Modification of Isoleucine-16 of Acetylated \( \delta \)-Chymotrypsin

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SUMMARY

Activation of acetylated chymotrypsinogen with trypsin leads to catalytically active acetylated \( \delta \)-chymotrypsin containing NH\(_2\)-terminal isoleucine. The importance of the cationic terminus to the control of the active conformation of acetylated \( \delta \)-chymotrypsin has been demonstrated (Oppenheimer, H. L., Labouesse, B., and Hess, G. P. (1966) J. Biol. Chem. 241, 2720). Later studies appeared to suggest that the modification of isoleucine-16 of \( \delta \)-chymotrypsin is not accompanied by the loss of catalytic activity as measured by the hydrolysis of N-acetyl-L-tyrosine ethyl ester (Agarwal, S. P., Martin, C. J., Blair, T. T., and Marini, M. A. (1971) Biochem. Biophys. Res. Commun. 43, 510; Blair, T. T., Marini, M. A., Agarwal, S. P., and Martin, C. J. (1971) FEBS Lett. 14, 86) or by the loss of active site content (Ghelis, C., Garel, J. R., and Labouesse, J. (1970) Biochemistry 9, 3902). In the present studies, controlled acetylation of the terminal \( \alpha \)-amino group of acetylated \( \delta \)-chymotrypsin with acetic anhydride led to a progressive loss of active sites of the enzyme. Determination of the catalytic and kinetic properties of the modified enzyme with the specific ester substrate N-acetyl-L-tyrosine ethyl ester or the nonspecific substrates \( \rho \)-nitrophenyl acetate and cinnamoyl imidazole gave nearly identical results. With N-acetyl-L-tyrosine ethyl ester as substrate, the acylation rate constants (0.01 s\(^{-1}\) at pH 8.5, 25\(^\circ\), in 3.3% acetonitrile) and the deacylation rate constants (0.01 s\(^{-1}\) at pH 8.5, 25\(^\circ\), in 3.3% acetonitrile) are identical for the acetyl isoleucine-16 and the isoleucine-16 species. This value correlates well with about 20% of the active site content of half-cysteine-1 is completely exposed and has a pK\(_a\) of 9.0 (6, 7). Participation of a group with a pK\(_a\) of about 9.0 in the activity of chymotrypsin has also been implicated from kinetic studies based on acyl-enzyme mechanism (8-11) as well as from the protoprotease equilibria studies of Hess et al. on diisopropylphosphoryl-\( \alpha \) -chymotrypsin (12, 13). Chemical and kinetic studies of both \( \alpha \)-chymotrypsin and acetylated \( \delta \)-chymotrypsin led Oppenheimer et al. (6) to propose that the ionization state of isoleucine-16 is important in the control of conformation and activity of chymotrypsins. Further support for this proposal has come from the x-ray crystallographic studies of Mathews et al. (14) and of Sigler et al. (1). These authors have shown that the salt linkage between isoleucine-16 and the aspartic acid residue adjacent to the active site serine residue maintains proper orientation of catalytically functional groups in \( \alpha \)-chymotrypsin.

Proteolytic activation of chymotrypsinogen to chymotrypsin leads to a new NH\(_2\)-terminal residue, isoleucine-16\(^\prime\) (2, 3). The activation phenomenon has been shown to be associated with conformational changes in the zymogen (4, 5). This observation is further supported by studies on the pH dependence of the specific rotation of zymogen and enzyme; the specific rotation of zymogen is independent of pH, whereas that of the active enzyme suggests a conformational transition with a pK\(_{\alpha}\) of about 9.0 (6, 7). Participation of a group with a pK\(_a\) of about 9.0 in the activity of chymotrypsin has also been implicated from kinetic studies based on acyl-enzyme mechanism (8-11) as well as from the protoprotease equilibria studies of Hess et al. on diisopropylphosphoryl-\( \alpha \)-chymotrypsin (12, 13). Chemical and kinetic studies of both \( \alpha \)-chymotrypsin and acetylated \( \delta \)-chymotrypsin led Oppenheimer et al. (6) to propose that the ionization state of isoleucine-16 is important in the control of conformation and activity of chymotrypsins. Further support for this proposal has come from the x-ray crystallographic studies of Mathews et al. (14) and of Sigler et al. (1). These authors have shown that the salt linkage between isoleucine-16 and the aspartic acid residue adjacent to the active site serine residue maintains proper orientation of catalytically functional groups in \( \alpha \)-chymotrypsin.

