Preparation and Kinetic Properties of a New Form of Chymotrypsin Which Is Active at Alkaline pH: $\alpha_\text{i}$-Chymotrypsin*

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SUMMARY

The preparation of a new stable and active form of chymotrypsin is described. The enzyme possesses threonine-147 instead of alanine-149 as the NH$_2$-terminal group of the C chain and has been called $\alpha_\text{i}$-chymotrypsin.

The conformational transition that affects chymotrypsins at alkaline pH was investigated and compared with that of $\alpha$- and $\delta$-chymotrypsins by studying the kinetic constants and their pH dependencies for the hydrolysis of various specific ester substrates. The $k_{cat}$ values obtained with $\alpha_\text{i}$-chymotrypsin are similar to those of $\alpha$- and $\delta$-chymotrypsins. The $K_\text{m}$ values showed a progressive increase toward the alkaline pH region. The shape of the $K_\text{m}$-pH profiles closely resemble those of $\delta$-chymotrypsin and differ considerably from the behavior of $\alpha$-chymotrypsin. The results strongly implicate the participation of the alanine-149 amino group in the reversible inactivation of $\alpha$-chymotrypsin at high pH.

This finding led us to suggest that the peculiar behavior of $\alpha$-chymotrypsin at alkaline pH may be caused by the ionization of the phenolic group of tyrosine-146 or the amino group of alanine-149, which are present as chain termini in $\alpha$-chymotrypsin, but not in $\delta$-chymotrypsin (7).

This paper reports the preparation, characterization, and kinetic properties at alkaline pH of a new stable and active form of chymotrypsin which possesses threonine-147 instead of alanine-149 as the NH$_2$-terminal group of the C chain. This enzyme, whose existence was first recognized in 1955 (9, 10), has been called $\alpha_\text{i}$-chymotrypsin following an early suggestion by Rovary et al. (11).

We have found that the kinetic properties of $\alpha_\text{i}$-chymotrypsin in the neutral and alkaline pH regions strongly resemble those of $\delta$-chymotrypsin. $\alpha_\text{i}$-Chymotrypsin is apparently able to bind substrates in this pH region, which makes it a considerably more active enzyme than $\alpha$-chymotrypsin. Our results strongly implicate the NH$_2$ terminus of alanine-149 as a participant in the reversible inactivation of $\alpha$-chymotrypsin at high pH. A preliminary account of this work has been reported (12).

EXPERIMENTAL PROCEDURE

Materials

Enzymes—The following bovine proteins used were obtained from Worthington Biochemical Corporation: salt-free, five-times crystallized, electrophoretically homogeneous bovine chymotrypsinogen A (Lot UGC-8CC); salt-free, three-times crystallized, chromatographically homogeneous $\alpha$-chymotrypsin (Lot CDS-6602); salt-free, three-times crystallized $\delta$-chymotrypsin (Lot CDD-6632); salt-free, two-times crystallized trypsin (Lot TRL 6256); diisopropyl phosphorofluoridate-treated carboxypeptidase A (crystalline, aqueous suspension Lot COADFP-SJA); and soybean trypsin inhibitor (crystalline, Lot S1-2CA).

Substrates—N-Acetyl-L-tryptophan methyl ester was a product from Cyclo Chemical Co., Lot 3-4735, and was crystallized twice from acetonitrile before use. N-(2-furyl)anilinomethyl-L-tryptophan methyl ester was a Cyclo Chemical Co. product and was crystallized twice from ethyl acetate-hexane before use.

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*$\alpha_\text{i}$-Chymotrypsin loses its ability to bind specific substrates or inhibitors in the alkaline pH region (1-3). This reversible inactivation has been associated with the disruption of an ion pair between the carboxyl group of aspartic acid-194 and the NH$_2$-terminal group of isoleucine-16, triggered by the deprotonation of this last residue (4-6).

