Chemical Derivatives of α-Chymotrypsinogen

III. REACTION WITH N-ACETYL-DL-HOMOCYSTEINE THIOLACTONE*

Djahanguir M. Abadi† and Philip E. Wilcox

From the Department of Biochemistry, University of Washington, Seattle 5, Washington

(Received for publication, October 13, 1959)

The reactions of α-chymotrypsinogen with carbon disulfide and with O-methyliosourea have been reported from this laboratory previously (1, 2). The present report concerns the reaction of this protein with a new acylating agent, N-acetyl-DL-homocysteine thiolactone. The use of the thiolactone for the introduction of sulphydryl groups into proteins was first suggested by Benesch and Benesch (3, 4) who carried out exploratory studies with glycine, glycylglycine, and gelatin. The relatively mild conditions employed for these reactions indicated that the thiolactone would acylate amino groups of proteins without damaging the native structure. By analogy with acetic anhydride, one would expect acylation to be directed specifically toward amino groups. Reaction of α-amino groups could be favored over reaction of ε-amino groups by control of pH and, consequently, the degree of ionization of amino groups, as was done in reactions with carbon disulfide (1).

The aim of the present work was to find experimental conditions for the synthesis and isolation of the monoacylated derivative of α-chymotrypsinogen in which only the single α-amino group was acylated. Studies of the protein were preceded by experiments on the hydrolysis of the thiolactone and on amidolysis by model compounds, diglycylglycine and ε-amino caproic acid, at various pH values. The reaction of α-chymotrypsinogen with thiolactone proved to be more complicated than anticipated. Chromatography on columns of carboxymethyl cellulose led to the separation of the reaction product into a number of components, some of which have been characterized.

EXPERIMENTAL

Materials

Bovine α-Chymotrypsinogen was supplied by Wilson Laboratories and by the Worthington Biochemical Corporation. The protein was purified further by recrystallization and was treated with diisopropylphosphorofluoridate as described previously (1). Before inhibition, the esterase activity of the purified protein corresponded to less than 1% chymotrypsin.

Trypsin, twice crystallized, was obtained from Worthington Biochemical Corporation and contained 50% MgSO₄.

N-Acetyl-DL-homocysteine thiolactone (m.p., 109-110°) was synthesized by acetylation of DL-homocysteine thiolactone chloride (3). The latter compound was purchased from Schwarz Laboratories, Inc.

Diglycylglycine was obtained from Hoffmann-La Roche, Inc. and ε-amino caproic acid was purchased from Distillation Products Industries.

Methylmercury nitrate was prepared as a 0.1 M solution by mixing equivalent weights of silver nitrate dissolved in water with methylmercury bromide dissolved in ethanol, filtering, and diluting to the appropriate volume with water. The methylmercury bromide was synthesized by the procedure of Hinkel and Angel (5).

Carboxymethyl cellulose was prepared from Whatman standard grade cellulose powder by the method of Ellis and Simpson (6). One treatment with chloroacetate in NaOH solution gave a product which contained 0.52 milliequivalent of acid per g. A second treatment gave a product which contained from 0.8 to 0.9 milliequivalent of acid per g, and it was this material which was used for chromatography in the present work.

N,N'-Diphenylthiocarbamide was obtained from G. Frederick Smith Chemical Company and was used without further purification.

Nitrogen gas from a commercial cylinder was freed of all oxygen by passage through a column of hot, freshly reduced copper wire.

All other chemicals were purchased commercially and were chemically pure, reagent, or analytical grade, whichever was appropriate for the particular use.

Methods

Hydrolysis of N-Acetyl-DL-homocysteine Thiolactone—The hydrolysis of the thiolactone at pH values near 7 or 8 results in the production of one equivalent of hydrogen ion:

\[
\text{CH₃CO-\text{NH-}\text{C-C=O}} \quad + \text{H₂O} \rightarrow \text{CH₃CO-\text{NH-}\text{C-C=}O⁻} + \text{H⁺}
\]

* The material presented in this paper is taken in part from the thesis of Djahanguir M. Abadi which was submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, University of Washington, Seattle, 1958.

† Present address, Pacific Northwest Research Foundation, Swedish Hospital, Seattle, Washington.
The rate of hydrolysis was determined by measuring the consumption of 0.02 N NaOH in a recording pH-stat (7). Due consideration was given to those factors which affect the accuracy of such measurements (8). The reaction vessel was similar to the one described by Dixon et al. (9), but had a capacity of 30 ml. Constant temperature water was circulated through the jacket. Hydrolysis was carried out in water or dilute KCl solution.