The technique of competitive labeling designed by Kaplan et al. (15) has been used to measure the ionization constants and reactivities of the three NH\(_2\) termini of \( \alpha \)-chymotrypsin. These results show that the NH\(_2\)-terminal half-cysteine-1 is completely exposed and has a pK\(_a\) of 7.9; the NH\(_2\)-terminal isoleucine has a pK\(_a\) of 8.9 and is buried, and alanine-149 has a pK\(_a\) of 8.5 and is partially buried (16). Furthermore, the reactivity of alanine-149 is apparently dependent on the ionization state of isoleucine-16. From their studies on chemical modification of \( \alpha \)-
and δ-chymotrypsins by nitrous acid, Dixon and Hofmann (17) assigned an abnormal pKα to the ε-amino group of isoleucine-16 and demonstrated that the modification of this amino group leads to an inactive enzyme. Recently, Fersht and Requena (18) and Fersht (19) have demonstrated that an equilibrium between active and inactive forms of α- and δ-chymotrypsins exists at neutral and alkaline pH values. The control of these conformations has been assigned to the ionization state of isoleucine-16.

In contrast to the observations reported above, prototropic titrations carried out by Marini and Martin (20) assigned normal ionization behavior to the ε- and γ-amino groups of δ-chymotrypsinogen. In later studies, succinylation (21) and amidination (22) of δ-chymotrypsin was found to bring about little or no change in the catalytic activities and active site contents of the modified enzymes. Ghelis et al. (23) in 1970 showed that the acetylation of the isoleucine-16 NH2 terminus led to the loss of enzyme activity when measured with the specific substrates L-AcTyr-Ort. However, the active site content determined by prolonged treatment with [1H]Pr2P-F pointed to almost total retention of the active sites of the modified enzyme.

In view of the controversy between these findings, we decided to reinvestigate the problem. The results of chemical and kinetic studies described in this communication disagree with the studies of trypsin and chymotrypsinogen. In later studies, succinylation of the α- and γ-amino groups of ω-chymotrypsin and chymotrypsinogen. In later studies, succinylation (21) and amidination (22) of δ-chymotrypsin was found to bring about little or no change in the catalytic activities and active site contents of the modified enzymes. Ghelis et al. (23) in 1970 showed that the acetylation of the isoleucine-16 NH2 terminus led to the loss of enzyme activity when measured with the specific substrates L-AcTyr-Ort. However, the active site content determined by prolonged treatment with [1H]Pr2P-F pointed to almost total retention of the active sites of the modified enzyme.

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**EXPERIMENTAL PROCEDURES**

**Materials**

Salt-free chymotrypsinogen was obtained from Worthington (Lots CG 4GA and CG 769) and from Mann (Lot T-1001). Solutions of the zymogen (10 mg per ml) were centrifuged at 10,000 rpm for 10 min. Salt-free, three times crystallized α-chymotrypsin was obtained from Worthington (Lots CD1 6148-9 and CD1 6LD); these α-chymotrypsin preparations were found to be 95 to 97% active, as determined with NPh-OAc, cinnamoyl imidazole, or AsTyr-Ort. Trypsin, pepsin and chymotrypsinogen, were obtained from Worthington (Lot TRL 71C).

Two lots of NPh-OAc (Mann C-2300 and C-2399), of melting point 77-78°, were used without further purification. AcTyr-Ort was obtained by a chromatographically pure preparation from Cyclo (Lot R-36069). N-Trans-cinnamoyl imidazole, with melting point of 153-154°, was prepared according to the method of Schonbaum et al. (24). Acetic anhydride, obtained from Mallinckrodt, was distilled before use. 1-3H]Acetic anhydride, with specified specific activity of 10 mCi per mmol, came from New England Nuclear; all dilutions prepared for experiments were counted for radioactivity, and in all cases the counts per minute were 35 to 50% of the expected levels. The liquid scintillator was Bray's solution (25). Sources of other chemicals have been described previously (6).

**Methods**

**Preparation of Chymotrypsin Derivatives—**Preparation of acetylated chymotrypsinogen in aqueous solution (about 10 mg per ml) was carried out as described in an earlier paper (6) except that in some experiments a different procedure was used for the deacylation of hydroxyl groups of tyrosine and serine residues. In these experiments, deacylation was effected with 0.5 M hydroxylamine at pH 7.5; the protein solution was brought to the desired concentration of hydroxylamine by the addition of 5 M or 2.5 M neutralized hydroxylamine-1HCl, the pH was adjusted, and the solution maintained at ice temperature for a period of 30 min.

