The Mechanism of the Specificity of Trypsin Catalysis

III. ACTIVATION OF THE CATALYTIC SITE OF TRYPSIN BY ALKYLAMMONIUM IONS IN THE HYDROLYSIS OF ACETYLGLYCINE ETHYL ESTER*

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It has been found that alkylammonium ions competitively inhibit the catalysis by trypsin of the hydrolysis of benzoyl-L-arginine ethyl ester (3). The inhibition can be considered to be a result of competition for the specificity-determining site of trypsin between the positively charged side chain of the substrate and the similarly charged alkylammonium ion. On the other hand, trypsin has been found to catalyze the hydrolysis of acetylglycine ethyl ester, probably without much participation of the specificity-determining site (2). If the specificity-determining site and the catalytic site are topographically distinct, it might be expected that a small alkylammonium ion would compete ineffectively, if at all, with the hydrolysis of acetylglycine ethyl ester; instead, the small alkylammonium ion and the acetylglycine ethyl ester molecule might be accommodated at the respective sites in place of a normal substrate such as α-N-acetyl-L-lysine ethyl ester.

The binding of the alkylammonium ions to the specificity-determining site and interaction of acetylglycine ethyl ester with the catalytic site have been studied separately (2, 3). However, there is a possibility that functioning of one site may influence action of the other site, and it is the purpose of the present investigation to study the possible interaction between these two parts of trypsin. This is made possible by the dissection, as it were, of a normal substrate, α-N-acetyl-L-lysine ethyl ester, into two parts, one interacting with the specificity-determining site and the other with the catalytic site. By so doing it has been possible to vary the concentrations of these two components independently of each other and hence to study the interaction of each site under different conditions of the other site.

EXPERIMENTAL PROCEDURE

Substrate—AGE1 was synthesized according to the method described in the preceding paper (2). It was used after two distillations without further recrystallization (m.p. 47.5-48.0°C).

Inhibitors—The alkylamines used were all commercially available preparations of reagent grade. Methylamine was a 30 to 35% aqueous solution. Ethylamine was a 70% aqueous solution and an anhydrous preparation, b.p. 19-20°C. The aqueous solutions were used without further purification. Anhydrous ethylamine, 1-propylamine, b.p. 48-50°C, and 1-n-butylamine, b.p. 76-78°C, were redistilled. The stock solutions of alkylammonium ions were prepared by diluting these amines with distilled water to appropriate concentrations, where they were determined by titration with standard HCl and methyl red as indicator and then neutralized to pH 6.5 with HCl. In the identification of the reaction products in the enzyme study, the concentration of the amine was determined spectrophotometrically by converting to a 2,4,6-trinitrophenyl derivative with 2,4,6-trinitrobenzene-1-sulfonic acid, m.p. 179-180°C, according to the method of Satake et al. (4). Soybean trypsin inhibitor was purchased from Worthington Biochemical Corporation and was used without further purification.

Enzyme—Trypsin was a twice recrystallized, salt-free Worthington preparation (Lot 6102). The concentration of a stock solution was determined from the optical density reading at 280 μm by using ε1% = 14.4 (5) and 24,000 as molecular weight (6).

Kinetic Method—Initial rates of hydrolysis were determined potentiometrically (7) with a Radiometer pH-Stat (model TTR1a) and recorder (model SBR2) assembly and are expressed by the absolute rate v/|E|, in sec⁻¹, where v is the initial rate in moles per liter per second and |E| is the concentration of the total enzyme in a reaction mixture; or by relative rate, vᵣ = v/vᵣ₀, where vᵣ₀ is the initial rate observed in the absence of an alkylammonium ion. All experiments were carried out at 25°C and pH 6.6. This pH, which is lower than the range of the maximal activity of trypsin, was chosen to eliminate the possibility of transfer of the acetylglycyl group of the substrate to the alkylamine, producing a corresponding alkylamide instead of hydrolysis products, and also to minimize complication due to the hydroxide ion-catalyzed hydrolysis of AGE at a higher pH (2).