Reaction of N-Acetyl-DL-homocysteine Thiolactone with Model Compounds—Aminolysis results in the formation of a new peptide bond and the generation of a sulfhydryl group.

\[
\text{CH}_2\text{CO}-\text{NH}-\text{C}-\text{CO} \rightarrow + \text{RNH}_2 \rightarrow \text{CH}_2\text{CO}-\text{NH}-\text{C}-\text{CO} - \text{NH}_2
\]

The reaction was followed by measuring the decrease in concentration of amino groups as determined by the colorimetric ninhydrin method. The reaction was carried out in 1 M phosphate buffers with the model compounds at a concentration of 5 \times 10^{-3} M and the thiolactone at a molar concentration 2 to 5 times greater. The pH of the buffers varied from 7 to 8. Aminolysis was allowed to proceed at 25°C under an atmosphere of nitrogen. From time to time duplicate samples of 0.100 ml were removed and the concentration of unreacted amine determined. As a control, the color yield of a standard amount of amine was determined with the same reagent. The ninhydrin reagent and the thiolactone at a molar concentration 2 to 5 times greater. The pH of the buffers varied from 7 to 8. Aminolysis was allowed to proceed at 25°C under an atmosphere of nitrogen. From time to time duplicate samples of 0.100 ml were removed and the concentration of unreacted amine determined. As a control, the color yield of a standard amount of amine was determined with the same reagent. The ninhydrin reagent and the procedure used in this laboratory have been described previously (10); the average deviation is approximately 1.5%.

Reaction of Thiolactone with \( \alpha \)-Chymotrypsinogen—Reactions with protein were carried out at constant pH under control of a pH-stat (7). A sample of lyophilized, salt-free chymotrypsinogen was dissolved in boiled distilled water which had been saturated with nitrogen gas. The pH was adjusted to 7.5 and the protein was treated with diisopropylphosphorofluoridate. The solution was clarified by centrifugation and the concentration was adjusted to the desired value. Protein concentration was determined routinely by measurements of absorbancy at 282 nm \((a_2 = 20.0, 1\% \text{ solution})\). The reaction vessel consisted of a jacketed beaker with a capacity of about 20 ml. Constant temperature water at 25°C was circulated through the jacket. For large scale preparations of the protein derivative, a covered wide-mouth jar (250 ml) was used as the reaction vessel and the reaction was performed at room temperature.

Weighed amounts of N-acetyl-DL-homocysteine thiolactone were added to the protein solution in the reaction vessel such that the final molar concentration of thiolactone was 10 to 15 times larger than the molar concentration of protein. The pH was adjusted and kept at the desired value by the automatic addition of a standardized NaOH solution. Throughout the course of the reaction the vessel was flushed with a small stream of nitrogen gas saturated with water vapor. At the termination of the reaction period, sulfhydryl groups produced by both aminolysis and hydrolysis were blocked by the addition of methylmercury nitrate in an amount which was estimated from the total base consumption of the pH-stat. Finally, the reaction mixture was exhaustively dialyzed for 3 to 4 days against 0.001 M HCl and the protein was lyophilized.

Estimation of Homocystine in Protein Derivatives—The number of moles of homocysteine incorporated per mole of protein was determined by oxidation of the protein derivative with performic acid, hydrolysis with 6 N HCl, and estimation of homocysteic acid in the hydrolysate. The procedure was similar to that described previously (10) except that the evaporation of water and acid in every instance was carried out in an all glass rotary evaporator under vacuum at room temperature. Homocysteic acid was estimated by the chromatographic method of Schram et al. (11); homocysteic acid is separated from cysteic acid and other acidic amino acids on column Dowex 2-X10 (0.9 \times 25 cm). The amount of amino acid in effluent fractions was determined by the colorimetric ninhydrin reaction. When a mixture of known amounts of L-cysteine and DL-homocysteine thiolactone were subjected to analysis, the yields of sulfonic acids were 97.5 and 96.5%, respectively. Since the recovery of cystine as cysteic acid from \( \alpha \)-chymotrypsinogen by the above procedure has been found to be 90 \pm 5%, the analytical values for homocysteine in protein derivatives have been corrected for an assumed loss equal to the loss for cystine.

The chromatographic method required about 1 \( \mu \)mole of protein for each analysis. A more sensitive but somewhat less accurate estimation of homocysteic acid was performed by high voltage electrophoresis (12) which required only 0.1 \( \mu \)mole of protein for each analysis. According to the procedure used in this laboratory (13, 14), homocysteic acid was separated from cysteic acid at pH 3.6 (acetic acid-pyridine-water; 1:10:89) after 30 minutes of electrophoresis at 1500 volts. The spots on the paper were eluted and were estimated by the colorimetric ninhydrin method (18). The mean deviation of six or more analyses was 3%, although the error in a single analysis was sometimes as much as 10%.