Excess hydroxylamine was eliminated by Sephadex filtration of the protein as described later. Acetylated δ-chymotrypsin and acetylated Pr2P-δ-chymotrypsin were prepared from the acetylated zymogen as has been described previously (6). Acetylated α-chymotrypsin was prepared from α-chymotrypsin according to the procedure used for the acetylation of the zymogen, except that the reaction was carried out in 90% sodium acetate solution as well as in water.

The materials termed recacetylated δ-chymotrypsin and recacetylated Pr2P-δ-chymotrypsin were prepared by treating the corresponding acetylated preparations with acetic anhydride and then affecting deacylation of hydroxyl groups with hydroxylamine. The usual procedure was to add 0.1 ml of acetic anhydride solution per 10 ml of acetylated protein (of concentration 4 to 10 mg per ml) at pH 5.7 over a period of 40 min to give a final acetic anhydride concentration of about 0.1 M. The reaction mixture was allowed to stand for another 40 min at pH 6.7 in order to permit complete acetylation of the protein and hydrolysis of excess anhydride, and then the deacylation of hydroxyl groups was effected by treatment with hydroxylamine as in the preparation of acetylated chymotrypsinogen. The extent of recacetylation could be controlled, however, by varying such conditions as the amount of acetic anhydride or the time period of the reaction. Specific radioactive activity of active site contents was calculated on the assumption that the incorporation of one acetyl group per mol of protein would yield a product exhibiting at least 500 counts per min per mg.

**Sephadex Filtration—**Acetylated chymotrypsinogen preparations were fractionated by passage through a Sephadex G-25 column following the deacylation treatment with hydroxylamine. A column (4 X 120 cm) was packed, equilibrated with water, and set up in a cold room (4°). The protein material, at a concentration of 4 to 10 mg per ml, was applied in aqueous solution and eluted with water at a flow rate of 40 to 50 ml per hour. The material was collected in 10-ml fractions with a total recovery of 85 to 90%. A smaller column (2.5 X 30 cm) was employed for fractionating recacetylated δ-chymotrypsin. Dilute protein solutions were concentrated at 133-134°, was prepared according to the method of Worthington (Lot TRL 71C).

**Quantitation of NH2-terminal Residues—**A procedure suggested by Spackman et al. (26) was used to identify NH2-terminal residues on the automatic amino acid analyzer. The analysis was preceded by a carboxymyation procedure described by Stark and Smyth (27). A solution containing about 2.5 mg of protein per ml (obtained from the Sephadex filtration) was made 8.5 M in urea and 0.62 M in KCNO at pH 8 (N-ethylmorpholine buffer), and allowed to react for 20 hours at 50°. The reaction mixture was dialyzed overnight against running water, and the protein concentration was determined by the Folin reaction with α-chymotrypsin as standard. Just prior to the cyclization of hydantoin, carboxamoyl-PL-norleucine in a concentration approximately that of chymotrypsin was added to the test solution as an internal standard.

**Assay of Active Site Content**

A. With Cinnamoyl Imidazole—Enzyme preparations were assayed for active site content by assay with cinnamoyl imidazole according to the method of Schonbaum et al. (24). Measurements were made at pH 5.0 and 25°. Initial velocity of the reaction was 3 to 15 μM protein, determined spectrophotometrically as described above, 30 μM cinnamoyl imidazole, 3.5% acetone.

* Acetylated e-chymotrypsin refers to the active enzyme obtained from zymogen, in which all free amino groups are acetylated. Recacetylated δ-chymotrypsin refers to acetylated δ-chymotrypsin with the NH2-terminal isoleucine blocked by an acetyl group. Unless mentioned otherwise, the active site concentration of recacetylated enzyme was about 0.2 mol/mol.
nitrile, and 0.1 M acetate buffer. Corrections were made for enzyme absorption at 310 nm.

