The Mechanism of the Specificity of Trypsin Catalysis

II. COMPARISON OF TRYPsin AND α CHYMOTRYPSIN IN THE NONSPECIFIC CATALYSES OF THE HYDROLYSIS OF ACETYLGLYCINE ETHYL ESTER*

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Two pancreatic endopeptidases, trypsin and chymotrypsin, are known to have many points in common, not only with respect to general properties as proteins but also in their enzymatic characteristics. Although these enzymes were originally discovered as endopeptidases, they have subsequently been found to hydrolyze both amide and esters of amino acids (2, 3), esters being hydrolyzed 100 to 1000 times faster than the corresponding amide (2–4) by either of the enzymes. The two enzymes are inhibited in a similar fashion by phosphate esters such as diisopropyl phosphorofluoridate (5, 6), and the inhibited enzymes have been found to contain a diisopropylphosphoryl group bound to the serine hydroxyl group of a common sequence, glycyl-L-seryl-L-aspartylglycine (7, 8), suggesting that this structure is commonly involved in the active sites of the two enzymes. The imidazole group of histidine has also been proposed to constitute a part of the active site of an enzyme. If the two enzymes react with the side chain of an amino acid, thus shown to be of quantitative rather than qualitative character, should be determined as a result of interaction between the side chain and a part of the active site of an enzyme. If the two enzymes react on a substrate without the amino acid side chain, such as acetylglucose ethyl ester, this interaction should be absent and the catalysis should be mediated by the interaction that involves the part which may mostly consist of the so-called catalytic site. This postulate will find experimental support if the catalyses of the hydrolysis of acetylglucose ethyl ester by the two enzymes are kinetically similar, which, in fact, has turned out to be the case, because the catalytic sites of the two enzymes seem to be similar while the specificity-determining sites are uniquely different as mentioned above. Thus study of such reactions, in which the contribution of the specificity-determining site is practically absent, seems to render possible the investigation of the action of the catalytic site separately from the part of the specificity-determining site. In turn it should also be possible to identify the effect of the specificity-determining site by comparing a specific catalysis with the nonspecific one.

Among the derivatives of glycine, methyl hippurate has been reported to be a fairly good substrate for α-chymotrypsin (20), which is not surprising since the molecule has a phenyl group that is 3 atoms removed from the carboxyl carbon atom of the glycine residue, thus nearly satisfying a structural requirement for specific substrates of chymotrypsin. On the other hand, the interaction of acetylglucose ethyl ester (21) or acetylglucose methyl ester (22, 23) with the specificity sites of either chymotrypsin or trypsin may be very weak if it occurs at all, since the acetamido structure does not contain a bulky hydrophobic or an electrically charged group. Thus acetylglucose ethyl ester seems to be a reasonable choice as nonspecific substrate for the present investigation.

EXPERIMENTAL PROCEDURE

Substrate—AGE was synthesized from acetylglucose, m.p. 207°, by esterification with anhydrous ethanol and thionyl chloride according to the general method (24). It was purified by two distillations under reduced pressure, b.p. 121° per 1.3 mm. The second distillation raised the melting point from 47.0–47.5° (uncorrected) to 47.5–48.0° (uncorrected) (reported m.p. 47.0–48.5° (21), 48° (25)).

1 The abbreviations used are: AGE, acetylglucose ethyl ester; AGMe, acetylglucose methyl ester; BAE, benzoyl-L-arginine ethyl ester.

2 This was prepared by acetylation of glycine ethyl ester by-
Enzymes—Beef pancreatic trypsin preparations were twice recrystallized and were obtained from Worthington Biochemical Corporation, Lots 6102, 6144-5, and 6147. The activities of these preparations were measured with BAE. Beef pancreatic α-chymotrypsin was salt-free, three times recrystallized, lyophilized preparations from the same source (Lots CDI-678.84R, CDI-6046-7, and CDI-6031). The activities of these preparations were measured with acetyl-n-tyrosine ethyl ester. Their concentration of an inhibitor solution was estimated from the optical density at 280 nm by using ε = 9.54 and a molecular weight of 24,000 (27) for trypsin and corresponding values of 20.0 (28) and 23,000 (28) for α-chymotrypsin.