Estimation of Methylmercury in Protein Derivatives—Two different methods were used for the estimation of methylmercury in samples of protein. The first method was a direct titration with a standard solution of \( N,N' \)-diphenylthiocarbazone (dithizone) carried out in homogeneous solution. In a typical analysis, 1 ml of an aqueous solution of the protein derivative containing about 1 \( \mu \)mole of methylmercury and up to 50 mg of protein was treated in a 15-ml test tube with one drop each of 0.2 M iodoacetamide, 0.1 M NaCN, and 1.0 M NaClO4. After dialysis of sulfhydryl groups had proceeded for 10 minutes at room temperature, one drop of 1.0 M trisodium ethylenediaminetetraacetate and 3.0 ml of \( N,N' \)-dimethylformamide-acetic acid (4:1 by volume) were added. The mixture was titrated with a standard solution of dithizone in n-butanol saturated with water. Fresh reagent at a concentration of 5 \times 10^{-4} M was prepared each day from a stock solution at 5 \times 10^{-3} M. The primary

1 The direct titration procedure was based on the method developed some years ago by Dr. Walter L. Hughes, Jr., at the Department of Physical Chemistry, Harvard Medical School, for the estimation of mercury and methylmercury in proteins, particularly mercury complexes of mercaptalbumin. A mixture of pyridine and acetic acid was not a suitable solvent in the present work and was replaced by \( N,N' \)-dimethylformamide-acetic acid in which the protein was soluble throughout the titration.
standard was a weighed sample of liquid mercury dissolved in nitric acid and diluted to 10^{-3} M. Dithizone solution was introduced from a semimicro buret and the end point in the orange solution was taken as the first perceptible brown discoloration caused by free dithizone. The precision of all titrations in the range of 1 \mu mole was within about \pm 2\%. It was assumed that at the respective end points each mercuric ion was bound to two dithizone molecules as the keto complex and each methylmercury ion was bound to one dithizone molecule as the enol complex. This assumption was substantiated by titration of solutions of methylmercury nitrate prepared from weighed amounts of methylmercury bromide.

The second method consisted of the complete oxidative digestion of the protein, the quantitative cleavage of methylmercury to give divalent mercuric ion, the extraction of the mercury with a chloroform solution of dithizone, and the estimation of the mercuric dithizone complex by the spectrophotometric method with the protein, the quantitative cleavage of methylmercury bromide.

Titrations of aliquots of the protein derivative containing about 1 \mu mole of methylmercury; 4 ml of a saturated solution of KMnO_4; and 2.0 ml of a mixture of concentrated sulfuric and nitric acids, equal parts by volume. Care was taken to leave the neck of the flask clean and dry. A tightly fitted cold finger was inserted through the neck of the flask and within 1 cm of the digestion mixture. The mixture was heated carefully to reflux and was boiled continuously for 2 hours. It was found that the precipitate of manganese dioxide, which formed in the mixture must persist throughout the digestion; otherwise an erroneously low amount of mercury was found in the spectrophotometric estimation and the use of more per-manganate was indicated. After the digestion mixture was cooled, it was treated with 3 ml of a hydroxylamine hydrochloride solution (concentration, 50 mg per ml). The colorless solution was transferred to a 100-ml volumetric flask, and the reaction flask was washed thoroughly with 2 ml of the hydroxylamine hydrochloride solution and sufficient water to bring the total combined volume to 100 ml. An aliquot of this latter solution, 20 to 40 ml, was diluted to 100 ml with water and was extracted in a separatory funnel with 25.0 ml of a cold chloroform solution of dithizone. Fresh dithizone solution at a concentration of 20 mg per liter was made up from a refrigerated stock solution 5 times more concentrated. Absorbancy of the extract was measured at 490 \text{ nm}. It was found that the precipitate of manganese dioxide, which formed in the mixture must persist throughout the digestion; otherwise an erroneously low amount of mercury was found in the spectrophotometric estimation and the use of more per-manganate was indicated. After the digestion mixture was cooled, it was treated with 3 ml of a hydroxylamine hydrochloride solution (concentration, 50 mg per ml). The colorless solution was transferred to a 100-ml volumetric flask, and the reaction flask was washed thoroughly with 2 ml of the hydroxylamine hydrochloride solution and sufficient water to bring the total combined volume to 100 ml. An aliquot of this latter solution, 20 to 40 ml, was diluted to 100 ml with water and was extracted in a separatory funnel with 25.0 ml of a cold chloroform solution of dithizone. Fresh dithizone solution at a concentration of 20 mg per liter was made up from a refrigerated stock solution 5 times more concentrated. Absorbancy of the extract was measured at 490 nm and 610 nm with a Beckman model B spectrophotometer with a light path of 10 mm. The amount of mercury in the unknown sample was estimated by comparing the absorbancy at 490 nm with the values of a standard curve plotted for known amounts of mercury between 0.04 to 0.4 \mu mole. The standard curve was obtained by extracting aliquots of the primary standard solution of mercuric nitrate from 100 ml of water containing 1.0 ml of concentrated sulfuric acid and 50 mg of hydroxyamine hydrochloride. Precautions were used to protect the dilute dithizone solution from decomposition by keeping the solution cold and away from light even during the extraction procedure. When absorbancy at 610 mg indicated excessive loss of dithizone resulting from light or from oxidation by nitric acid, the results of the assay were discarded. Under carefully controlled conditions, the error of the method was less than 2% at the level of 1 \mu mole of mercuric mercury in the digested protein sample. In control experiments, the recovery of mercury from an equimolar mixture of \alpha-chymotrypsigen and methylmercury nitrate was 98 \pm 1%.