B. With NPh-OAc—The general procedure was to enter 2.5 ml of buffer solution (0.05 M potassium pyrophosphate, pH 8.5) into sample and reference cells maintained at 25°. Then 0.1 ml of NPh-OAc stock (3.47 mM in dry acetonitrile) was added to the sample cell, and optical density at 400 nm was recorded for 2 min. Then 0.5 ml of enzyme stock solution (0.3 to 3.0 mg per ml) was quickly transferred to the sample cell. The solution was rapidly mixed and its absorbance change recorded for 2 to 5 min. Measurements were made of an initial "burst" due to formation of nitrophenolate ion at pH 8.5. Initial concentrations in the experimental solutions were 0.11 mM NPh-OAc and 0.2 to 20 mM enzyme in 3.5% (v/v) acetonitrile. Concentration of nitrophenolate ion ($\epsilon_{400} = 1.8 \times 10^4 M^{-1} cm^{-1}$) during the "burst" was calculated with allowances made for dilution effects and for time delay during addition of enzyme (this was less than 10 s). Typical results of nitrophenolate "burst" against enzyme concentration showed the expected linear relationship. In cases when the enzyme or active site concentrations were low (as in the case of reacetylated $\delta$-chymotrypsin), the reaction was initiated by the addition of NPh-OAc to the solution containing enzyme and buffer. NPh-OAc blanks were run concurrently.

**Steady State Kinetic Measurements of Acetylated and Reacetylated $\delta$-Chymotrypsins**

Measurements of the enzyme-catalyzed hydrolysis of AcTyr-OEt were performed at pH 8.5, 25° in 0.1 M KCl with the use of the pH-stat. Corrections for nonenzyme substrate hydrolysis were made. Initial substrate concentration varied from 0.6 to 14 mM, and initial enzyme concentration was 0.02 to 1.8 $\times 10^{-4}$ M. At all times, a stream of wet nitrogen was passed over the substrate solution being hydrolyzed. The data were computed according to the Lineweaver-Burk form of the Michaelis-Menten equation by means of a digital computer program. Data weighing and calculation of standard errors were carried out according to the method of Wilkinson (28). For routine activity assays, 11 mM AcTyr-OEt and about 1.0 $\mu$M enzyme were used.

Enzyme-catalyzed hydrolysis of NPh-OAc was monitored at 400 nm on a Cary 14 spectrophotometer, with liberation of nitrophenolate recorded automatically as a function of time. The steady state deacylation rate constant, $k_{2s}$, was calculated in terms of the observed deacylation rate and the magnitude of the "burst" of nitrophenolate, which is a measure of enzyme active sites. The magnitude of the "burst" was calculated as $\Delta A_{400}$, and the nitrophenolate burst was extracted in units of $\Delta A_{400}$. Concentration of nitrophenolate ion ($\epsilon_{400} = 1.8 \times 10^4 M^{-1} cm^{-1}$) during the "burst" was calculated with allowances made for dilution effects and for time delay during addition of enzyme (this was less than 10 s). Typical results of nitrophenolate "burst" against enzyme concentration showed the expected linear relationship. In cases when the enzyme or active site concentrations were low (as in the case of reacetylated $\delta$-chymotrypsin), the reaction was initiated by the addition of NPh-OAc to the solution containing enzyme and buffer. NPh-OAc blanks were run concurrently.

**Steady State and Presteady State Kinetic Measurements of AcTyr-OEt and NPh-OAc Hydrolysis Catalyzed by Acetylated and Reacetylated $\delta$-Chymotrypsins**

Acetylation kinetics were conducted at 25° in 0.1 M potassium pyrophosphate, pH 6.9, at varying NPh-OAc concentrations of 0.05 to 0.02 mM. Deacylation rate of acetylation was followed by nitrophenol release at 405 nm, the isosbestic point for nitrophenol and nitrophenolate. Rate constants were calculated from simple first order plots or by the Guggenheim method (29). Corrections for NPh-OAc hydrolysis in the absence of enzyme were carried out. Concentration of active sites in these studies was 5 to 10 $\mu$M. In some experiments the proflavin displacement method of Brandt and Hess (30) was used and gave nearly identical values. Enzyme, NPh-OAc, and proflavin concentrations were 10, 100, and 50 $\mu$M, respectively.

**RESULTS**

**Enzyme Activity of Reacetylated $\delta$-Chymotrypsin**

Chromatography of reacetylated $\delta$-chymotrypsin on a Sephadex G-25 column (2.5 $\times$ 30 cm) revealed one major absorbance peak at 280 nm (Fig. 1). A small trailing peak was observed in some preparations which probably represents small peptide fragments. Activity analysis of individual fractions around the peak of optical density was carried out in order to determine if the enzyme species is homogeneous, heterogeneous, or uniformly heterogeneous. Based on catalytic rate constant obtained for AcTyr-OEt with acetylated $\delta$-chymotrypsin, the reacetylated enzyme was found to be about 14% active when assayed with AcTyr-OEt. Activity content of NPh-OAc was shown to be about 14% active over the range of high optical densities. Active site titrations with NPh-OAc at pH 8.5, which measures the rapid liberation of p-nitrophenol, gave identical results. The calculated deacylation rate constant with NPh-OAc at pH 8.5, with six different protein fractions, gave a range of values from 0.008 s$^{-1}$ to 0.012 s$^{-1}$, which are nearly identical with the values obtained with acetylated $\delta$-chymotrypsin (see legend to Fig. 1). These results appear to indicate that protein fractions are either homogeneous or uniformly heterogeneous.
Enzyme activity and active site content of reacetylated \( \delta \)-chymotrypsin