Recent studies on the pH dependence of $\delta$-chymotrypsin-catalyzed reactions indicated that the binding ability of this enzyme is remarkably less dependent on pH when compared with $\alpha$-chymotrypsin (3, 7). Although there is evidence for the existence of the same ionic bond in crystals of phenylmethane-sulfonyl-$\delta$-chymotrypsin (8), our previous results clearly indicated that the deprotonation of the isoleucine-16 amino group causes only a minor effect on the binding ability of this enzyme.

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This work was supported by Grant ROI-HLO-5726 from the National Institutes of Health, United States Public Health Service.
N-Trans(2-furyl)acycloxy-L-phenylalanine methyl ester was synthesized from L-phenylalanine methyl ester (Mann Research Laboratories) and furalcarboxaldehyde. After three crystallizations from ethyl acetate-hexane the melting point was constant at 100–101°C, lit. m.p. 99.0–100.8°C (13). N-Trans-cinnamoylimidazole, recrystallized four times from hexane, was a gift from Dr. Y. Nakagawa. N-Acetyl-d-trypophanamide (Cylo Chemical Co., Lot K 5723) was used as delivered. Tryptophol was obtained from Sigma Chemical Co. and was crystallized two times before use, m.p. 56°C. Benzy alcohol was purified as described before (3). Stock solutions of substrates were prepared in either acetonitrile (Malincrodt, nanograde) or dimethyl sulfoxide (Matheson, Coleman and Bell). All buffer solutions had an ionic strength of 0.1 M and were prepared from analytical reagent grade materials.

**Methods**

**Kinetic Runs**—The kinetics of hydrolysis were determined using a Cary 14 PM recording spectrophotometer equipped with a thermostatted cell compartment at 25 ± 0.2°C. The hydrolysis of N-acetyl-d-trypophan methyl ester was followed at 400 nm as described before (7). The hydrolysis of N-trans(2-furyl)acycloxy-L-trypophan methyl ester was followed at 335 nm as described before (7). The hydrolysis of N-trans(2-furyl)acycloxy-L-phenylalanine methyl ester was followed at 335 nm. The absorbance data were converted into rate data using \( AE = \frac{\lambda}{1 \pm \epsilon} \) as described before (3). The hydrolysis of N-trans(2-furyl)acycloxy-L-trypophan methyl ester was followed at 335 nm. The absorbance data were converted into rate data using \( AE = \frac{\lambda}{1 \pm \epsilon} \) as described before (3).

**Preparation of Affinity Chromatography Columns**—When necessary enzyme preparations were purified from inactive material by affinity chromatography on Sepharose-epsilonaminocaproyl-d-trypophan methyl ester (18). epsilon-Aminocaproyl-d-trypophan methyl ester was synthesized as follows. 1.1 Equivalents of D-trypophan methyl ester (Cylo Chemical Co.), 1.1 eq of triethylamine and 1 eq of dicyclohexylcarbodiimide were added to a solution of 1 eq of N’-Z-aminoacaproyl acid (Cylo Chemical Co.) in dichloromethane solution (Z = benzyloxy-carbonyl). After overnight stirring at room temperature, the precipitated amino acids were filtered and washed with 1 N HCl, water, 1 N NaHCO₃, and water. The material showed only one spot (ultraviolet, iodine, ninhydrin-positive, RF 0.13) by thin layer chromatography on silica gel using chloroform-methanol (9:1) as solvent. The Z group was removed by catalytic hydrogenation with 10% Pd on charcoal, in ethyl alcohol, at 40 mm above atmospheric pressure for 3 hours. The material obtained after filtration and removal of the solvent showed a single spot by thin layer chromatography on silica gel using chloroform-methanol (9:1) as solvent. The peptide was linked to Sepharose 4B (Pharmacia Fine Chemicals) as described by Cuatrecasas et al. (19).