Inhibitors—Soybean trypsin inhibitor was obtained from Worthington Biochemical Corporation (Lot 5478). The molar concentration of an inhibitor solution was estimated from the optical density at 280 nm by using ε = 9.54 and a molecular weight of 21,500 (29). Indole was of the highest reagent grade (m.p. 52–53).

Kinetic Methods—The initial rate of hydrolysis of AGE was determined by using a Radiometer model TTT1a pH-Stat and either an Ole Dich recorder or a model SDR2 recorder with a reaction volume of 10 ml and 0.02 N NaOH as titrating agent. A 30-ml reaction vessel was placed in a thermostated water bath under a stream of nitrogen that had been previously bubbled through water of the reaction temperature, and the solution was stirred with a magnetic stirrer. The pH of the solution could be maintained within ±0.01 pH unit of a desired value. All measurements were carried out at or below pH 7.0, since nonenzymatic hydrolysis of the substrate reaches a level comparable with that of the enzymatic catalyses at this pH region. Since this situation should mitigate the precision of the measurement which is required for the intended comparison, a lower pH range had to be chosen. Even below pH 7, the nonenzymatic hydrolysis was detectable, and each measurement had to be corrected for it. Most of the data have been obtained with twice distilled AGE, and it has been confirmed in several representative measurements that the recrystallization of the substrate from diisopropyl ether does not affect the kinetic results.

Inhibition—In order to confirm that the hydrolysis is really catalyzed by the active sites of the enzymes, it has been shown that the trypsin catalysis is inhibited almost stoichiometrically by soybean trypsin inhibitor. The catalysis by α-chymotrypsin is competitively inhibited by indole, as shown in Fig. 1. The plots for 1 mM indole (open circles) give an inhibition constant, Ki, of 0.86 mM and those for 2 mM (open squares) give a Ki value of 0.97 mM. Another set of experiments, in which the concentration of AGE was kept constant (0.149 M) while the concentration of indole was varied from 6 to 0.1 mM, gave a Ki of 1.0 mM (at pH 7.0). These results are not far from the value of 0.80 mM obtained for competitive inhibition at pH 7.9 (32).

## RESULTS

A comparison of catalyses by trypsin and by α-chymotrypsin of the hydrolysis of a common, nonspecific substrate, AGE, under steady state conditions has been made by investigating the dependence on pH, ionic strength, and temperature of the apparent Michaelis-Menten constants, Kₘ, and the apparent first order rate constants of product formation, kₐ, for the two enzymes. Although it is normally desirable to employ the pH of the maximal activity, probably above pH 8.4, the rate of the nonenzymatic hydrolysis of the substrate reaches a level comparable with that of the enzymatic catalyses at this pH region. Since this situation should mitigate the precision of the measurement which is required for the intended comparison, a lower pH range had to be chosen. Even below pH 7, the nonenzymatic hydrolysis was detectable, and each measurement had to be corrected for it. Most of the data have been obtained with twice distilled AGE, and it has been confirmed in several representative measurements that the recrystallization of the substrate from diisopropyl ether does not affect the kinetic results.

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The main part of the data consists of results of two independent series of experiments carried out 1 year apart. Results of preliminary and confirmatory experiments are also incorporated. Therefore the data represent the average of two, three, or four values, except for the experiments on the effect of salt concentration.

## FIG. 1. Inhibition by indole of the α-chymotrypsin-catalyzed hydrolysis of AGE in 0.1 M KCl at 25 °C, pH 6.8. The initial absolute rate v₀/[E]₀ is plotted against v₀/[E], AGE at 1 mM indole (open circles) and 2 mM indole (open squares).

