Modification of a Single Disulfide Bond in Trypsinogen and the Activation of the Carboxymethyl Derivative*

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SUMMARY

Sodium borohydride reduction of native trypsinogen produced a specific cleavage of only 1 of the 6 disulfide bonds. The partially reduced protein was converted to the 14C-carboxymethyl derivative, and tryptic hydrolysis produced two unique peptides in 72% yield which contained the radioactive label. The composition of the two purified peptides from a completely carboxymethylated sample agreed with the amino acid sequence around Disulfide 179 to 203. (Residue numbers refer to the amino acid sequence of trypsinogen.) Activation of carboxymethyl trypsinogen with trypsin and with enteropeptidase formed almost fully active molecules toward the active site titrant 3-nitrophenyl-p'-guanidinobenzoate. However, the rate of activation of the modified zymogen was much lower than with trypsinogen. Furthermore, the relative activity of the modified enzyme toward synthetic ester substrates was no greater than one-fifth of the normal rate. Disulfide 179 to 203 is the disulfide loop containing the active site serine of trypsin, and the altered enzyme activity suggests that a small change occurred in the orientation of amino acid residues which are part of the active site.

Reagents which modify disulfide bonds are generally highly specific and provide a way to evaluate the reactivity of disulfides in proteins as a means to probe tertiary structure and map topology, and to detect conformational changes (1). The cleavage of a limited number of disulfide bonds in proteins was earlier attempted with chemical agents in the absence of denaturing conditions (2). In recent work, greater emphasis was placed on the identification of the reactive disulfide and the effect on biological activity and properties of the altered protein. Thus, one or two specific disulfides were cleaved in trypsinogen inhibitors (3-6), ribonuclease (7), and papain (8, 9) with complete retention of biological activity. Therefore, one or two specific disulfides were cleaved in trypsin in-ing conditions (2). In recent work, greater emphasis was placed on the identification of the reactive disulfide and the effect on biological activity and properties of the altered protein. Thus, one or two specific disulfides were cleaved in trypsinogen inhibitors (3-6), ribonuclease (7), and papain (8, 9) with complete retention of biological activity. We recently reported on the reductive cleavage of a nonessential disulfide in trypsinogen (10) and on a difference in chemical reactivity of the disulfide bonds of trypsin and chymotrypsin (11). The study of two enzymes of homologous structure provided an example of differing disulfide reactivity applied as a probe of tertiary structure. Further consideration must now be given to the effect of disulfide modification on the structure and properties of a protein. We report here on the preparation of a reduced and carboxymethylated trypsinogen, the identification of the reactive disulfide, and the activation of S-carboxymethyl trypsinogen.

EXPERIMENTAL PROCEDURE

Materials

Crystalline trypsinogen, trypsin, and L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone-trypsin were obtained from Worthington. Enterokinase was a product of Pentex, Inc. (Kankakee, Illinois), Nα-tosyl-L-arginine methyl ester and Nα-benzoyl-L-arginine ethyl ester were obtained from Mann, and p-nitrophenyl-p'-guanidinobenzoate and dihydroxyethylthio were purchased from the Cyclo Chemical Corporation (Los Angeles, California). Sephadex products were purchased from Pharmacia. Sodium borohydride was obtained from Metal Hydrides, Inc. (Beverly, Massachusetts), and it was stored at room temperature in a desiccator over anhydrous calcium chloride. Mercaptoethanol and iodoacetic acid were obtained from Matheson, Coleman and Bell (East Rutherford, New Jersey), and 5,5'-dithiobis(2-nitrobenzoic acid) was from Aldrich. Glutathione was purchased from Schwarz. The oxidized forms of glutathione and dihydroxyethylthio were prepared by the procedure of Spackman, Stein, and Moore (12). Oxytocin was a gift from Dr. Vincent du Vigneaud. 14C-Iodoacetic acid was obtained from New England Nuclear.

Methods

Amino Acid Analyses—Protein and peptide samples hydrolyzed in 6 N HCl at 110° for 20 to 24 hours in a vacuum (13) were analyzed on a Spinco model 120B analyzer. Calibration mixtures included S-carboxymethylcycteine when necessary. The composition of protein samples was expressed as molar ratios based on the average recovery of all amino acids omitting S-carboxymethylcysteine, methionine, valine, and isoleucine from the average.