**Preparation of thr-Neochymotrypsinogen A—Chymotrypsinogen A (100 mg) was treated with 5 mg of \( \delta \)-chymotrypsin and 2 mg of soybean trypsin inhibitor in 0.1 M phosphate buffer pH 7.6, 0.3 M ammonium sulfate. After 12 hours at 25°C the solution was made 4 \( \times 10^{-4} \) M in diisopropyl phosphorofluoridate, incubated 90 min at 0°C, dialyzed extensively against 5 \( \times 10^{-4} \) M HCl, and lyophilized. A second procedure used involved the same incubation mixture as above but in 0.1 M citrate buffer, pH 3.1, 0.3 M ammonium sulfate for 44 hours at 25°C. The proteins obtained were devoid of enzymatic activity. They were characterized by analysis of their NH₂-terminal and COOH-terminal groups (Table I).**

**Activation of thr-Neochymotrypsinogen A to \( \alpha_1 \)-Chymotrypsin—**

One hundred milligrams of thr-neochymotrypsinogen A were treated with 5 mg of trypsin in 0.1 M Tris-HCl buffer, pH 8.0, 0.005 M CaCl₂ at 0°C. After 1 hour, the mixture was dialyzed against 5 \( \times 10^{-4} \) M HCl and purified by chromatography on a Sepharose-epsilonaminocaproyl-d-trypophan methyl ester column.

### Table I

<table>
<thead>
<tr>
<th>NH₂-terminal residues</th>
<th>( \delta )-Neochymotrypsinogen</th>
<th>( \delta )-Neochymotrypsinogen</th>
<th>( \alpha_1 )-Chymotrypsin</th>
<th>( \alpha_1 )-Chymotrypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>0.11</td>
<td>0.08</td>
<td>0.11</td>
<td>0.14</td>
</tr>
<tr>
<td>Thr</td>
<td>0.85</td>
<td>0.82</td>
<td>0.82</td>
<td>0.06</td>
</tr>
<tr>
<td>Ser</td>
<td>0.08</td>
<td>Traces</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>Asp</td>
<td>Traces</td>
<td>Traces</td>
<td>Traces</td>
<td>0.09</td>
</tr>
<tr>
<td>Ile</td>
<td>0.09</td>
<td>Traces</td>
<td>0.82</td>
<td>0.82</td>
</tr>
</tbody>
</table>

* The values are averages of two determinations. An accuracy greater than 15% is not claimed for the present determinations.
* Prepared at pH 7.6, 12 hours at 25°C (see details under "Methods").
* Prepared at pH 3.1, 44 hours at 25°C (see details under "Methods").
* Prepared from \( \delta \)-chymotrypsin by the procedure of Miller et al. (20).
* Fractional values relative to the measured amount of isoleucine.

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**Methods**

**Kinetic Runs**—The kinetics of hydrolysis were determined using a Cary 14 PM recording spectrophotometer equipped with a thermostatted cell compartment at 25 ± 0.2°C. The hydrolysis of N-acetyl-L-trypophan methyl ester was followed at 400 nm as described before (7). The hydrolysis of N-trans(2-furyl)acycloxy-L-trypophan methyl ester was followed at 335 nm as described before (7). The hydrolysis of N-trans(2-furyl)acycloxy-L-phenylalanine methyl ester was followed at 335 nm. The absorbance data were converted into rate data using \( AE = \frac{\lambda}{1 \pm \epsilon} \) as described before (3). The hydrolysis of N-trans(2-furyl)acycloxy-L-trypophan methyl ester was followed at 335 nm. The absorbance data were converted into rate data using \( AE = \frac{\lambda}{1 \pm \epsilon} \) as described before (3).

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One hundred milligrams of thr-neochymotrypsinogen A were treated with 5 mg of trypsin in 0.1 M Tris-HCl buffer, pH 8.0, 0.005 M CaCl₂ at 0°C. After 1 hour, the mixture was dialyzed against 5 \( \times 10^{-4} \) M HCl and purified by chromatography on a Sepharose-epsilon-aminocaproyl-d-trypophan methyl ester column.
The sample was applied as a concentrated solution in $5 \times 10^{-4}$ M HCl. After washing the column with 0.1 M Tris-HCl pH 8.0, the active enzyme was eluted with 0.1 M acetic acid, pH 3.0 (Fig. 1), dialyzed against $5 \times 10^{-4}$ M HCl and lyophilized. The protein was further characterized by quantitative NH$_2$-terminal and COOH-terminal analyses. Results are shown in Table I.