[Graph of inhibition by indole of the α-chymotrypsin-catalyzed hydrolysis of AGE in 0.1 M KCl at 25 °C, pH 6.8. The initial absolute rate v₀/[E]₀ is plotted against v₀/[E], AGE at 1 mM indole (open circles) and 2 mM indole (open squares).]
can be considered to be constant over the pH range studied for both of the enzymes. The averaged values are also listed. It is interesting to note here again that $K_m$ is practically unaffected by the temperature change, just as in the case of other nonspecific catalyses (19).

Effects of Temperature and pH on $k_2$–$k_3$ has been obtained at both 25° and 35° below pH 7.0, and the values obtained are shown in Figs. 4 and 5 for trypsin and $\alpha$-chymotrypsin, respectively. It can be seen that each set of $k_2$ values falls on a single

\[
\begin{array}{c|c|c|c|c|c|c|c|c|c|c}
\text{pH} & \text{Trypsin} & \text{Trypsin} & \text{Trypsin} & \text{Trypsin} & \text{Trypsin} & \text{Trypsin} & \text{Trypsin} & \text{Trypsin} & \text{Trypsin} & \text{Trypsin} \\
6.8 & 0.77 ± 0.12 & 0.81 ± 0.15 & 0.43 ± 0.06 & 0.56 ± 0.06 & 0.46 ± 0.05 & 0.50 ± 0.05 & 0.40 ± 0.09 & 0.50 ± 0.09 & 0.40 ± 0.09 & 0.46 ± 0.09 \\
6.6 & 0.88 ± 0.12 & 0.90 ± 0.11 & 0.46 ± 0.06 & 0.46 ± 0.06 & 0.46 ± 0.06 & 0.46 ± 0.06 & 0.46 ± 0.06 & 0.46 ± 0.06 & 0.46 ± 0.06 & 0.46 ± 0.06 \\
6.4 & 0.88 ± 0.17 & 0.85 ± 0.09 & 0.36 ± 0.06 & 0.34 ± 0.12 & 0.34 ± 0.12 & 0.34 ± 0.12 & 0.34 ± 0.12 & 0.34 ± 0.12 & 0.34 ± 0.12 & 0.34 ± 0.12 \\
6.2 & 0.69 ± 0.74 & 0.74 ± 0.13 & 0.42 ± 0.08 & 0.41 ± 0.10 & 0.41 ± 0.10 & 0.41 ± 0.10 & 0.41 ± 0.10 & 0.41 ± 0.10 & 0.41 ± 0.10 & 0.41 ± 0.10 \\
6.0 & 0.88 ± 0.11 & 0.46 ± 0.09 & 0.50 ± 0.09 & 0.50 ± 0.09 & 0.50 ± 0.09 & 0.50 ± 0.09 & 0.50 ± 0.09 & 0.50 ± 0.09 & 0.50 ± 0.09 & 0.50 ± 0.09 \\
\text{Average} & 0.79 ± 0.14 & 0.83 ± 0.12 & 0.41 ± 0.07 & 0.46 ± 0.09 & 0.46 ± 0.09 & 0.46 ± 0.09 & 0.46 ± 0.09 & 0.46 ± 0.09 & 0.46 ± 0.09 & 0.46 ± 0.09 \\
\end{array}
\]

which was the lowest limit for a reasonably accurate measurement.

Effects of Temperature and pH on $K_m$–$K_m$ has been determined at several pH values between 6 and 7 at 25° and 35°, and these values are listed in Table I. Within experimental error,
In drawing the titration curves, each pK value was obtained from a series of rate measurements at a constant substrate concentration, instead of being computed from the $k_3$ values obtained from the Eadie plots. This procedure is justified because $K_m$ remains constant over the region of the $k_3$ change, and hence the observed rate is related to $k_3$ by a constant proportionality factor. Better accuracy can be expected by this method than by using $k_3$ values, because the computation of $k_3$ already involves a large extrapolation error. The product of the initial absolute rate and hydrogen ion concentration, $v[H^+]/[E]_i$, was plotted against the rates for trypsin and $\alpha$-chymotrypsin in Figs. 6 and 7, respectively, and the ionization constant, $K_I$, was read from the slope. For convenience of comparison between the two enzymes in the hydrolysis of AGE as well as other substrates, the plateau or maximal values of $k_3$ in a high pH region have been computed from the $K_I$ values, and all the $k_3$ values for each of the enzymes have been obtained by extrapolating the titration curve above pH 7.0. These values were averaged to obtain the $k_3$ max values listed in Table II. These procedures for determining the $K_I$ and $k_3$ max values are based on the assumption that $K_m$ is independent of pH and that there is only one ionizable group that affects the enzyme activity. It must be noted that in these nonspecific catalyses, pK values of trypsin and $\alpha$-chymotrypsin are very close, in contrast to the case for specific catalyses.