Enzyme concentrations were in the vicinity of 4 × 10⁻⁶ M in a reaction volume of 10 ml containing either 0.05 M CaCl₂ or 0.1 M KCl. The initial rate could be determined within an experimental error of approximately ±3%.

RESULTS

Effects of Alkylammonium Ions on Rate of AGE Hydrolysis—The presence of methyl-, ethyl-, or 1-propylammonium ion has been found to increase the initial rate of the trypsin-catalyzed hydrolysis of AGE at constant concentration of the substrate. Plots in Fig. 1, left and right, illustrate the effects of the alkylammonium ion concentrations on the relative initial rate, vᵣ, in...
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0.0 0.2 0.4 0.6 0.6

\[ \text{ALKYL AMMONIUM}^+, \text{M} \]

FIG. 1. Effects of methyl- (filled squares), ethyl- (open squares), 1-propyl- (open circles), and 1-n-butylammonium (filled circles) ions on the initial relative rate, \( v_r \), of the trypsin-catalyzed hydrolysis of AGE in 0.1 M KCl at pH 6.6 and 25° at AGE concentrations of 0.276 M, 0.376 M, 0.377 M, and 0.355 M, respectively. The initial rate in the absence of the alkylammonium ion is taken as unity in each case.

0.1 M KCl, with the rate in the absence of the alkylammonium ion taken as unity. Methylammonium ion (filled squares) at 0.85 M increases the rate 3.18-fold, and ethylammonium ion (open squares) at 0.34 M causes a 9.54-fold increase. 1-Propylammonium ion (open circles) is effective at lower concentrations in increasing the rate, but at higher concentrations the effect is reversed, resulting in a maximum at 0.024 M, where the relative rate is 2.37. Calcium ion has been known to have a favorable effect on the trypsin catalysis. Effects of ethylammonium ion and 1-propylammonium ion in 0.05 M CaCl\(_2\) are shown in Fig. 2 by open squares and open circles, respectively. In CaCl\(_2\) these ammonium ions are effective at lower concentrations than in KCl, but the extent of the increase is not larger. However, the complex effect of 1-propylammonium ion in KCl is replaced by a plain curve representing a saturation of the accelerating effect. Contrary to these homologues of smaller size, 1-n-butylammonium ion inhibits the AGE hydrolysis, as shown by the filled circles in Fig. 1 (right). This inhibition was further studied at a constant concentration (5 mM) of 1-n-butylammonium ion, while the concentration of AGE was changed from 0.2 M to 2 M. The Eadie plots of the results (filled circles) are presented in Fig. 8 together with the plots for the results obtained in the absence of the inhibitor (open circles).

Effects of Alkylammonium Ions on \( K_m \) of AGE Hydrolysis—In steady state enzyme kinetics, due observed increase of the rate can be the result of either a decreased apparent Michaelis-Menten constant, \( K_m \), or an increased apparent first order rate constant, \( k_a \), or a combined effect of both. To analyze the mechanism, Eadie plots (8) have been made at several different concentrations of ethylammonium ion (Fig. 3) and 1-propylammonium ion (Fig. 4). The \( K_m \) values as read from the slope of the plots are 0.72 M (0.79 \( \pm \) 0.14 M (3)) in the absence of the ammonium ion, and 0.80 M, 0.78 M, and 0.84 M with ethylammonium ion at concentrations of 0.048 M, 0.096 M, and 0.19 M, respectively. With 1-propylammonium ion at 0.0072 M and 0.040 M, the \( K_m \) values are 0.73 M and 0.92 M, respectively. It is apparent that \( K_m \) remains practically constant while the concentration of the alkylammonium ions is changed, and that these ions exert their accelerating effects solely through an increase in \( k_a \).