Preparation of $\alpha_1$-Chymotrypsin—Two different procedures were employed to prepare this form of chymotrypsin. The first procedure is based on the observations of Roversi et al. (9, 12). They reported that the action of $\delta$-chymotrypsin on chymotrypsinogen A in the absence of trypsin activity resulted in an inactive protein with threonine as NH$_2$-terminal amino acid instead of the expected alanine. They suggested that the attack of trypsin on this protein could give rise to an active enzyme having threonine and isoleucine as NH$_2$-terminal residues (11). The name $\alpha_1$-chymotrypsin was suggested (11, 12).

We have succeeded in preparing this new active form of chymotrypsin. Several experimental conditions were examined for the preparation of $\delta$-chymotrypsinogen (Fig. 1, Compound II). Two sets of conditions were found to be optimal regarding the yield of NH$_2$-terminal threonine and the lack of nonspecific autolysis during the reaction. One of them involves incubation of chymotrypsinogen with $\delta$-chymotrypsin at pH 7.6 for 12 hours at 25$^\circ$ in the presence of trypsin inhibitor. The second involves the incubation of the same protein mixture at pH 3.1 for 11 hours at 25$^\circ$. The use of lower incubation temperatures like 4$^\circ$ or 0$^\circ$ for longer periods of time resulted in low yields regardless of the incubation time. This indicates that temperature plays an important role in the reaction. In both cases the reaction is stopped by the addition of diisopropyl phosphorofluoridate.

The identification of the proteins obtained as $\delta$-chymotrypsinogen is based on the results of the end group analyses shown in the first two columns of Table I. The results show that the proteins contain mainly threonine as NH$_2$-terminal residue and tyrosine as COOH-terminal residue, as expected from the structure of $\delta$-chymotrypsinogen (Fig. 1, Compound II). A small amount of NH$_2$-terminal alanine was consistently found in all preparations. This analysis indicates that the preparation contains at least 10% of the contaminant $\alpha_{11}$-chymotrypsinogen. As expected, the proteins were found to be completely inactive when assayed with specific chymotrypsin substrates prior to the addition of diisopropyl phosphorofluoridate.

Activation of $\delta$-chymotrypsinogen to $\alpha_1$-chymotrypsin was accomplished by incubating the zymogen with 5% (w/w) trypsin at 0$^\circ$. Under these conditions, which resemble the classical "rapid activation" of chymotrypsinogen (21), 90% of the theoretical amount of active enzyme was obtained. This enzyme, after purification by affinity chromatography contains mainly threonine and isoleucine as NH$_2$-terminal residues and tyrosine and leucine as COOH-terminal residues, as shown in the third column of Table I. These values are expected from the structure of $\alpha_1$-chymotrypsin (Fig. 1, Compound III). The existence of a small amount of NH$_2$-terminal alanine indicates the presence of about 10% of $\alpha_{11}$-chymotrypsin, which probably arises from the activation of the 10% $\alpha_{11}$-chymotrypsinogen contaminant.

The affinity chromatography step was found to be particularly useful, since it removes the undesirable proteins remaining in the preparation: unreactive zymogen, diisopropylphosphoryl-$\delta$-chymotrypsin and trypsin. The chromatography was carried out at 4$^\circ$ and the sample applied in a small volume of $1 \times 10^{-4}$ M HCl. A typical chromatogram is shown in Fig. 2.