**Effects of Salt Concentration on $k_3$ and $K_m$—Effects of high salt**

<table>
<thead>
<tr>
<th></th>
<th>$k_3$ max</th>
<th>pH</th>
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</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td></td>
<td></td>
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<tr>
<td>25°C</td>
<td>2.8 ± 0.29</td>
<td>7.10 ± 0.05*</td>
</tr>
<tr>
<td>35°C</td>
<td>5.2 ± 0.24</td>
<td>6.86 ± 0.02†</td>
</tr>
<tr>
<td>$\alpha$-Chymotrypsin</td>
<td></td>
<td></td>
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<tr>
<td>25°C</td>
<td>6.6 ± 0.46</td>
<td>7.05 ± 0.07‡</td>
</tr>
<tr>
<td>35°C</td>
<td>11.2 ± 1.7</td>
<td>6.89 ± 0.07‡</td>
</tr>
</tbody>
</table>

* Obtained at an AGE concentration of 0.427 M.
† Obtained at an AGE concentration of 0.176 M.
‡ Obtained at an AGE concentration of 0.214 M.
enzymes are both slowly changing functions of the KCl concentration. As shown in Fig. 8 (left), the k₃ values of the two enzymes are both slowly changing functions of the KCl concentration within the concentration range studied, showing a gradual decrease toward a higher concentration. The Kₘ values seem to be affected more strongly by the salt concentration, as shown in Fig. 8 (right). Here again the two enzymes show similar behavior, decreasing toward a higher concentration with approximately the same slope. The decrease seems to mean that the high salt concentration stabilizes the enzyme-AGE complex.

**DISCUSSION**

The foregoing results clearly demonstrate that trypsin and α-chymotrypsin catalyze the hydrolysis of AGE nonspecifically. However, the rates of the catalyses are three orders of magnitude lower than specific reactions, even at a substrate concentration as high as 1.0 M.

In the catalyses of the hydrolysis of AGE, trypsin and α-chymotrypsin show a close similarity in many points. Firstly, the Kₘ values are not different by more than a factor of 2 (Table I). This can be considered as a very minor difference in view of the fact that the Kₘ values of trypsin and α-chymotrypsin vary by several orders of magnitude depending on substrate and reaction conditions (2-4, 19). Secondly, the k₃ max values of the two enzymes are similar, being different only by a factor of 2 (Table II). When one considers that k₃ of the nonspecific hydrolysis of AGE is decreased by factors of more than 1000 from the specific cases, that the values of the specific catalyses are different by an order of magnitude between the two enzymes, and that k₃ of each enzyme varies by several orders of magnitude depending on substrates (2-4, 19), the two k₃ max values obtained, 2.6 × 10⁻⁶ sec⁻¹ for trypsin and 6.6 × 10⁻⁶ sec⁻¹ for α-chymotrypsin at 25° and high pH, can be considered remarkably close. Thirdly, the agreement in the pK values of the pH profile of k₃ (Table II) is noteworthy, since the pK values for specific catalyses by trypsin and α-chymotrypsin are in distinctly different ranges: the hydrolysis of BAE by trypsin has a pK of 6.25 (10) while pK values in the vicinity of 6.8 ± 0.1 have been found for α-chymotrypsin for most of its specific substrates (11, 28, 34). Fourthly, the temperature dependence values of the pK profiles show good agreement (Table II). As the temperature is raised from 25° to 35°, the pK value of trypsin is decreased by 0.24 pH unit from 7.10 to 6.86, while that of α-chymotrypsin is lowered by 0.11 pH unit from 7.63 to 6.72, representing heats of ionization of 10° and 8.5 kcal per mole, respectively, for the group responsible for the pH profile. Fifthly, the temperature-dependent changes of k₃ max agree very well (Table II); elevation of the temperature by 10° increases k₃ max for trypsin and α-chymotrypsin by factors of 1.9 and 1.7, respectively, representing 11° and 10 kcal per mole in heat of activation. Sixthly, the Kₘ values of neither enzymes are affected by the temperature change. This insensitivity of Kₘ to the temperature change has been encountered in the case of other nonspecific catalyses by the two enzymes (19) and seems to be a feature of a nonspecific catalysis. Finally, the manner in which Kₘ and k₃ slowly change with KCl concentration in the ranges studied is the same for the two enzymes.

