Preparation and Kinetic Properties of a New Form of Chymotrypsin Which Is Active at Alkaline pH: \( \alpha_l \)-Chymotrypsin*

(Pablo Valenzuela and Myron L. Bender)

**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 \( \alpha_l \)-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 \( \delta \)-chymotrypsin are similar to those of \( \alpha \)- and \( \delta \)-chymotrypsins. The \( K_m \) values showed a progressive increase toward the alkaline pH region. The shape of the \( K_m \)-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 amine 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 \( \alpha_l \)-chymotrypsin following an early suggestion by Roversi et al. (11).

We have found that the kinetic properties of \( \alpha_l \)-chymotrypsin in the neutral and alkaline pH regions strongly resemble those of \( \delta \)-chymotrypsin. \( \alpha_l \)-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 CFC-SCC); 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 acetone/methanol before use. N-Trans(2-furyl)acryloyl-L-tryptophan methyl ester was a Cyclo Chemical Co. product and was crystallized twice from ethyl acetate-hexane before use.

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*This work was supported by Grant ROI-HLO-5726 from the National Institutes of Health, United States Public Health Service. 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 \( \alpha_l \)-chymotrypsin following an early suggestion by Roversi et al. (11). We have found that the kinetic properties of \( \alpha_l \)-chymotrypsin in the neutral and alkaline pH regions strongly resemble those of \( \delta \)-chymotrypsin. \( \alpha_l \)-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).
N-trans(2-furyl)acryloyl-L-phenylalanine methyl ester was synthesized from L-phenylalanine methyl ester (Mann Research Laboratories) and furoyl chloride. 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-L-trypophanamine (Cyto 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. Benzyl alcohol was purified as described before (3). Stock solutions of substrates were prepared in either acetone-triethylamine or dimethyl sulfoxide (Matheson, Coleman and Bell). All buffer solutions had an ion 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-L-trypophan methyl ester was followed at 300 nm as described before (7). The hydrolysis of N-trans-(2-furyl)acryloyl-L-trypophan methyl ester was followed at 335 nm as described before (3). The hydrolysis of N-trans-(2-furyl)acryloyl-L-phenylalanine methyl ester was followed at 335 nm. The absorbance data were converted into rate data using $\Delta A = 1220 \text{ m}^{-1} \text{cm}^{-1}$ as the difference in molar absorptivities between the ester and the acid. The results were analyzed using a onerun digital computer program based on a least squares analysis of $v/s$ versus $r$ (14). Stock solutions of all enzymes were prepared in 0.1 M KCl-0.001 M HCl immediately before use. The normality of active enzyme solutions was determined by spectrophotometric titration with N-trans-cinnamoylimidazole (15). The pH was determined at the beginning and at the end of each reaction using a Radiometer 4C or a Corning model 12 pH meter. Competitive inhibition constants were determined using N-trans-(2-furyl)acryloyl-L-trypophan methyl ester as substrate, as described elsewhere (3).

**End Group Determinations**—Quantitative NH$_2$-terminal analysis was performed by the method of Sanger (16) after first inhibiting the enzymes with $4 \times 10^{-4}$ M disopropyl phosphorofluoridate at pH 7.0, 0°C for 90 min. Dinitrophenyl amino acids were determined spectrophotometrically after separation by thin layer chromatography as described by Labouesse and Gervais (11). Quantitative COOH-terminal residue determinations were carried out using disopropyl phosphorofluoridate-treated carboxypeptidases A as described by Potte (18). Peptides were incubated with 1 mg per ml of carboxypeptidase A at 1:20 ratio by weight to the substrate in 0.2 M NH$_4$HCO$_3$. Control tubes with carboxypeptidase A and protein substrate alone were also prepared. Incubation was continued for 2 hours during which 200-μl aliquots were removed at appropriate time periods, the protein was removed from them, then they were lyophilized and applied to an amino acid analyzer. Amino acid analyses were performed using Spineo amino acid analyzers model 120-B and 120-C with regular Spineo AA-15 and AA-28 resin.