**Effect of acetylation of isoleucine-16 on enzymic activity of acetylated \( \delta \)-chymotrypsin**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Active sites per molecule</th>
<th>Catalytic activity, AcTyr-OEt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinnamoyl imidazole pH 8.5</td>
<td>0.90</td>
<td>220</td>
</tr>
<tr>
<td>NPh-OAc pH 7.5</td>
<td>0.79</td>
<td>189</td>
</tr>
<tr>
<td>Acetylated ( \delta )-chymotrypsin</td>
<td>0.75</td>
<td>164</td>
</tr>
<tr>
<td>Reacetylated ( \delta )-chymotrypsin</td>
<td>0.34</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>0.11</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>19</td>
</tr>
</tbody>
</table>

* The details of the assay conditions for different methods are described under “Experimental Procedures.”
* Three different preparations of acetylated \( \delta \)-chymotrypsin.

### Relationships between Enzyme Activity and Active Site Content of Reacetylated \( \delta \)-Chymotrypsin Preparations

A comparison of the activities of various preparations of acetylated and reacetylated \( \delta \)-chymotrypsins was carried out using AcTyr-OEt, cinnamoyl imidazole, and NPh-OAc at pH 8.5. Acetylation with acetic anhydride was controlled by either varying the concentration of the reagent or the time of reaction. The deacylation of hydroxyl groups after reacetylation was carried out at pH 7.5 with 0.5 mM hydroxylamine for 30 min at 0°C. The protein solution was then filtered through Sephadex G-25, with >90% recovery.

The deacylation of hydroxyl groups after reacetylation was carried out at pH 8.5 with 0.1 mM hydroxylamine for 60 min at 0°C. The protein solution was then filtered through Sephadex G-25, with >90% recovery.

The deacylation rate constant for each of the substrate concentrations was subtracted from each observed acylation rate constant prior to the construction of Eadie plots (\( k_{on} \) versus \( k_{cat}/k_S \)). The data for acetylated and reacetylated \( \delta \)-chymotrypsins fit the same line (graph not shown here) thus indicating identical kinetic behavior of both enzyme species. The values of \( k_0 \) and \( K_m \) (app) were found to be 0.12 \pm 0.04 s\(^{-1}\) and 0.3 \pm 0.05 mM, respectively. These numbers appear to be in the same range as those calculated for AcTyr-OEt by Kezdy and Bender (at pH 5.91, \( k_0 \), 0.18 s\(^{-1}\) and \( K_m \) (app), 0.37 mM) (31).
Relationship between Enzyme Activity and NH$_2$-terminal Isoleucine Content

A. NH$_2$-terminal Analyses—The effect of reacetylation on content of NH$_2$-terminal residues was analyzed by the automatic amino acid analyzer and compared with the concentration of enzyme-active sites. Only a single isoleucine residue having a free $\alpha$-amino group is expected when acetylated chymotrypsinogen is activated under conditions which yield acetylated $\delta$-chymotrypsin. Thus, analysis of NH$_2$-terminal groups of the acetylated and reacetylated $\delta$-chymotrypsin preparations provides an indication of the extent to which this free $\alpha$-amino group has reacted during the reacetylation procedure. The values obtained confirm the presence of a single NH$_2$-terminal isoleucine residue in acetylated $\delta$-chymotrypsin, and show that it is this $\alpha$-amino group that is acetylated during the reacetylation process (Table II). Active site determinations show that decrease in NH$_2$-terminal isoleucine content is accompanied by loss of activity. Data obtained with $\alpha$-chymotrypsin and with the preparation termed reacetylated iPr$_2$P-$\delta$-chymotrypsin are included in Table II for purposes of comparison.

Acetylation of $\alpha$-chymotrypsin in the presence and absence of 60% sodium acetate is also shown in Table II. In the sodium acetate solution, NH$_2$-terminal alanine-149 is almost completely acetylated whereas 0.7 mol of isoleucine-16 per mol of the enzyme are still available for acetylation. Corresponding to this acetylation, 0.62 active sites per mol of the enzyme are still catalytically functional. In contrast, acetylation of the enzyme in aqueous solution results in nearly complete acetylation of isoleucine-16 with a concomitant loss of the active site content of the molecule.