The kinetic results show that trypsin and α-chymotrypsin catalyze in a similar fashion the hydrolysis of AGE, which, with the possible exception of AGMe, is the smallest amino acid ester substrate for these enzymes. Because of this small size and due apparently weak binding of substrate as indicated by the high Kₘ values, it might be considered that the catalysis of AGE is centered in only part of the total active region of each of the enzymes, and that this part has primarily a catalytic function. The similarity of the kinetics for the two enzymes indicates that the action of such a catalytic site is to a limited extent isolated from the effects of the rest of the active region, and that the actual structure of the catalytic site might be the same for both enzymes. The latter observation is supported by a number of organic chemical studies (5-8) as well as some previous kinetic studies (10-12).

With knowledge of the contribution from the catalytic site, one can estimate the contribution from the interaction between the side chain of a specific, or good, substrate and the other part of the active site, which may be called the specificity-determining site. For example, the Kₘ of the hydrolysis of BAE by trypsin, 1.0 × 10⁻⁴ M (10), is 1.25 × 10⁻⁴ times smaller than that for AGE, which may be identified as the contribution from the specificity-determining site. Such an attempt to dissect the enzyme-amino acid substrate interaction seems to find a support in the report of Cohn and Edsall (35) that the free energy of interaction between a solvent and various parts of an amino acid is additive, if it is assumed that the substrate amino acid is buried in the active site of enzyme.

In the actual catalysis with a specific substrate, however, the action of the catalytic site is not independent of that of the specificity-determining site because k₃, which may be an index of the action of the former, is much larger for a specific, strongly bound substrate than for AGE. For example, in the specific hydrolysis of AGE by trypsin, k₃ is approximately 500 times as high as it is for AGE, and the specific hydrolysis of acetyl-L-tyrosine ethyl ester by α-chymotrypsin is almost 3000 times as rapid as that of AGE. In this connection, it is interesting to note a correlation between the pK values of the pH profile of k₃ and logarithms of the Kₘ values as shown in Fig. 9. It indicates...
that the lower the $K_m$ value is, the lower becomes the pK value. The points for chymotrypsin (open circles) seem to fall on a straight line, and two points for trypsin (open squares) determine a line with slope not much different from that for chymotrypsin. This may be explained either by a kinetic mechanism similar to that proposed by Bruice and Sturtevant (36) for a model enzyme system, or as an indication that substrate binding at the specificity-determining site induces transformation of the catalytic site. The true meaning of $K_m$ for the $\alpha$-chymotrypsin-catalyzed esterolysis is not yet clear. However, if one naively assumes that the difference in $RT$ in $K_m$ within the same type of reactions is due to the difference in $\Delta F$ of the substrate binding, and that the pH profile of $k_2$ is directly related to the ionization of the imidazole group of the histidine residue at the catalytic site, the relationship shown in Fig. 9 may be interpreted as a linear relationship between $\Delta F$ of the substrate binding and $\Delta F$ of the ionization of the imidazole group. It has been proposed that the imidazole group is hydrogen-bonded to the hydroxyl group of the serine residue of the catalytic site (for example (37)), yet the histidine residue has not been found in the same peptide chain with the serine residue. It is tempting to interpret the above result as an indication that the binding of substrate brings the histidine and the serine residues close to each other to the hydrogen-bonded state, and that the stronger the binding, the stronger the hydrogen bonding between the two residues. This hydrogen bonding may shift the pK value of the imidazole group. However, there is no uniform relationship between the $k_2$ values and the pK values among the substrates in question. This absence of correlation between the catalytic rate and the strength of the hydrogen bonding leaves a doubt in the above interpretation.