**Table I**

<table>
<thead>
<tr>
<th>Columns</th>
<th>Cross section</th>
<th>Height</th>
<th>Mixing volume</th>
<th>Sample weight</th>
<th>Flow rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cm^2</td>
<td>cm</td>
<td>liters</td>
<td>g</td>
<td>ml hr.^-1</td>
</tr>
<tr>
<td>Analytical</td>
<td>0.64</td>
<td>40</td>
<td>0.30</td>
<td>0.00-0.10</td>
<td>0</td>
</tr>
<tr>
<td>Intermediate</td>
<td>3.1</td>
<td>60</td>
<td>3.00</td>
<td>0.50-1.00</td>
<td>30</td>
</tr>
<tr>
<td>Preparative</td>
<td>28.0</td>
<td>60</td>
<td>20.00</td>
<td>5.0 -10.00</td>
<td>300</td>
</tr>
</tbody>
</table>

**RESULTS**

**Hydrolysis of N-Acetyl-DL-homocysteine Thiolactone**—Typical results of experiments on the rate of hydrolysis of thiolactone at various pH values are shown in Fig. 1. The concentration of thiolactone was about 3 times that used in reactions with \alpha-chymotrypsigen. Since in all cases less than 0.1% of the thiolactone was hydrolyzed, the slope of each linear plot gives the initial rate of the reaction. Measurements over a pH range of 7.0 to 8.5 and at concentrations of thiolactone between 4 and 7 \times 10^{-3} M demonstrated that the reaction is first order with
FIG. 1. Hydrolysis of N-acetyl-DL-homocysteine thiolactone as measured by the consumption of 0.208 N NaOH in a pH-stat at 25°. Data was read directly from recorder chart. Initial concentration of thiolactone was 6.71 X 10⁻³ M; A, pH 8.0; B, pH 7.6; C, pH 7.3; and D, pH 7.0.

Reaction of N-Acetyl-DL-Homocysteine Thiolactone with Model Compounds—The molar concentration of diglycyglycine was chosen to be approximately the same as the molar concentration of protein which was used in subsequent experiments. Fig. 2 shows the gradual decrease in concentration of the α-amino groups at pH 7.52 for various initial concentrations of thiolactone. From the initial slopes of the rate curves, apparent second order rate constants were calculated. As a result of the experiments on hydrolysis at pH 7.5 it was known that the small loss of thiolactone by hydrolysis would have no significant effect on these calculations. A constant value, 5.8 X 10⁻⁴ M⁻¹ sec⁻¹ (mean deviation, 0.3 X 10⁻⁹), was obtained over a range of concentrations of thiolactone from 1 to 3 X 10⁻³ M. This apparent rate constant was calculated with the assumption that all molecules of peptide were equally reactive, regardless of their charge. Actually, the rate of acylation is pH dependent, as shown in Table III. The alternative assumption that reactivity of the peptide is due exclusively to the unprotonated species is not supported by the data, since correction of k' for degree of dis-

### Table II

<table>
<thead>
<tr>
<th>Concentration of Thiolactone</th>
<th>Solvent</th>
<th>x</th>
<th>k</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.71 X 10⁻³ M</td>
<td>water</td>
<td>0.97</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td>4.99 X 10⁻³ M</td>
<td>water</td>
<td>1.03</td>
<td>4.5 ± 0.6</td>
</tr>
<tr>
<td>6.66 X 10⁻³ M</td>
<td>0.10 M KCl</td>
<td>1.04</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>4.99 X 10⁻³ M</td>
<td>0.10 M KCl</td>
<td>1.03</td>
<td>2.8 ± 0.2</td>
</tr>
</tbody>
</table>

Each value of the rate constant, k, is the average of at least five independently determined rate constants over a range of pH from 7.0 to 8.3. The rate constant k is defined by the equation, 

$$\frac{da}{dt} = k_a[OH^-]^x$$

where a is the concentration of thiolactone and x is the order with respect to hydroxyl ion.

Values of x, the order with respect to hydroxyl ion, are also averages, and the average deviation in k is indicated.

The basic hydrolysis of simple carboxylic esters is also first order with respect to hydroxyl ion and to ester, and salt likewise causes a decrease in rate of hydrolysis. Although the second order rate constant for the hydrolysis of the thiolactone is considerably larger than the rate constant for simple esters (10), it is plausible to assume that both reactions have similar mechanisms, i.e., the hydrolysis of thiolactone is initiated by a nucleophilic attack of a hydroxyl ion on the carbonyl carbon.
The dissociation of the amino group, as shown in the last column of Table III, does not give a constant which is invariable with pH.

Although the present data are not extensive enough to elucidate the mechanism of acylation of an amino group by N-acetyl-DL-homocysteine thiolactone, it appears that although the reactivity of the protonated amine is less than that of the unprotonated amine, the unprotonated species is not solely responsible for reaction with thiolactone.