**Preparation of Affinity Chromatography Columns**—When necessary enzyme preparations were purified from inactive material by affinity chromatography on Sepharose-ε-amincaproyl-L-trypophan methyl ester (18). ε-Aminocaproyl-L-trypophan methyl ester was synthesized as follows. 1.1 Equivalents of L-trypophan methyl ester (Cyto Chemical Co.), 1.1 eq of triethylamine and 1 eq of dicyclohexylcarbodimide were added to a solution of 1 eq of N'-Z-aminocaproic acid (Cyto Chemical Co.) in dichloromethane solution (Z = benzoyloxycarbonyl). After overnight stirring at room temperature, the precipitated material was filtered and the filtrate washed with 1 N HCl, water, 1 N NaHCO$_3$, and water. The material showed only one spot (ultraviolet and I$_2$-positive, ninhydrin-negative, 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 ethanol, at 40 mm above atmospheric pressure for 3 hours. The material obtained after filtration and removal of the solvent showed one spot (ultraviolet, I$_2$ and ninhydrin-positive, RF 0.83) 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 N'-Neochymotrypsinogen A—Chymotrypsinogen A (100 mg) was treated with 5 mg of δ-chymotrypsin and 2 mg of soybean trypsin inhibitor in 0.1 M phosent a 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 disopropyl 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 enzymic activity. They were characterized by analysis of their NH$_2$-terminal and COOH-terminal groups (Table I).

**Activation of N'-Neochymotrypsinogen A to α-Chymotrypsin**—One hundred milligrams of N'-neochymotrypsinogen A were treated with 5 mg of trypsin in 0.1 M Tris-HCl buffer, pH 8.0, 0.065 M CaCl$_2$ at 0°C. After 1 hour, the mixture was dialyzed against 5 \times 10^{-4} M HCl and purified by chromatography on a Sepharose-ε-amincaproyl-L-trypophan methyl ester column.

### Table I

Equivalent of chain terminal amino acids per mole of α-Chymotrypsin and related proteins

<table>
<thead>
<tr>
<th>NH$_2$-terminal residues</th>
<th>ε-neochymotrypsinogen$^a$</th>
<th>Neochymotrypsinogen$^a$</th>
<th>α-Chymotrypsin$^b$</th>
<th>δ-Chymotrypsin$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>0.11; 0.08</td>
<td>0.09; 0.11</td>
<td>0.11; 0.14</td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>0.85; 0.82</td>
<td>0.82; 1.0</td>
<td>0.77; 0.96</td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>0.08; traces</td>
<td>0.08; 0.10</td>
<td>0.08; 0.10</td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>traces</td>
<td>traces</td>
<td>traces</td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>0.09; traces</td>
<td>0.82; 1.0</td>
<td>0.80; 1.0</td>
<td></td>
</tr>
<tr>
<td>COOH-terminal residues</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td>1.15; 1.10</td>
<td>1.13; 1.13</td>
<td>1.10</td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>0.15; 0.12</td>
<td>1.2; 1.2</td>
<td>1.10</td>
<td></td>
</tr>
</tbody>
</table>

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*a* 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 δ-chymotrypsin by the procedure of Miller et al. (20).

*Fractional relative to the measured amount of isoleucine.
The sample was applied as a concentrated solution in 5 x 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 x 10^{-4} M HCl and lyophilized. The protein was further characterized by quantitative NH2-terminal and COOH-terminal analyses. Results are shown in Table I.