Effects of Ethylammonium Ion on pH Profile of Catalysis—The pH profile of \( k_2 \) of the AGE hydrolysis by trypsin in the absence of the alkylammonium ion has been found to be represented by a single group titration curve of \( pK \) 7.1 \( \pm \) 0.05, while \( K_m \) is constant between pH 6.0 and 7.0 (2). \( K_m \) of the hydrolysis in the presence of ethylammonium ion also does not change with pH, the value being 0.74 \( \pm \) 0.11 at pH 6.0 and 0.70 \( \pm \) 0.09 at pH 7.0. This constancy of \( K_m \) justifies the use of a single concentration of substrate in the study of the pH profile of \( k_a \). Also the binding of the alkylammonium ions is not affected by pH between pH

FIG. 3. Eadie plots of the trypsin-catalyzed hydrolysis of AGE in the presence and absence of ethylammonium ion in 0.1 M KCl at pH 6.6 and 25°.
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FIG. 4. Eadie plots of the trypsin-catalyzed hydrolysis of AGE in the presence and absence of 1-propylammonium ion in 0.1 M KCl at pH 6.6 and 25°.

FIG. 5. pH profile of the initial rate of the trypsin-catalyzed hydrolysis of AGE at 0.166 M in the presence of 0.160 M ethylammonium ion at 25°. The rate is in an arbitrary unit.

6.0 and 7.2. Therefore the pH profile obtained with 0.166 M AGE and 0.160 M ethylammonium ion at 25° (Fig. 5) can be considered as the pH profile of \( k_s \). These points can be fitted by a single group titration curve of pK 6.94 ± 0.04, which is shown in Fig. 5.

Identification of Reaction Product—The possibility of the transfer of an acetylglycyl group mediated by trypsin to the alkylammonium ion to form a corresponding alkylamide has been disproved by the following experiment. Approximately 1 μmole of trypsin and 3 mmoles of AGE were allowed to react in 10 ml of 0.1 M KCl solution at 25° and pH 6.6, in the presence of 72 μmoles of 1-propylammonium ion. At 0, 4, and 8 hours of reaction, aliquots of the reaction mixture were extracted, the enzyme was inactivated by soybean trypsin inhibitor, and the concentration of 1-propylamine was determined by the method of Satake et al. (4). More than 80 μmoles of AGE were hydrolyzed during this experiment, and the concentration of 1-propylamine was found unchanged within an experimental error of 3%.

Discussion

As shown by the example of 1-propylammonium ion, an alkylammonium ion is not acylated during the hydrolysis of AGE by trypsin. The increase in the rate caused by the presence of methyl-, ethyl-, or 1-propylammonium ion cannot be attributed to the direct participation of the alkylammonium ion in the reaction as acceptor for the acetylglycyl group, leading to the formation of a corresponding acetylglycyl alkylamide. The possibility that alkylamide formation is the cause of the increased rate is also incompatible with the fact that 1-n-butylammonium ion inhibits the reaction whereas smaller members of the homologues accelerate it. The absence of acyl transfer to the alkylammonium ion has also been established in the case of BAE hydrolysis by trypsin at pH 6.6 (3). Even at pH 7.6, transacylation to the \( \varepsilon \)-amino group of lysine was not observed (9). On the other hand, the alkylammonium ions have been found to inhibit the hydrolysis of BAE competitively (3). Based on these facts, the effects on the AGE hydrolysis may be considered as a result of the binding of the ions to the specificity-determining site of trypsin.

Consider two equilibria,

\[
E + A \rightleftharpoons K_s EA
\]

(1)

\[
ES + A \rightleftharpoons K_s ESA
\]

(2)

where an alkylammonium ion, \( A \), is bound to enzyme, \( E \), or to an enzyme-substrate complex, \( ES \), forming complexes \( EA \) or \( ESA \), respectively. Let us assume that the two equilibria have an identical dissociation constant, \( K_s \), and that the binding of \( A \) and the binding of \( S \) to the enzyme are independent of each other, which seems to be the case as is stated below. Denote the entire alkylammonium ion-bound species (\( EA \) and \( ESA \)) by \( E' \) and the entire unbound species (\( E \) and \( ES \)) by \( E'' \). Then the concentrations of \( EA' \) and \( E'' \) are related by

\[
\frac{[E']}{[EA']} = \frac{[E'']}{[EA'']} = K_s
\]