Similar data were obtained for the reaction between ε-amino-caproic acid and N-acetyl-DL-homocysteine thiolactone. At pH 7.52 and 25°, the apparent second order rate constant was found to be 1.2 x 10-4 M-cm² sec⁻¹. In other words, the rate of reaction of the ε-amino group was 5 times less than the rate of reaction of the α-amino group of the peptide under similar conditions. At pH 8.0 the factor was only 3. As in the case of the peptide, no simple relation could be found between the concentration of the unprotonated species of ε-amino caproic acid and the rate of acylation of the amino group.

Reaction of N-Acetyl-DL-homocysteine Thiolactone with α-Chymotrypsinogen—In the early experiments considerable precipitation occurred when the concentration of chymotrypsinogen was 10% or higher and the thiolactone was present in 10-fold molar excess. In the later experiments in which lower concentrations (3 to 6%) of the protein were used, little or no precipitation occurred. Analysis of the protein recovered from various reaction mixtures indicated gradual incorporation of N-acetylhomocysteine into the molecule of α-chymotrypsinogen. Typical results are given in Table IV.

At pH 7.2 the rate of acylation of α-chymotrypsinogen was found to be too slow for the practical preparation of derivatives. Even at pH 7.5, four days at 25° were required to reach a level of incorporation of homocysteine approaching 2 moles per mole of protein. At pH 8.0 the rate was appreciably higher; in 96 hours, 5.6 moles of homocysteine per mole were incorporated.

Electrophoretic analyses of the various preparations also gave evidence for the gradual acylation of the protein as evidenced by the appearance of components with lower mobility. It was apparent from electrophoretic patterns such as those shown in Fig. 3 that reaction with the thiolactone resulted in the synthesis of a number of distinct derivatives. The major peak in these patterns for reaction at pH 7.5 represents unreacted α-chymotrypsinogen as judged by its electrophoretic mobility, 3.74 x 10⁻⁵ cm² volt⁻¹ sec⁻¹ (20). The electrophoretic pattern of the reaction product obtained at pH 8 containing 5.6 moles of homocysteine per mole shows many components, although none corresponded to unreacted protein, and none amounted to a large fraction of the whole.

Chromatography of Reaction Products—Preliminary investigations of various preparations by means of chromatography on the small column of carboxymethyl cellulose confirmed the presence of a number of components in each. Again the major component in reaction products prepared at pH 7.5 corresponded to unreacted α-chymotrypsinogen, as judged by its electrophoretic mobility, 3.74 x 10⁻⁵ cm² volt⁻¹ sec⁻¹ (20). The electrophoretic pattern of the reaction product obtained at pH 8 containing 5.6 moles of homocysteine per mole shows many components, although none corresponded to unreacted protein, and none amounted to a large fraction of the whole.

From these preliminary analyses it appeared that monosubstituted derivatives occurred in the largest relative amounts in preparations for which the degree of acylation lay between 1.3 and 2. Further discussion will be concerned with such preparations.

Analyses of the major chromatographic fractions of the preparation shown in Fig. 4 by the method which uses high voltage paper electrophoresis gave the following results in moles of homocysteic acid per mole of protein: A, trace; B, 0.9; C, 1.0; D, 1.5; and E, 2.4. Fraction A is identified conclusively as unreacted α-chymotrypsinogen. Both Fractions B and C are largely mono-

---

**Table III**

Variation of apparent second order rate constants with pH for reaction of N-acetyl-DL-homocysteine thiolactone with diglycylglycine

Each reaction mixture contained 5 x 10⁻³ M diglycylglycine and 25 x 10⁻³ M thiolactone in 0.1 M phosphate buffer. Initial rates of reaction were determined in such a way that loss of thiolactone by hydrolysis had no significant effect on the apparent rate constant k'. In calculating a, the degree of dissociation of the peptide hydrolysis had no significant effect on the apparent rate constant k'. In calculating a, the degree of dissociation of the peptide hydrolysis had no significant effect on the apparent rate constant k'.

<table>
<thead>
<tr>
<th>pH</th>
<th>a</th>
<th>k' x 10⁻¹</th>
<th>k'/a x 10⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.00</td>
<td>0.11</td>
<td>3.2</td>
<td>20</td>
</tr>
<tr>
<td>7.20</td>
<td>0.17</td>
<td>4.8</td>
<td>28</td>
</tr>
<tr>
<td>7.48</td>
<td>0.27</td>
<td>5.7</td>
<td>23</td>
</tr>
<tr>
<td>7.74</td>
<td>0.43</td>
<td>9.0</td>
<td>21</td>
</tr>
<tr>
<td>8.02</td>
<td>0.55</td>
<td>10.7</td>
<td>19</td>
</tr>
</tbody>
</table>

---

**Table IV**

Extent of acylation of various preparations of α-chymotrypsinogen derivatives at pH 7.5