The second procedure employed to prepare $\alpha_1$ chymotrypsin used in our kinetic experiments was that described by Miller et al. for the conversion of $\delta$-chymotrypsin into an enzyme form called by them $\kappa$-chymotrypsin (20). According to the results on the NH$_2$-terminal analysis that they reported, $\kappa$-chymotrypsin is identical with the previously prepared and described $\alpha_1$-chymotrypsin (12). End group analysis of our preparation confirmed these findings of Miller et al. (20). As shown in the

![Figure 1](http://www.jbc.org/)

**Fig. 1.** Schematic representation of the structures of chymotrypsinogen A (I), $\delta$-neochromeotrypsinogen (II), and $\alpha_1$-chymotrypsinogen (III).

**Fig. 2.** Chromatography of an $\alpha_1$-chymotrypsin preparation on Sepharose-aminocaproyl-$\delta$-tryptophan methyl ester in 0.04 M Tris-HCl pH 8.0. Fractions of 1.2 ml were collected from a column size 0.8 X 20 cm. The sample was applied in a small volume of $1 \times 10^{-4}$ M HCl. The protein Peak A is eluted in the wash through and it is inactive. The active enzyme is eluted in Peak B by 0.1 M acetic acid, pH 3.0.
seen that the \( K_i \) values for the \( \alpha_1 \)-chymotrypsin-catalyzed re-
trypsin were employed. Fig. 3 shows the results obtained using
S-acetyl-L-tryptophan methyl ester as substrate. It can be
and 6-chymotrypsin. Two different preparations of cui-chymo-
trypsin. The shape of the curves resembles
behavior has been found in all chymotrypsins examined to date,
increases toward the alkaline pH region. Although this be-
increases significantly less above pH 9 com-
pared to cw-chymotrypsin. The preparation seems to
contain about 10% of c\( \gamma \)-chymotrypsin as suggested by the pres-
ence of a small amount of N\(_{\text{Hz}}\)-terminal alanine.

The behavior of \( \alpha_1 \)-chymotrypsin can be seen in more detail
in Figs. 3, 4, and 5 where the \( K_i \) values for the three
inhibitors benzyl alcohol, tryptophol and N-
trypsin are consistent with a dependence on a
group of the enzyme with an apparent \( pK_e \) of 9.3, which shifts
upon binding of substrate to a \( pK_e^{ES} \) of 10.2. These values
indicate that \( K_m \) values should level off above pH 11.

The \( K_m \)-pH profiles obtained using \( N\)-trans-(2-furyl)acryloyl-
L-tryptophan methyl ester as substrate are shown in Fig. 4. The \( K_m \) values for both preparations of \( \alpha_1 \)-chymotrypsin increase
above pH 9 showing the same profile as the one obtained with \( \delta \)-chymotrypsin. The data for \( \delta \)-chymotrypsin and \( \alpha_1 \)-chymo-
trypsin are consistent with a dependence on a group of the en-
zyme with an apparent \( pK_e \) of 9.4, which again shifts upon
substrate binding to a \( pK_e^{ES} \) of 10.2.

Fig. 5 shows the results obtained with \( N\)-trans-(2-furyl)
acryloyl-L-phenylalanine methyl ester as substrate. Again the
same general results are obtained. The behavior of \( \alpha_1 \)-chymo-
trypsin is very different than that of \( \alpha_\text{chymotrypsin} \) and re-
sembles closely that of \( \delta \)-chymotrypsin. The profile obtained
with \( \alpha_1 \)-chymotrypsin indicates a dependence on a group of the
enzyme with an apparent \( pK_e \) of 9.0 that changes to 10.1 in the
enzyme-substrate complex.

### Binding of Competitive Inhibitors at Alkaline pH

The binding of the competitive inhibitors benzyl alcohol, tryptophol and \( N\)-acetyl-L-tryptophanamide to \( \alpha_1 \)-chymotrypsin was studied at
pH 10.5 by competitive inhibition kinetics using \( N\)-trans-(2-furyl)
acryloyl-L-tryptophan methyl ester as substrate. The results
are shown in Table III where a comparison is made with values
previously reported for \( \alpha \) and \( \delta \)-chymotrypsin (3). The results
show \( K_i \) values for the three inhibitors significantly lower than the
values of \( \alpha_1 \)-chymotrypsin and which are essentially the same
as those of \( \delta \) chymotrypsin.