Preparation of α1-Chymotrypsin—Two different procedures were employed to prepare this form of chymotrypsin. The first procedure is based on the observations of Roversy et al. (9, 12). They reported that the action of δ-chymotrypsin on chymotrypsinogen A in the absence of trypsin activity resulted in an inactive protein with threonine as NH2-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 NH2-terminal residues (11). The name α1-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 thr-neochymotrypsinogen (Fig. 1, Compound II). Two sets of conditions were found to be optimal regarding the yield of NH2-terminal threonine and the lack of nonspecific autolysis during the reaction. One of them involves incubation of chymotrypsinogen with δ-chymotrypsin at pH 7.6 for 12 hours at 25° 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°. The use of lower incubation temperature like 4° or 0° 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 thr-neochymotrypsinogen 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 NH2-terminal residue and tyrosine as COOH-terminal residue, as expected from the structure of thr-neochymotrypsinogen (Fig. 1, Compound II). A small amount of NH2-terminal alanine was consistently found in all preparations. This analysis indicates that the preparation contains at least 10% of the contaminant α1a-neochymotrypsinogen. 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 thr-neochymotrypsinogen to α1-chymotrypsin was accomplished by incubating the zymogen with 5% (w/w) trypsin at 0°. 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 NH2-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 α1-chymotrypsin (Fig. 1, Compound III). The existence of a small amount of NH2-terminal alanine indicates the presence of about 10% of α-chymotrypsin, which probably arises from the activation of the 10% α1a-neochymotrypsinogen contaminant.

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

The second procedure employed to prepare α1-chymotrypsin used in our kinetic experiments was that described by Miller et al. for the conversion of δ-chymotrypsin into an enzyme form called by them κ-chymotrypsin (20). According to the results on the NH2-terminal analysis that they reported, κ-chymotrypsin is identical with the previously prepared and described α1-chymotrypsin (12). End group analysis of our preparation confirmed these findings of Miller et al. (20). As shown in the
Kinetic constants for the hydrolysis of specific ester substrates catalyzed by $\alpha$-chymotrypsin at different pH values