<table>
<thead>
<tr>
<th>No.</th>
<th>Protein concentration</th>
<th>Thiolactone concentration</th>
<th>Reaction time</th>
<th>Homocysteine per mole protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µ x 10⁻³</td>
<td>µ x 10⁻³</td>
<td>hrs.</td>
<td>moles</td>
</tr>
<tr>
<td>1</td>
<td>1.3</td>
<td>13</td>
<td>6</td>
<td>trace</td>
</tr>
<tr>
<td>2</td>
<td>1.3</td>
<td>13</td>
<td>12</td>
<td>trace</td>
</tr>
<tr>
<td>3</td>
<td>1.3</td>
<td>13</td>
<td>26</td>
<td>0.3</td>
</tr>
<tr>
<td>4</td>
<td>1.3</td>
<td>21</td>
<td>72</td>
<td>1.2</td>
</tr>
<tr>
<td>5</td>
<td>1.3</td>
<td>21</td>
<td>74</td>
<td>1.1</td>
</tr>
<tr>
<td>6</td>
<td>1.3</td>
<td>26</td>
<td>98</td>
<td>1.8</td>
</tr>
<tr>
<td>7</td>
<td>1.5</td>
<td>21</td>
<td>61</td>
<td>0.9</td>
</tr>
<tr>
<td>8</td>
<td>2.0</td>
<td>20</td>
<td>96</td>
<td>1.7</td>
</tr>
</tbody>
</table>

---

For Fig. 3, the electrophoretic patterns of reaction products of α-chymotrypsinogen with different contents of homocysteine: a, 1.1 and b, 1.7 moles per mole of protein, both reactions at pH 7.5; c, 5.6 moles per mole of protein, reaction at pH 8. Analyses were performed in 0.1 M acetate buffer at pH 4.97 with the use of the Spinco model H apparatus. Time of electrophoresis is indicated.
substituted, whereas di- and trisubstituted derivatives appear in the subsequent effluent fractions.

Chromatography on columns of intermediate size with the elution gradient between 0.01 M and 0.25 M buffer gave patterns closely similar to those obtained with analytical columns. The larger column provided enough material for analysis by the method of oxidation, hydrolysis, and column chromatography. The elution diagram for a typical estimation of homocysteic acid is shown in Fig. 5. Fraction C was found to contain 1.16 moles of homocysteine per mole protein by this method. Rechromatography of this fraction on a column 60 cm long resulted in its separation into two components, C1 and C2, in a ratio of about 3 to 1 as shown in Fig. 6. Analysis of component C1 gave a value of 0.99 mole of homocysteine per mole. Presumably fraction C2 is a disubstituted derivative.

Reaction with Carbon Disulfide—Further information about the chemical nature of Fraction C was obtained by testing its reactivity toward CS2. It had been shown previously that CS2 at pH 6.9 reacts specifically with the single α-amino group of α-chymotrypsinogen. Complete substitution of the α-amino group may be achieved with less than 5% reaction at ε-amino groups. When Fraction C was treated with CS2 and the product was analyzed, only 0.07 mole of CS2 per mole of protein was recovered. In a parallel experiment under identical conditions, unacylated α-chymotrypsinogen gave 1.04 moles of CS2 per mole. It may be concluded that Fraction C and, therefore, C1 and C2 are completely acylated on the α-amino group.

Physical Properties of Fraction C1—The electrophoretic pattern of fraction C1 is shown in Fig. 7. Under the conditions of the experiment, this fraction behaves as a single electrophoretic component with a mobility of 3.2 × 10^-4 cm^2 volt^{-1} sec^{-1}. The reduction in mobility from the value for α-chymotrypsinogen (3.8 × 10^-4 cm^2 volt^{-1} sec^{-1}, 0.1 M acetate buffer, pH 4.97) is consistent with the reduction of the net positive charge by one unit as a result of the acylation of the α-amino group.

Fraction C1 also behaved as a single component in the ultracentrifuge. The sedimentation diagrams in Fig. 8 were used to calculate a sedimentation constant of s_{20,w} = 2.98 S. At the same concentration and in the same solvent α-chymotrypsinogen has a sedimentation constant of s_{20,w} = 2.45 S (20). The derivative would be expected to have a slightly larger constant because of the increase in molecular weight by 375 units.

A comparison of the ultraviolet absorbancy of Fraction C1 and α-chymotrypsinogen, either at pH 3 or in 0.1 N NaOH, showed no significant differences. The molar absorbancy index of the derivative was slightly higher than that of the parent protein at wave lengths below 260 μm and this relationship was reversed in the region of maximum absorbance around 280 μm. However the maximum difference was less than 4%.

Rapid Activation of Chromatographic Fractions—Each of the Fractions A, C, D, and E of the preparation shown in Fig. 4 was treated with trypsin under conditions of rapid activation along with a sample of the original α-chymotrypsinogen acting as a control. The results of the measurements of esterase activity are presented in Table V where the rates of the enzymatic reactions are compared. The final level of activity is the same within experimental error for each fraction and is about 90% of the activity obtained from the α-chymotrypsinogen control. Therefore, it is probable that chemical changes have had no effect on the activity of the derivatives. The fact that the fractions from the column, including Fraction A which is unreacted protein, become activated somewhat more slowly, and the final activity is somewhat less than that of the original chymotrypsinogen remain unexplained. Similar behavior has been shown by other α-chymotrypsinogen derivatives (2).