B. [1-$^{14}$C]Acetyl Groups Incorporated per Mol and Enzyme Activity—For experiments in which [1-$^{14}$C]acetic anhydride was used for reacetylation, the protein fractions of Fig. 1 were concentrated and further purified by passage through a second Sephadex column (2.5 x 30 cm). The elution pattern from this column revealed (Fig. 2) a single peak comprising 50 to 90% of the total applied protein. Each eluate fraction was analyzed for protein content, activity (as determined by cinnamoyl imidazole and AcTyr-OEt assays), and incorporation of [1-$^{14}$C]acetyl groups. Protein-containing fractions were found to contain about 0.2 mol of active sites per mol of the enzyme. Incorporation of [1-$^{14}$C]acetyl groups was determined on the basis of radioactive counting of each protein-containing fraction, corrected for subtraction of a background count, measured for a fraction which precedes the protein by four fractions. A calculation of moles of acetyl groups incorporated per mol of protein, made on the basis of the known level of specific radioactivity of the reagent, showed that approximately 1 to 1.2 acetyl groups per mol of protein had been incorporated during the reacetylation procedure (Fig. 3).

Some comparable results for acetylated iPr$_2$P-$\delta$-chymotrypsin that had been reacetylated with radioactive acetic anhydride are given in Table II; acetylated iPr$_2$P-$\delta$-chymotrypsin showed no activity when tested with specific or nonspecific substrates, but incorporated about 0.4 mol of [1-$^{14}$C]acetyl groups per mol of the protein (Table II). The differences in the moles of [1-$^{14}$C]acetyl groups incorporated between reacetylated $\delta$-chymotrypsin and acetylated iPr$_2$P-$\delta$-chymotrypsin amounts to 0.7 to 0.8 mol per mol of the protein and is consistent with 70 to 80% loss of activity of the enzyme.

The correspondence between the incorporation of [1-$^{14}$C]acetyl groups into acetylated $\delta$-chymotrypsin and the decrease in the content of active sites as well as of the free $\alpha$-amino group of isoleucine-16 is quite apparent. Under conditions where the acetylation is only partially complete, higher amounts of the isoleucine end group compare well with the content of intact active sites. Acetylated iPr$_2$P-$\delta$-chymotrypsin showed appreciable incorporation of [1-$^{14}$C]acetyl groups when subjected to treatment identical with acetylated $\delta$-chymotrypsin. The incorporation of radioactivity, however, did not correlate with the acetylation of isoleucine-16 as 0.90 mol per mol of protein could still be determined as NH$_2$-terminal residue. This abnormal behavior of isoleucine-16 of acetylated iPr$_2$P-$\delta$-chymotrypsin towards

<table>
<thead>
<tr>
<th>Material</th>
<th>NH$_2$-terminal amino acid content</th>
<th>[1-$^{14}$C]Acetyl groups incorporated during reacetylation</th>
<th>Active sites per molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylated $\delta$-chymotrypsin</td>
<td>1.09</td>
<td>1.13</td>
<td>0.17</td>
</tr>
<tr>
<td>Reacetylated $\delta$-chymotrypsin</td>
<td>0.2</td>
<td>0.41</td>
<td>0.01</td>
</tr>
<tr>
<td>Reacetylated iPr$_2$P-$\delta$-chymotrypsin</td>
<td>0.9</td>
<td>1.6</td>
<td>0.98</td>
</tr>
<tr>
<td>$\alpha$-Chymotrypsin</td>
<td>1.24</td>
<td>2.3</td>
<td>0.62</td>
</tr>
<tr>
<td>$\alpha$-Chymotrypsin acetylated in sodium acetate solution</td>
<td>0.70</td>
<td>0.23</td>
<td>0.19</td>
</tr>
<tr>
<td>$\alpha$-Chymotrypsin acetylated in water solution</td>
<td>0.25</td>
<td>0.25</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Amino acid analyses were carried out by a Technicon amino acid analyzer. The quantitation of NH$_2$-terminal amino acid contents is based on norleucine as internal standard.

* The acetylation of isoleucine-16 of the acetylated $\delta$-chymotrypsin was carried out by the addition of 0.1 ml of radioactive acetic anhydride to 10 ml of the protein solution, over a period of 10 min.

* Measured by the cinnamoyl imidazole method.