<table>
<thead>
<tr>
<th>pH</th>
<th>Buffer</th>
<th>$k_{cat}$ sec$^{-1}$</th>
<th>$K_m \times 10^5$M</th>
<th>$k_{cat}$ sec$^{-1}$</th>
<th>$K_m \times 10^6$M</th>
<th>$k_{cat}$ sec$^{-1}$</th>
<th>$K_m \times 10^6$M</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.83</td>
<td>Phosphate</td>
<td>26.8 ± 0.3</td>
<td>7.5 ± 0.4</td>
<td>23.9 ± 1.2</td>
<td>5.0 ± 1.0</td>
<td>17.1 ± 0.5</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>7.00</td>
<td>Phosphate</td>
<td>37.1 ± 0.4</td>
<td>7.4 ± 0.6</td>
<td>31.4 ± 0.8</td>
<td>5.6 ± 0.5</td>
<td>36.3 ± 0.4</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>7.81</td>
<td>Phosphate</td>
<td>43.3 ± 0.4</td>
<td>6.4 ± 0.5</td>
<td>32.4 ± 1.3</td>
<td>4.6 ± 0.7</td>
<td>46.0 ± 1.2</td>
<td>4.0 ± 0.2</td>
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<tr>
<td>8.20</td>
<td>Tris-HCl</td>
<td>46.8 ± 0.8</td>
<td>6.9 ± 0.2</td>
<td>36.2 ± 1.4</td>
<td>6.2 ± 0.3</td>
<td>45.6 ± 0.9</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>9.83</td>
<td>Veronal</td>
<td>52.3 ± 0.5</td>
<td>6.9 ± 0.5</td>
<td>36.8 ± 0.0</td>
<td>7.6 ± 1.2</td>
<td>50.0 ± 1.3</td>
<td>4.0 ± 0.5</td>
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<tr>
<td>9.05</td>
<td>Veronal</td>
<td>51.5 ± 0.4</td>
<td>10.3 ± 0.7</td>
<td>36.9 ± 0.5</td>
<td>7.9 ± 0.8</td>
<td>52.8 ± 2.1</td>
<td>6.1 ± 0.6</td>
</tr>
<tr>
<td>9.30</td>
<td>Carbonate</td>
<td>53.8 ± 0.8</td>
<td>13.0 ± 1.1</td>
<td>42.9 ± 1.0</td>
<td>9.8 ± 0.6</td>
<td>60.0 ± 0.8</td>
<td>10.1 ± 0.5</td>
</tr>
<tr>
<td>9.61</td>
<td>Carbonate</td>
<td>54.6 ± 1.0</td>
<td>16.3 ± 2.8</td>
<td>39.9 ± 1.0</td>
<td>12.4 ± 0.8</td>
<td>63.3 ± 0.6</td>
<td>13.0 ± 0.4</td>
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<tr>
<td>9.80</td>
<td>Carbonate</td>
<td>55.2 ± 1.2</td>
<td>18.5 ± 0.5</td>
<td>38.5 ± 0.8</td>
<td>14.2 ± 1.7</td>
<td>63.1 ± 1.2</td>
<td>15.3 ± 0.6</td>
</tr>
<tr>
<td>10.02</td>
<td>Carbonate</td>
<td>52.3 ± 1.1</td>
<td>20.6 ± 1.0</td>
<td>40.8 ± 2.1</td>
<td>18.3 ± 1.5</td>
<td>61.6 ± 1.5</td>
<td>18.1 ± 1.0</td>
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<tr>
<td>10.22</td>
<td>Carbonate</td>
<td>51.7 ± 0.8</td>
<td>23.0 ± 2.0</td>
<td>39.6 ± 2.1</td>
<td>23.3 ± 2.1</td>
<td>59.9 ± 1.3</td>
<td>21.7 ± 0.6</td>
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<tr>
<td>10.40</td>
<td>Carbonate</td>
<td>51.5 ± 1.2</td>
<td>29.9 ± 2.1</td>
<td>39.7 ± 1.8</td>
<td>27.5 ± 1.8</td>
<td>67.5 ± 3.8</td>
<td>29.0 ± 2.0</td>
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<tr>
<td>10.60</td>
<td>Carbonate</td>
<td>47.6 ± 1.3</td>
<td>31.9 ± 1.6</td>
<td>37.6 ± 1.5</td>
<td>30.6 ± 2.1</td>
<td>62.5 ± 2.1</td>
<td>37.3 ± 1.8</td>
</tr>
<tr>
<td>10.80</td>
<td>Carbonate</td>
<td>45.8 ± 1.5</td>
<td>35.5 ± 1.5</td>
<td>35.2 ± 1.9</td>
<td>34.0 ± 4.5</td>
<td>63.2 ± 2.5</td>
<td>35.0 ± 1.9</td>
</tr>
<tr>
<td>11.02</td>
<td>Carbonate</td>
<td>45.8 ± 1.8</td>
<td>40.0 ± 4.0</td>
<td>33.1 ± 2.1</td>
<td>35.0 ± 3.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>11.23</td>
<td>Carbonate</td>
<td>46.1 ± 1.8</td>
<td>41.0 ± 4.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

$^{1}$pK$_E$ is a constant for a given enzyme. Within the experimental error, the pK$_E$ values used in fitting the $\alpha$-chymotrypsin curves are very similar and we do not know which is correct.

The behavior of $\alpha$-chymotrypsin can be seen in more detail in Figs. 3, 4, and 5 where the $K_m$-pH profiles obtained with this enzyme are compared with those obtained with $\alpha$-chymotrypsin and $\delta$-chymotrypsin. Two different preparations of $\alpha$-chymotrypsin were employed. Fig. 3 shows the results obtained using $\gamma$-acetyl-L-tryptophan methyl ester as substrate. It can be seen that the $K_m$ values for the $\alpha$-chymotrypsin-catalyzed reaction (triangles) increase significantly less above pH 9 compared to $\alpha$-chymotrypsin. The shape of the curves resembles closely that of $\delta$-chymotrypsin, although the $K_m$ values are a bit higher. When these data are treated according to the method of Dixon (22), the results 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$_E$ of 10.2. These values indicate that $K_m$ values should level off above pH 11.

The $K_m$-pH profiles obtained using $\gamma$-trans-(2-furyl)acryloyl-L-tryptophan methyl ester as substrate are shown in Fig. 4. The $K_m$ values for both preparations of $\alpha$-chymotrypsin increase above pH 9 showing the same profile as the one obtained with $\delta$-chymotrypsin. The data for $\delta$-chymotrypsin and $\alpha$-chymotrypsin are consistent with a dependence on a group of the enzyme with an apparent pK$_E$ of 9.4, which again shifts upon substrate binding to a pK$_E$ of 10.2.