Large Scale Preparation of Monoacylated Derivative—In order to obtain quantities of a single component such as C1 weighing up to 1 g, it was necessary to increase the scale of preparation and fractionation. For example, in one large preparation, 9.8 g of protein in 200 ml of water were treated with 0.65 g of thioglycolic acid (2 × 10^{-4} M and 20 × 10^{-4} M, respectively) at pH 7.5 for 98 hours. At the termination of the reaction, 1.0 × 10^{-3} mole of sodium thiocyanate was added. The yield of lyophilized product, after small amounts of insoluble protein had been removed by centrifugation at the beginning and end of the reaction, was 94%.

Attempts to fractionate the reaction product by precipitation

![Fig. 4. Chromatographic elution diagram for an analysis of 50 mg of a reaction product containing 1.7 moles of homocysteic acid per mole of protein (——). The elution diagram for α-chymotrypsinogen is shown for comparison (-----). Elution of the analytical column of carboxymethyl cellulose was performed with a nonlinear gradient of phosphate buffer between 0.01 M and 0.25 M.

![Fig. 5. Chromatographic analysis of Fraction C on a column of Dowex 2 ion exchange resin (0.9 X 25 cm) after oxidation and hydrolysis according to the method of Schram et al. (10). Absorbancy at 570 μm is plotted on the ordinate for each effluent fraction after treatment with ninhydrin reagent.]}
Fig. 6. Chromatographic elution diagram of Fraction C from an intermediate column of carboxymethyl cellulose 60 cm long. Elution by nonlinear gradient of phosphate buffer between 0.01 M and 0.25 M.

Fig. 7. The electrophoretic pattern of Fraction C1. Analysis was carried out for 200 minutes in 0.1 M acetate buffer at pH 4.97 with the use of the Spinco model H apparatus.

Fig. 8. Sedimentation patterns for Fraction C1, 1.0% solution in 0.1 M glycine buffer at pH 3.0. The pictures shown here were taken at 8, 24, 48, and 88 minutes. Analysis was carried out at 58,268 r.p.m. in a Spinco model E analytical ultracentrifuge at 20°.

with graded concentrations of (NH₄)₂SO₄ were unsuccessful. The ratios of the principal chromatographic fractions, A through E, were not altered to a useful degree in the insoluble or soluble phases. Therefore, it was necessary to resort to many separations on the carboxymethyl cellulose columns of intermediate size or to employ the larger 6.0-cm preparative column. Resolution was increased with the use of a lower gradient between 0.01 M and 0.20 M phosphate buffer. A typical elution diagram is shown in Fig. 9. It may be noted that a partial resolution of Fractions C1 and C2 was achieved and that these fractions are well separated from unreacted protein. A summation of absorbancy under each of the various peaks indicated the following yields as % of the total product: A plus B, 55; C1, 11; C2, 5; and D, 14.

Methylmercury Content of Derivatives—Analyses for methylmercury in the total reaction product and in various fractions of a large scale preparation (see Fig. 9) are presented in Table VI. At first, when direct titration indicated the ratio of methylmercury to homocysteine in the product to be less than 1.0, it was thought that possibly not all of the methylmercury in the protein was titrated with dithizone. Analyses of samples which had been completely digested with sulfuric and nitric acids plus permanganate proved that the titration method had given the correct value for total methylmercury.

It may be noted from Table VI that Fraction A contained essentially no mercury, in accord with the fact that it contained no homocysteine. Fraction X (see Fig. 9) contained the most mercury. Since this fraction emerged from the column after unsubstituted protein, it is regarded as a degradation product which had reacted with excess methylmercury.

Fraction C1, which contained 0.99 mole of homocysteine per mole of protein but only 0.3 mole of methylmercury, gave no positive test for sulfhydryl group with nitroprusside. Removal of methylmercury by dialysis against cysteine liberated only about 0.3 equivalent of sulfhydryl per mole of protein.

Methylmercury may have been found in nonstoichiometric amounts in the derivatives because it was lost from mercaptide linkage with the protein during dialysis and chromatography. This assumption would also imply that the sulfhydryl groups which were thus liberated were then blocked by some unknown intramolecular reaction or process of oxidation.

**TABLE V**

Comparison of activation of chromatographic fractions with activation of native α-chymotrypsinogen

Esterase activity obtained from the same amount of α-chymotrypsinogen under identical conditions is equated to 100.

<table>
<thead>
<tr>
<th>Time of activation</th>
<th>Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>min.</td>
<td>A</td>
</tr>
<tr>
<td>15</td>
<td>70</td>
</tr>
<tr>
<td>30</td>
<td>83</td>
</tr>
<tr>
<td>60</td>
<td>82</td>
</tr>
<tr>
<td>120</td>
<td>88</td>
</tr>
</tbody>
</table>
February 1960

D. M. Abadi and P. E. Wilcox

403

2% 4% mo 14w loo0 lo00 m zzoo 24%

15 ml. EACH

FIG. 9. Chromatographic elution diagram for the preparative fractionation of 5.0 g. of a reaction product containing 1.3 moles of homocysteine per mole of protein. The linear elution gradient of phosphate buffer is shown by the broken line.