* Radioactive acetic anhydride, 0.1 ml per 10 ml of solution, was added over a period of 10 min to the acetylated iPr$_2$P-$\delta$-chymotrypsin preparation.

* Sodium acetate concentration was 60%.

![Fig. 3. Elution diagram for the second Sephadex G-25 filtration of radioactive reacetylated $\delta$-chymotrypsin. O, protein content; $\Delta$, activity determined by AcTyr-OEt method; $\Delta$, active site assay with cinnamoyl imidazole; and $\square$, incorporation of [1-$^{14}$C]$\alpha$-acetyl groups (mol/mol of the protein). For other details, see under "Experimental Procedures."](http://www.jbc.org/content/117/1/3597/F1.large.jpg)
acetylation is consistent with data obtained previously with iPr2P-α-chymotrypsin (12).

**Discussion**

Our data on the catalytic and physical properties of the re-acetylated enzyme are in agreement with the earlier studies of Ghelis et al. (23). However, one significant difference between these two studies is in the active site content of the modified enzyme. These authors showed that even though the catalytic efficiency of the active enzyme is lost on acetylation of isoleucine-16, the active site content of the modified enzyme, as measured by the incorporation of 3H-labeled iPr2P group, is nearly 0.8 mol per mol compared to 1.1 mol per mol for the unacetylated enzyme. A possible explanation of this discrepancy may be in the method for the determination of the active site content of the modified enzyme. We measured the rapid liberation of p-nitrophenol or the rapid formation of cystamino enzyme, whereas Ghelis et al. (23) used prolonged incubation (15 hours) with [3H]iPr2P-F to realize the full active site content of 0.8 mol per mol. Incubation for shorter time periods (about 20 min) with [3H]iPr2P-F or radioactively p-nitrophenylanthranilate (23) gave a value of about 0.2 mol per mol of active site content which is similar to the values obtained by us (Table I). Therefore, it seems that the higher active site content obtained on prolonged incubation with iPr2P-F represents the intrinsic activity of the modified enzyme, which is similar to the intrinsic activity of chymotrypsinogen reported by Gertler et al. (33). The contribution from intrinsic activity for rapid measurement of active site content would not be sufficient to explain our data even if we accept their values of acylation and deacylation constants of the modified enzyme as 2 × 10^{-7} s^{-1} and 1 × 10^{-2} s^{-1}, respectively (see below concerning our reservations about these values). The corrected active site content of enzyme species listed last in Table I (based on N = Ea(k2 + k3)p) would only amount to 0.2 mol per mol which is much lower than the value reported by these authors.

The value of the acylation rate constant, ka (0.13 s^{-1} at pH 6.0) and the deacylation rate constant, kd (0.01 s^{-1} at pH 8.5) obtained in our experiments with the modified enzyme compares favorably with those of Kessler and Bender (31) for the α-chymotrypsin-NPh-OAc system: ka = 0.18 s^{-1} at pH 5.91, and kd = 5.6 × 10^{-3} s^{-1} at pH 7.8. Therefore, these values of the kinetic constants can be assigned to the presence of the unmodified enzyme in the reacylated δ-chymotrypsin preparations. The ka and kd values of 2 × 10^{-7} s^{-1} and 1 × 10^{-2} s^{-1}, respectively, obtained by Ghelis et al. (23) most probably refer to the intrinsic activity of the modified enzyme, though the kd value appears to be similar to the a-chymotrypsin-NPh-OAc (31) and the acetylated δ-chymotrypsin-NPh-OAc systems (the present data).

Our results are in disagreement with the later studies of Agarwal et al. (22) and of Blair et al. (21). These authors have shown that the α-amino group of isoleucine-16 of δ-chymotrypsin can be succinylated or amidinated without appreciable loss in the catalytic activity or active site content. Introduction of a negatively charged succinylamino group is likely to generate charge-charge repulsions with aspartate-194 and thereby lead to a destabilized and inactive conformation. Additionally, the contention of Ghelis et al. (23) and of Marini and Martin (20) that the α-amino group of isoleucine-16 has a normal pKa of about 7.8 appears to be in conflict with the wealth of evidence assigning an abnormal pKa of about 9.0 to this group (16-19).