### Table II

<table>
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<tr>
<th>Buffer</th>
<th>( \text{N-acyl-L-tryptophan methyl ester} )</th>
<th>( \text{N-trans-(2-furyl)acryloyl-L-tryptophan methyl ester} )</th>
<th>( \text{N-trans-(2-furyl)acryloyl-L-phenylalanine methyl ester} )</th>
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</thead>
<tbody>
<tr>
<td>( \text{pH} )</td>
<td>( k_\text{cat} ) sec(^{-1})</td>
<td>( K_m \times 10^6 ) M</td>
<td>( k_\text{cat} ) sec(^{-1})</td>
</tr>
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<td>6.83</td>
<td>26.8 ± 0.3</td>
<td>7.5 ± 0.4</td>
<td>23.9 ± 1.2</td>
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<td>7.30</td>
<td>37.1 ± 0.4</td>
<td>7.4 ± 0.6</td>
<td>31.4 ± 0.8</td>
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<tr>
<td>7.81</td>
<td>43.3 ± 0.4</td>
<td>6.4 ± 0.5</td>
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<tr>
<td>8.20</td>
<td>46.8 ± 0.8</td>
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<td>36.2 ± 1.4</td>
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<tr>
<td>8.53</td>
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<td>6.9 ± 0.5</td>
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<tr>
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<td>9.61</td>
<td>54.6 ± 1.0</td>
<td>16.3 ± 2.8</td>
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<td>9.80</td>
<td>55.2 ± 1.2</td>
<td>18.5 ± 0.5</td>
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<td>10.02</td>
<td>52.3 ± 1.1</td>
<td>20.6 ± 1.0</td>
<td>40.8 ± 2.1</td>
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<td>10.22</td>
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<td>23.0 ± 2.0</td>
<td>39.0 ± 2.1</td>
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<td>10.40</td>
<td>51.5 ± 1.2</td>
<td>29.0 ± 2.1</td>
<td>39.7 ± 1.8</td>
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<td>10.60</td>
<td>47.6 ± 1.3</td>
<td>31.0 ± 1.6</td>
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<tr>
<td>10.80</td>
<td>45.8 ± 1.5</td>
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<td>35.2 ± 1.9</td>
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<td>11.02</td>
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<td>40.0 ± 4.0</td>
<td>33.1 ± 2.1</td>
</tr>
<tr>
<td>11.23</td>
<td>46.1 ± 1.8</td>
<td>41.0 ± 4.0</td>
<td>——</td>
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</table>

\[ \text{pK}_e \] is a constant for a given enzyme. Within the experi-
mental error, the \( pK_e^{ES} \) values used in fitting the \( \alpha_1 \)-chymotrypsin
curves are very similar and we do not know which is correct.

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Note: The table entries and calculations are based on the understanding of the text and the provided data. The values represent kinetic constants obtained under specific pH conditions and with different substrates and inhibitors.
FIG. 3 (left). $K_m$-$pH$ profiles for α-chymotrypsin (○); α₁-chymotrypsin (prepared according to the procedure described in this paper) (Δ); α₂-chymotrypsin (prepared according to Miller et al. (20)) (▲); and δ-chymotrypsin-catalyzed (●) hydrolyses of $N$-acetyl-$L$-tryptophan methyl ester. The solid lines are calculated from the equation:

$$K_m = K_m^{1^{st}} \frac{1 + \frac{K_e}{[H^+]} + \frac{K_e^S}{[H^+]}}{1 + \frac{K_{e^S}}{[H^+]}}$$

using the values $pK_e = 9.0$, $pK_{e^S} > 11$ for α-chymotrypsin; $pK_e = 9.3$, $pK_{e^S} = 10.2$ for α₁-chymotrypsin (Δ) and $pK_e = 9.25$, $pK_{e^S} = 9.75$ for δ-chymotrypsin.