(3)

Let us further assume that the bound species of the enzyme, \( EA' \), attain an elevated rate of catalysis of the AGE hydrolysis, \( v_a \), per mole of enzyme, and that the unbound species, \( E'' \), have the original rate, \( v_b \), per mole of enzyme. Then the observed rate, \( v \), per mole of the total enzyme, catalyzed by a mixture of \( E' \) and \( EA' \), is represented by

\[
v = \frac{[E']v_a + [EA']v_a}{[E'] + [EA']}
\]

(4)

Elimination of \([E']\) and \([EA']\) from Equations 3 and 4 and rearrangements give the following equation for \( v_a \)

\[
v = -K_s(v - v_b)/[A] + v_a
\]

(5)

or, dividing both sides by \( v_b \), the relative initial rate, \( v_r \), is

\[
v_r = -K_s(v_r - 1)/[A] + v_r/v_b
\]

(6)

Plots of \( v_r \) against \((v_r - 1)/[A]\) are shown in Fig. 6, A to C, for

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Fig. 6. Plots according to Equation 6 for the increase of the rate of the trypsin-catalyzed hydrolysis of AGE caused by methyl- (A), ethyl- (B), and 1-propylammonium (C) ions in 0.1 M KCl at AGE concentrations of 0.279 M, 0.376 M, and 0.374 M, respectively, at pH 6.6 and 25°.

Fig. 7. Plots according to Equation 6 for the increase of the rate of the trypsin-catalyzed hydrolysis of AGE caused by ethyl- (open squares) and 1-propylammonium (open circles) ions in 0.05 M CaCl₂ at an AGE concentration of 0.322 M at pH 6.6 and 25°.

TABLE I

Dissociation constants, Kₐ and Kᵢ, of trypsin-alkylammonium ion complexes at pH 6.6 and 25°.

<table>
<thead>
<tr>
<th>Alkylammonium ion</th>
<th>Salt</th>
<th>Kₐ x 10²</th>
<th>Kᵢ x 10²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl-</td>
<td>0.1 M KCl</td>
<td>38</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>0.05 M CaCl₂</td>
<td>6.9</td>
<td>6.2</td>
</tr>
<tr>
<td>Ethyl-</td>
<td>0.1 M KCl</td>
<td>4.1</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>0.05 M CaCl₂</td>
<td>0.86</td>
<td>0.87</td>
</tr>
<tr>
<td>1-Propyl-</td>
<td>0.1 M KCl</td>
<td>0.69</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>0.05 M CaCl₂</td>
<td>0.69</td>
<td>0.61</td>
</tr>
</tbody>
</table>

 Reported in our previous paper (3) are listed in Table I for comparison. Very good agreement between Kₐ and Kᵢ in all cases shows that the increase in the rate of the AGE hydrolysis is a result of the binding of the alkylammonium ion to the specificity-determining site of trypsin. These facts also indicate that the strength of the binding of the alkylammonium ions is not affected by whether the substrate is the small AGE molecule or the large, electrically charged BAE molecule.

Although the meaning of the apparent Michaelis-Menten constant, Kₐ, for the trypsin-catalyzed esterolysis is still debatable in detail at present, there is evidence that suggests that the strength of the initial substrate binding is the major factor in determining its value (10, 11). The independence of Kₐ for AGE hydrolysis on the concentration of the alkylammonium ions shows that the binding of the latter to the specificity-determining site does not affect the binding of AGE. Therefore, the accelerating effect of the alkylammonium ions is exclusively due to an enhanced activity of the catalytic site as reflected by the increased apparent first order rate constant.