TABLE VI

Methylmercury in reaction products of a-chymotrypsinogen, N-acetyl-DL-homocysteine thiolactone and methylmercury nitrate

The total product contained 1.3 moles of homocysteine per mole of protein.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Direct titration (μ mole)</th>
<th>Analysis of digest (μ mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total product</td>
<td>0.58, 0.63, 0.59</td>
<td>0.61, 0.58, 0.49</td>
</tr>
<tr>
<td>A</td>
<td>0.00</td>
<td>trace</td>
</tr>
<tr>
<td>C1</td>
<td>0.26</td>
<td>0.27, 0.25</td>
</tr>
<tr>
<td>C2</td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>D</td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>D + E</td>
<td>0.32</td>
<td>0.15</td>
</tr>
<tr>
<td>X</td>
<td>0.32</td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

By the criteria of electrophoresis, sedimentation, and chromatography as carried out in the present work, Fraction C1 is a homogeneous protein. The results of chemical analysis for homocysteine and the absence of reactivity of the α-amino group toward CS2 leave little doubt that this derivative consists of σ-chymotrypsinogen substituted on the α-amino nitrogen with an N-acetyl-homocysteinyll grouping. Since only 0.3 equivalent of methylmercury per mole of protein was found in this fraction but no free sulfhydryl could be detected, the characterization of the sulfhydryl group is incomplete. A change in the procedure for blocking this group may possibly give a derivative with a stoichiometric amount of mercury.

Several important problems remain. One which may bear some relation to the binding of methylmercury involves the stereochemistry of the reaction and of the products. Since racemic thiolactone was employed in the present work, partial asymmetric synthesis is possible, and fractionation of derivatives containing D- and L-residues of homocysteine must be considered. Furthermore, differences in chemical properties of diastereoisomeric derivatives would be anticipated.

An inspection of the chromatographic elution diagrams suggests that positional isomers may be separated by carboxymethyl cellulose columns. The principal fractions A, C, D, and E decrease in net positive charge on the protein in the order of elution; charge must play a large role in determining the affinity of the adsorbent for the protein. However, within the di- and tri-substituted fractions, resolution into subcomponents, presumably positional isomers, is evident. And yet, the potential resolving powers of protein chromatography have not been fully exploited.

Attention should be called to the fact that the separation of modified protein into a series of derivatives, such as those which appear in Fig. 9, may be expected to aid in the determination of protein structure by the isomorphic replacement method of x-ray diffraction analysis.

A comparison between the reaction of CS2 (1) and N-acetyl-DL-homocysteine thiolactone shows striking differences which may be related to the relative rates of reaction of α- and ε-amino groups and to the effect of pH. The former reagent is specific for the α-amino group of α-chymotrypsinogen at pH 6.9 where the ratio of reaction rates for α-amino and ε-amino groups is 200:1. The fact that the thiolactone gives a large variety of products throughout the useful range of pH is explained by the observations with the model compounds, diglycylglycine and ε-amino caproic acid. For these compounds the ratio of reaction rates is about 5:1 in favor of the α-amino group at pH 7.5. Amino groups apparently react at an appreciable rate with the thiolactone even when they are largely in the charged form. Thus, the thiolactone resembles acetic anhydride which acetylates α- and ε-amino groups at pH 6 to 7, the former type somewhat more rapidly than the latter (21–23).

SUMMARY

The use of N-acetyl-DL-homocysteine thiolactone for the preparation of derivatives of α-chymotrypsinogen has been investigated. The rates of hydrolysis and aminolysis of the thiolactone by diglycylglycine and ε-amino caproic acid were determined under conditions similar to those used for reaction with the protein.

Reactions with protein were carried out in a pH-stat. At the termination of the reaction, methylmercury nitrate was added to block the sulfhydryl groups which had been generated. Under all conditions studied, the reaction product consisted of many
chemically distinct components as revealed by chromatography on columns of carboxymethyl cellulose. Conditions were found which gave a monoacylated derivative as a principal component. The derivative, isolated by chromatography, was shown to be homogeneous in electrophoresis and sedimentation, and chemical evidence established that it differed from native α-chymotrypsinogen by the substitution of an N-acetyl homocysteine residue on the α-amino group.

Each of the principal chromatographic fractions of the reaction product could be activated by trypsin to enzymes with esterase activities comparable to that of δ-chymotrypsin.

Acknowledgments—This investigation was carried out with the support of the National Institutes of Health, United States Public Health Service (Grant 508037), and the people of the State of Washington (Initiative 171 Funds for Research in Biology and Medicine). The authors wish to thank Mr. Wen Tan and Mr. Bryan Gordon for valuable technical assistance.

REFERENCES
Chemical Derivatives of α-Chymotrypsinogen: III. REACTION WITH N-ACETYLDI-HOMOCYSTEINE THIOLACTONE
Djahanguir M. Abadi and Philip E. Wilcox


Access the most updated version of this article at
http://www.jbc.org/content/235/2/396.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/235/2/396.citation.full.html#ref-list-1