FIG. 4 (center). $K_m$-$pH$ profiles for the α₁-chymotrypsin (○); α₂-chymotrypsin (prepared according to the procedure described in this paper) (△); α₁-chymotrypsin (prepared according to Miller et al. (20)) (▲); and δ-chymotrypsin-catalyzed (●) hydrolyses of $N$-trans (2-furyl)acryloyl-$L$-tryptophan methyl ester. The solid lines are calculated as in Fig. 3 using the values $pK_e = 9.0$, $pK_{e^S} > 11$ for α₁-chymotrypsin and $pK_e = 9.4$, $pK_{e^S} = 10.2$ for α₁- and δ-chymotrypsins.

FIG. 5 (right). $K_m$-$pH$ profiles for the α₁-chymotrypsin (○); α₂-chymotrypsin (prepared according to the procedure described in this paper) (△); α₁-chymotrypsin (prepared according to Miller et al. (20)) (▲); and δ-chymotrypsin-catalyzed (●) hydrolyses of $N$-trans (2-furyl)acryloyl-$L$-phenylalanine methyl ester. The solid lines are calculated as in Fig. 3 using the values $pK_e = 9.0$, $pK_{e^S} > 11$ for α₁-chymotrypsin, $pK_e = 9.0$, $pK_{e^S} = 10.1$ for α₂-chymotrypsin and $pK_e = 9.2$, $pK_{e^S} = 10.1$ for δ-chymotrypsin.

Our original strategy in preparing α₁-chymotrypsin was based on the observations of Rovery et al. (11). Our results show that under a particular set of conditions, α₁-chymotrypsin can be prepared with a 80 to 90% yield by controlled proteolysis of chymotrypsinogen A at 25°C followed by rapid activation by trypsin at 0°C.

α₁-Chymotrypsin also has been prepared recently by Miller et al. (20) using a different approach. Based on the early observations of Bettelheim and Neurath (10), by autolyzing concentrated solutions of δ-chymotrypsin, they have isolated and partially characterized an active enzyme with amino-terminal groups corresponding to those of α₁-chymotrypsin. The enzyme was called α₁-chymotrypsin, ignoring the fact that the same enzyme has been called α₂-chymotrypsin before (11, 12).

Our preparation of α₁-chymotrypsin is quite stable. We found no change in the nature of its end group after 2 weeks storage at 2°C, pH 3.9 at a concentration of 1 mM or less. Similar conclusions have been reached by Miller et al. (20) with their α₁-chymotrypsin (α₁-chymotrypsin) preparation.

It is quite possible that the set of reactions by which we have prepared α₁-chymotrypsin occur under physiological conditions. We would like to suggest that the pathway, chymotrypsinogen A $\rightarrow$ thr-neochymotrypsinogen $\rightarrow$ α₁-chymotrypsin, should be included in future chymotrypsinogen A activation schemes as an alternative route to α₁-chymotrypsin or related enzymes. Whether this pathway, or the classical one: chymotrypsinogen A $\rightarrow$ nla-neochymotrypsinogen $\rightarrow$ α₁-chymotrypsin prevails, will depend on whether the dipeptide Thr-Asp is split out before or

### TABLE III

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$</th>
<th>α₁-Chymotrypsin</th>
<th>δ-Chymotrypsin</th>
<th>α₂-Chymotrypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzyl alcohol</td>
<td>≥300</td>
<td>49.0 ± 2.1</td>
<td>52.0 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Tryptophol</td>
<td>≥30</td>
<td>3.0 ± 0.2</td>
<td>3.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-$d$-tryptophanamide</td>
<td>≥70</td>
<td>7.1 ± 0.3</td>
<td>8.0 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

a Carbonate buffer, 25°C in 3.1% (v/v) dimethyl sulfoxide-water. The values represent the average of three $K_i$ determinations.
b Values from Valenzuela and Bender (3).