Fig. 5 shows the results obtained with $\gamma$-trans-(2-furyl)acryloyl-L-phenylalanine methyl ester as substrate. Again the same general results are obtained. The behavior of $\alpha$-chymotrypsin is very different than that of $\alpha$-chymotrypsin and resembles closely that of $\delta$-chymotrypsin. The profile obtained with $\alpha$-chymotrypsin indicates a dependence on a group of the enzyme with an apparent pK$_E$ of 9.0, which changes up 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 $\alpha$-chymotrypsin was studied at pH 10.5 by competitive inhibition kinetics using $\gamma$-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 $\alpha$-chymotrypsin and which are essentially the same as those of $\delta$-chymotrypsin.

*Note: The table above includes kinetic constants for the hydrolysis of specific ester substrates catalyzed by $\alpha$-chymotrypsin at different pH values. The values are presented in a tabular format with pH levels, buffer types, $k_{cat}$ sec$^{-1}$ values, and $K_m \times 10^5$M and $K_m \times 10^6$M values. The behavior of $\alpha$-chymotrypsin is compared with other forms of chymotrypsin, and the results 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$_E$ of 10.2. The $K_m$ values indicate that $K_m$ values should level off above pH 11.

*References (3, 7)*
FIG. 3 (left). $K_m$-pH profiles for α-chymotrypsin (○); αI-chymotrypsin (prepared according to the procedure described in this paper) (Δ); αL-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:

$$K_m = \frac{1 + K_{\text{app}}}{1 + \frac{K_{\text{app}}}{[H^+]}},$$

using the values $pK_{\text{a}} = 9.0$, $pK_{\text{a}} > 11$ for α-chymotrypsin; $pK_{\text{a}} = 9.3$, $pK_{\text{a}} > 10.2$ for αI-chymotrypsin (Δ) and $pK_{\text{a}} = 9.25$, $pK_{\text{a}} > 9.75$ for δ-chymotrypsin.

FIG. 5 (right). $K_m$-pH profiles for the α-chymotrypsin (○); αI-chymotrypsin (prepared according to the procedure described in this paper) (Δ); αI-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 $pK_{\text{a}} = 9.0$, $pK_{\text{a}} > 11$ for α-chymotrypsin and $pK_{\text{a}} = 9.4$, $pK_{\text{a}} > 10.2$ for αI- and δ-chymotrypsins.

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

αI-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 αI-chymotrypsin. The enzyme was called αI-chymotrypsin, ignoring the fact that the same enzyme has been called αI-chymotrypsin before (11, 12).

Our preparation of αI-chymotrypsin is quite stable. We found no change in the nature of its end group after 2 weeks storage at 2°C, pH 3.0 at a concentration of 1 mM or less. Similar conclusions have been reached by Miller et al. (20) with their αI-chymotrypsin (αI-chymotrypsin) preparation.

It is quite possible that the set of reactions by which we have prepared αI-chymotrypsin occur under physiological conditions. We would like to suggest that the pathway, chymotrypsinogen A → Thr-neochymotrypsinogen → αI-chymotrypsin, should be included in future chymotrypsinogen A activation schemes as an alternative route to α-chymotrypsin or related enzymes. Whether this pathway, or the classical one: chymotrypsinogen A → Ala-neochymotrypsinogen → α-chymotrypsin prevails, will depend on whether the dipeptide Thr-Asp is split out before or not.
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 \( \beta \)-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 indicate 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, 23). 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 \( \chi \)-chymotrypsins. The results indicate that \( \alpha \)-chymotrypsin, in contrast to \( \alpha \)-chymotrypsin, is able to bind substrate and therefore 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 irreversible inactivation of \( \alpha \)-chymotrypsin at high pH.

Recent observations of Kaplan (21) 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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