Extensive quantitative evidence presented here lends further support to the hypothesis that a positively charged NH2-terminal isoleucine is important in the control of the conformation and activity of chymotrypsins. A direct relationship between acetylation of isoleucine and the loss of enzyme-active sites is maintained whether the enzyme is assayed with cinnaomyl imidazole, NPh-OAc, or AcTyr-OEt (Table I). For example, acetylation of 0.8 mol per mol of isoleucine-16 in acetylated δ-chymotrypsin reduces the active site concentration by 80% (Table II). Similarly, a 37% reduction in the active site content of α-chymotrypsin compared favorably with the acetylation of the α-amino group of isoleucine-16 (about 44%) but not with the acetylation of the other NH2-terminal residue, alanine-149 (about 85%, Table II). These results are in accord with the studies of Dixon and Hofmann (17) on the nitrosation of α-amino group of alanine-149.

The data obtained with [1-3H]acetate (Table II) further supports the above argument. The number of acetyl groups incorporated into acetylated δ-chymotrypsin can be essentially accounted for by the acetylation of isoleucine-16. Slightly higher values obtained for acetyl groups (1.13 mol per mol) in Table I and Fig. 3 may be attributed to acetylation of some peptide impurity. As shown in Fig. 3, we were unable to achieve complete inactivation corresponding to the incorporation of a mole of [1-3H]acetyl groups per mol of the acetylated δ-chymotrypsin. The activity of the protein thus modified was always 10 to 20% of the original value. A prolonged treatment (2 to 3 hours) with acetic anhydride could be used to produce inactive enzyme, but such species had a considerably greater amount of acetyl groups/mol of the enzyme. The additional covalently bound acetyl moiety could not be removed by hydroxylamine and was probably attached to the autoexsision fragments produced during prolonged incubation (34). Therefore, we did not carry out complete acetylation of acetylated δ-chymotrypsin.

The possibility, however, exists that acetylation of the α-amino group of isoleucine-16 is only indirectly related to the loss of active sites of the enzyme. A critical group of the enzyme (Group X, Fig. 4) could be acetylated, causing an irreversible unfolding of the molecule accompanied by inactivation and exposure of the α-amino group of isoleucine-16 (Fig. 4, Mechanism A). In a subsequent reaction with acetic anhydride, the

**Fig. 4.** Possible mechanisms leading to the acetylation of isoleucine-16 of acetylated δ-chymotrypsin. In Mechanism I, [A1] represents acetylated but active conformation which is irreversibly converted to [A2], isoleucine-16 is then acetylated to further convert A1 into reacylated δ-chymotrypsin [Ac-CT]. In Mechanism II, [B1] and [B2] are in prototropic equilibrium with each other as well as with the active conformation. Acetylation of B2 either directly or through an intermediate [B'2] leads to inactive reacylated δ-chymotrypsin [Ac-CT].
isoleucine α-amino group is acetylated, and during the purification procedure the acetyl group on X is hydrolyzed, yielding the inactive protein designated Ac-CT. This mechanism is not consistent with the data presented here (Tables I and II). The relationship between the acetylation of the NH₂-terminal isoleucine α-amino group and the loss of enzyme activity indicates that the acetylation procedure does not per se inactivate the protein irreversibly. If irreversible inactivation had taken place, one would expect the percentage of intact active site to be lower than the percentage of free α-amino group of isoleucine-16. However, this is not the case (see Tables I and II). In Mechanism B, an inactive or partially active conformation (B₂ in Fig. 4) is in equilibrium with the active protein conformation. On acetylation, B₂ is rapidly converted to the inactive form Ac-CT either directly or through another intermediate, B', which could be considered akin to the high pH form of δ-chymotrypsin demonstrated by Fersht (19).

A considerable amount of evidence supports Mechanism B. In chymotrypsinogen, which possesses intrinsic activity and binds substrates less efficiently (33), the α-amino group of isoleucine-16 is in an amide linkage and is, therefore, unable to carry a positive charge at any pH. Activation of thezymogen produces the NH₂-terminal isoleucine which appears to lock the enzyme in a catalytically active conformation. Recently Fersht and Requena (18) and Fersht (19) have obtained evidence for a conformational equilibrium between the two forms of δ-chymotrypsin and have demonstrated that the acetylation procedure does not per se inactivate the protein irreversibly. If irreversible inactivation had taken place, one would expect the percentage of intact active site to be lower than the percentage of free α-amino group of isoleucine-16. However, this is not the case (see Tables I and II). In Mechanism B, an inactive or partially active conformation (B₂ in Fig. 4) is in equilibrium with the active protein conformation. On acetylation, B₂ is rapidly converted to the inactive form Ac-CT either directly or through another intermediate, B', which could be considered akin to the high pH form of δ-chymotrypsin demonstrated by Fersht (19).

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