhibited in the normal reaction. Some ammonium ions inhibit the normal reaction and also the accelerated reaction. The effect with 2 of the ions is slight, so that we could not conclude with reasonable certainty whether or not acceleration or inhibition occurs. One of these was tested and found to inhibit the accelerated reaction. The concentrations needed for acceleration are appropriate for binding at the anionic site, but no reasonably certain conclusion could be drawn concerning a site of acceleration.

REFERENCES

Acceleration of the Rate of Reaction of Methanesulfonyl Fluoride and Acetylcholinesterase by Substituted Ammonium Ions
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