The fact that reasonably straight lines are obtained in the plots according to Equation 6 (Figs. 6 and 7) shows that Kₐ remains constant while the concentration of the alkylammonium ions is increased.
ions is changed. In other words, in the hydrolysis of AGE, the binding of the alkylammonium ion to the specificity-determining site of trypsin is not affected by whether the catalytic site is functioning or not, whereas the binding at the specificity-determining site increases the activity of the catalytic site. In the case of trypsin, therefore, the induced fit of enzyme to substrate proposed by Thoma and Koshland (12) seems to take place at the catalytic site as a result of the binding of the alkylammonium ion. When one considers a normal substrate of trypsin such as ALE, which comprises both alkylammonium and AGE structures in a single molecule, it would be reasonable to assume that each part may enter a similar interaction as a separate species with the respective sites of the enzyme, although the effect would be more pronounced in this case than with the separate species. The initial binding of the side chain of ALE to the specificity-determining site should obviously be a specific process. The binding will, then, induce a change in the structure of the catalytic site and increase its activity. Thus the sharp specificity of trypsin may be interpreted as a result of a double action of the specificity-determining site. The value of $k_3$ of the AGE hydrolysis at its optimal pH has been estimated to be 0.028 sec$^{-1}$ (2). The presence of ethylammonium ion will increase it to approximately 0.3 sec$^{-1}$, which, however, is far lower than the $k_3$ values of most of the normal ester substrates of trypsin, which are usually in the vicinity of 10 sec$^{-1}$ (13). The specific interaction of the side chain of the normal substrate thus seems to cause an increase in the reactivity of the catalytic site by a factor of 300. This large factor seems to be sufficient to explain the conspicuous specificity of the trypsin catalysis.

In connection with the present work, it is interesting to note the recent discovery by Trowbridge, Krehbiel, and Laskowski (14) that a high concentration of $\alpha$-$N$-toluenesulfonyl-$L$-arginine methyl ester increases the rate of the trypsin-catalyzed hydrolysis of the same substrate to a level approximately 6 times as high as that predicted by simple Michaelis-Menten kinetics. Elucidation of whether this so-called substrate activation and the type of activation observed in the present study involve the same mechanism awaits a further investigation.

Fig. 8 shows the Eadie plots for the inhibition of the AGE hydrolysis by 1-n-butylammonium ion (filled circles) in comparison with the uninhibited catalysis (open circles). The plots with a break indicate that the inhibition does not fit in any simple pattern, although the lower part of the plots suggests a certain degree of competitive inhibition. On the other hand, the data of Fig. 6B, which have been obtained by varying the concentration of the inhibitor at a fixed concentration of substrate, give a straight line when the relative is plotted against the product of the relative rate times the inhibitor concentration (Fig. 9). Both competitive and noncompetitive mechanisms predict a straight line in this plot with slopes equal to $-K_i/K_i ([S] + K_i)$ for the former and $-1/K_i$ for the latter. If the competitive inhibition is assumed, $K_i = 7$ mM is obtained, and the assumption of noncompetitive inhibition gives $K_i = 10$ mM. Both are much higher than the true inhibition constant, 1.7 mM, obtained previously with BAE as substrate (3). This shows again that the inhibition in question is not of a simple nature. Whatever the mechanism may be, the trypsin-catalyzed hydrolysis of AGE is inhibited by 1-n-butylammonium ion and accelerated by 1-propylammonium ion. Trypsin has been shown to be very specific with regard to the length of the hydrocarbon chain of its substrate. The esters and amides of $\alpha$-$N$-benzoyl-$L$-homo-arginine and 1-$\alpha$-$N$-benzamido-$\gamma$-guanidinobutyric acid are attacked at much lower rates than the corresponding derivatives of arginine (15). Similarly, $\alpha$-$N$-benzoyl-$L$-ornithine amide is a very poor substrate of trypsin, whereas $\alpha$-$N$-benzoyl-$L$-lysine amide is a good substrate (16). As illustrated in Scheme 1 by the examples of BAE and ALE, the distances between the center of the positive charge on the side chains and the carboxyl carbon...
atom are approximately identical with arginine and lysine. When 1-n-butylammonium ion and AGE molecule are arranged in a series, the corresponding distance may be only slightly longer, owing to the possible overlapping of C—H bonds at the junction of the 2 molecules. This slight difference may be the cause of the inhibition. These facts indicate that there is a high degree of precision in the binding of substrate to trypsin, and this precision seems to be an important factor for the sharp specificity of this enzyme. In this connection, it is interesting to note a contrasting situation with $\alpha$-chymotrypsin. This enzyme has a much broader pattern of specificity, and the following findings seem to suggest a lower degree of precision in the binding of substrate by this enzyme. Indole inhibits the $\alpha$-chymotrypsin-catalyzed hydrolysis of AGE in an unequivocally competitive way (2). When indole and AGE are arranged in a series, the combined length is definitely shorter than a corresponding normal substrate, acetyltryptophan ethyl ester. Therefore, if indole and AGE were bound exactly to the sites where the corresponding parts of acetyltryptophan ethyl ester are normally bound, no interference, or inhibition, would take place. The observed inhibition suggests that the bindings are not exact. A slight acceleration of deacylation of acetyl group from monoacetylchymotrypsin was observed in the presence of indole (17). Thus it is only when the size of substrate is reduced to an acetyl group that the interference in the binding between indole and a substrate disappears.