Wolf and Niemann (21) have studied the $\alpha$-chymotrypsin-catalyzed hydrolysis of AGE in 0.5 M NaCl at pH 7.9 and 25°C, and have obtained a $K_m$ of 0.096 M and a $k_3$ of 0.013 sec$^{-1}$. If the present data can be extrapolated to a higher pH, as we have assumed above, the corresponding values will be 0.41 M and 0.066 sec$^{-1}$ in 0.1 M KCl at 25°C at the pH of maximal activity of the enzyme. A direct comparison of these two results may not be significant because the conditions of the two experiments are different and the assumption necessary for the extrapolation may not be valid. On the other hand, the study of the $\alpha$-chymotrypsin-catalyzed hydrolysis of AGMe by the above authors (23) gave a $K_m$ of 0.03 M and a $k_3$ of 0.013 sec$^{-1}$. They have also reported (22) that the Eadie plots of the hydrolysis of AGMe consist of two straight lines. The upper limb of the plots has a high value of $K_m$ comparable with our value for AGE, and the $K_m$ of the lower limb is closer to their value for AGE. In view of the kinetic similarity of AGE and AGMe hydrolysies observed by Wolf and Niemann (21, 23) and the structural similarity of the two substrates, it may be possible that an analogous break in Eadie plots might be obtained at pH 7.9, and that their data were for the lower limb of the plots, which could have been too low to be detected at lower pH, while the present study deals with the upper limb at lower pH. However, as stated under "Results" above, the break in the Eadie plots could not be detected over the range of AGE concentration from 0.012 to 1.0 M in the present experiment in 0.1 M KCl at pH 6.6 and 25°C, which indicates that the present observations are not the result of the substrate activation. As the pH is raised from 6.6 to 7.9, additional functional groups of $\alpha$-chymotrypsin dissociate. The possibility that some of these groups exert a base catalysis on the hydrolysis of AGE or AGMe may not be excluded in view of the fact that these substrates are very susceptible to the hydroxide ion catalysis. The above authors have not presented any evidence that the observed reaction was really catalyzed by the active site of the enzyme. It is only unfortunate that at the high AGE concentration employed in the present experiment, the rate of the nonenzymatic hydrolysis of AGE was too high at pH 7.9 to confirm the above interpretation with reasonable accuracy.

**SUMMARY**

1. Acetylglycine ethyl ester was chosen as a nonspecific substrate for trypsin and $\alpha$-chymotrypsin to minimize the specific interaction of the amino acid side chain of substrate with enzyme.

2. Comparisons at 25°C with respect to $K_m$, 0.79 M for trypsin and 0.41 M for $\alpha$-chymotrypsin; the maximal first order rate constant, 0.028 sec$^{-1}$ and 0.066 sec$^{-1}$, and its temperature dependence; the pH profile of the rate, pK = 7.10 and 7.03, and its temperature dependence; and the dependence of $K_m$ and $k_3$ on salt concentration showed a close similarity of the catalyses by the two enzymes.

3. It was concluded that the specific interaction involving the substrate with the specificity site of the enzymes is practically absent and that the catalyses are mediated mostly by the catalytic sites alone. In turn, the kinetic similarity was interpreted as evidence for a structural similarity of the catalytic sites of the two enzymes.

4. A linear relationship between logarithm of $K_m$ and pK of the pH profile of the rate was discussed.

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