### DISCUSSION

α₁-Chymotrypsin was recognized by Rovery et al. (9) and Bettelheim and Neurath (10) as being present in mixtures obtained from activation of chymotrypsinogen A by trypsin. Bettelheim and Neurath (10) showed that when a rapid activation mixture (zymogen-trypsin, 20:1) was left standing at 5°C for 56 hours, the product had only 0.21 residue of NH₂-terminal alanine but the same COOH-terminal residues as α₁-chymotrypsin. Rovery et al. (11) obtained under certain conditions a neochymotrypsinogen with threonine instead of alanine as NH₂-terminal group. It was suggested that this neochymotrypsinogen could eventually give rise to an active enzyme by the attack of trypsin.
after the action of trypsin. This is probably a function of the time of action and relative concentration of trypsin in the activation site.

The main objective of the present work was to study the kinetic properties of $\alpha$-chymotrypsin at alkaline pH. Because of its peculiar structure, having threonine instead of alanine in the C-chain terminus, the properties of this enzyme are particularly important in trying to explain the reason for the different alkaline pH behavior between $\alpha$- and $\delta$-chymotrypsin. This phenomenon has not yet been explained.

During the course of our investigations on the kinetic properties of $\delta$-chymotrypsin, we have found several significant differences between this enzyme and $\alpha$-chymotrypsin. The binding ability of $\delta$-chymotrypsin does not decrease as much at alkaline pH as it does in $\alpha$-chymotrypsin. $K_m$ values for the $\delta$-chymotrypsin-catalyzed hydrolysis of specific ester and amide substrates are considerably lower than those of $\alpha$-chymotrypsin and show only a small increase at alkaline pH (7). The binding of competitive inhibitors to both enzymes indicates the same difference (3). Kinetic measurements and proton uptake studies upon binding of substrates and inhibitors show that the $pK_a$ shifts occurring in $\delta$-chymotrypsin upon binding of substrate are significantly smaller (0.5 to 1.0 $pK_a$ units) than those found with $\alpha$-chymotrypsin (2.0 or more $pK_a$ units).

There is much evidence available indicating that there is an equilibrium between active and inactive forms of $\alpha$-chymotrypsin at alkaline pH (1, 5, 6). It is believed that this transition is governed by the ionization state of the amino group if isoleucine-16 (1, 6, 22). Our investigations have shown that this transition is different in both enzymes. The alkaline pH form of $\alpha$-chymotrypsin is unable to bind substrate and therefore completely inactive, whereas the alkaline form of $\delta$-chymotrypsin is able to bind substrate and therefore active. The difference on the $pK_a$ shifts is also an indication of a difference in the conformation of the enzyme-substrate complex at alkaline pH between $\alpha$- and $\delta$-chymotrypsin.

In this investigation a substantially different behavior at alkaline pH was found between $\alpha$- and $\alpha$-chymotrypsin. The results indicate that $\alpha$-chymotrypsin, in contrast to $\alpha$-chymotrypsin, is able to bind substrate and is active at alkaline pH. Since the main structural difference relevant to the alkaline pH dependence between the two enzymes is the presence of a free amino group of alanine-149 in the former, our results can be taken as strong evidence for the participation of the alanine-149 amino group in the reversible inactivation of $\alpha$-chymotrypsin at high pH.

Recent observations of Kaplan (24) seem to confirm the postulated involvement of alanine-149. Studying the properties of the isoleucine-16 amino group by a competitive labeling technique, Kaplan found that above pH 9.8 $\alpha$-chymotrypsin undergoes an additional conformational change not controlled by the isoleucyl NH$_2$ terminus (24). Furthermore, experiments with the alanine-149 amino group suggest that this group is involved in a conformational change on deprotonation of the isoleucyl NH$_2$ terminus (24).

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Preparation and Kinetic Properties of a New Form of Chymotrypsin Which Is Active at Alkaline pH: $\alpha_1$-Chymotrypsin

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