The binding of alkylammonium ion causes a lowering of the pH value for the catalytic site, as shown by the example of ethylammonium ion. This lowering may seem to explain, at least in part, the observed increase of the catalytic rate at pH 6.6. However, the observed shift of pH from 7.10 in the absence of ethylammonium ion to 6.94 in its presence can bring about only a 20% increase in the catalytic rate at pH 6.6. Thus it is clear that the shift of the pH value is only a very minor factor in the observed acceleration of the catalysis caused by the alkylammonium ion.

It has been noted in our previous report (2) that there is a simple relationship between $\log K_m$ and the pH of the pH profile of $K_a$ in the $\alpha$-chymotrypsin-catalyzed hydrolyses of various ester substrates. For trypsin catalysis, similar plots are shown in Fig. 10 for BAE (18, 11), AGE (2), and ethylammonium ion-accelerated hydrolysis of AGE. For the $K_m$ value of this last reaction, the product of $K_m$ of AGE and $K_a$ of ethylammonium ion is used, on the assumption that the additivity of $\log K_m$ from various parts of an inhibitor molecule (3) can be extended to the present case. The plots again fall on a straight line, indicating a close correlation between $K_m$ and pH. This is in accord with the fact that the binding of the side chain of a substrate seems to induce an increase in the activity of the catalytic site, although its detailed mechanism is yet to be elucidated.

**SUMMARY**

1. The rate of the trypsin-catalyzed hydrolysis of acetylglycine ethyl ester is increased approximately 3-, 9-, and 2-fold by methyl-, ethyl-, and 1-propylammonium ions, respectively, at pH 6.6 and 25°, whereas 1-n-butylammonium ion inhibits the catalysis.

2. Formation of an enzyme-alkylammonium ion complex with a higher rate of catalysis than the free enzyme explains the relationship between the rate and the alkylammonium ion concentration. The dissociation constants of the complexes agree with values obtained previously from inhibition experiments.

3. The observed increase in the rate of hydrolysis of acetylglycine ethyl ester is due to an increased first order rate constant, whereas the Michaelis-Menten constant remains unchanged.

4. It is proposed that the binding of the alkylammonium ion induces activation of the catalytic site of trypsin, and hence that the sharp specificity of this enzyme may be caused by double action of the alkylammonium moiety of its normal substrates, which first selectively binds to the specificity-determining site and then induces the activation of the catalytic site.

5. A direct correlation between the logarithm of the Michaelis-Menten constant and the pH of the pH profile of the first order rate constant has been found with two different ester substrates and acetylglycine ethyl ester with ethylammonium ion.

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