Radioactivity Measurements—Radioactively labeled peptides separated on paper systems were detected with a Packard model 7201 radioluminogram scanner. Specific radioactivity (counts per min per μ mole of sample) was determined on acid hydrolysates of protein or peptide samples with a Packard Tri-Carb model 3200 liquid spectrometer. The amount of sample counted was known from amino acid analysis of the hydrolysate. The
specific radioactivity of the 1-14C-iidoacetate used to label sulfhydryl groups was 126,000 cpm per pmole.

Trypsin Activity—The rate of hydrolysis of N-α-tosyl-l-arginine methyl ester and N-α-benzoyl-l-arginine ethyl ester was measured at pH 8.1 and 25° with a Radiometer pH-stat assembly. The conditions routinely used were 0.01 M substrate, 0.02 M CaCl2, 0.10 M NaCl, and 1 to 5 × 10−7 M trypsin or 3 to 10 × 10−8 M trypsin derivative. The specific activity was calculated as micromoles of substrate hydrolyzed per sec per pmole of active site and expressed as a first order rate constant. The active site titration was performed spectrophotometrically according to the method of Chase and Shaw with p-nitrophenyl-p′-guanidinobenzoate (14). Trypsin concentration was determined spectrophotometrically at 280 mg with E1%280 of 15.6. The same value was also used for the S-carboxymethyltrypsin.

Activation of Zymogen—The conversion of zymogen to active enzyme was performed at pH 5.0 and 25°. The reaction mixture contained 30 mg of zymogen; 15 ml of 0.1 M tris buffer, pH 5; 0.05 M CaCl2; and 1.5 mg of trypsin. The solution was clarified by centrifugation at 0°. To the cold protein solution, the partial reduction of the protein was performed in a buffered system, and the procedure was a modification of our earlier work (11). A freshly prepared solution of 25 mg of trypsinogen was added in 0.2 ml of 0.001 M HCl and 0.05 M CaCl2 was clarified by centrifugation at 0°. To the cold protein solution, 2.0 ml of 0.2 M borate buffer, pH 9.1, were added, followed by the slow addition of 0.5 ml of 1.0 M NaBH4 in 0.2 M borate, pH 9.1. The reaction mixture was kept in an ice water bath and maintained under a nitrogen barrier. The sample was stirred at slow speed with a magnetic stirrer. Foaming occurred only to a limited extent, and no attempt was made to eliminate it.

A 0.2 ml aliquot removed as a function of time was added to 2.0 ml of 0.1 M HCl. The pH accelerated the hydrolysis of excess NaBH4, producing hydrogen gas. Foaming was minimized by adding a trace amount of Anti-fom A (Dow-Corning Corporation, Midland, Michigan) to the acidified sample. After 30 min at room temperature, foaming ceased; the protein concentration of the solution was then determined spectrophotometrically on a 2-ml sample, and the concentration of sulfhydryl groups was estimated by the method of Ellman (15).

Reduction of Model Compounds—The kinetic constants for the reduction of trypsinogen and several disulfide-containing compounds were estimated with the following modification of the above procedure. To each of a series of 8 volumetric flasks with a capacity of 10 ml, 0.1 ml of a 2.4 mM solution of the test sample was added, followed by 0.8 ml of a 0.2 M borate buffer, pH 9.1; 0.9 ml of water; and 0.2 ml of 1 M sodium borohydride in borate buffer. The reaction vessels were maintained in an ice water bath, and the contents of individual flasks were analyzed for sulfhydryl groups as a function of reaction time. The reaction was terminated on the addition of 1 ml of 0.5 M HCl, and the foaming produced was completely contained within the flask. The Ellman procedure was performed with the addition of 0.05 ml of 0.01 M 5,5'-dithioba-s(2-nitrobenzoic acid), followed by 2 ml of 1 M Tris, pH 8.5, and diluting to the 10-ml mark.

Alkylation of Partially and Completely Reduced Trypsinogen—In a typical experiment, trypsinogen was partially reduced with sodium borohydride for 60 to 90 min. The reaction was terminated on lowering the pH to 3 with the slow addition of 1 M HCl. After foaming ceased, the pH was raised to pH 8.5 and 1-14C-iidoacetate in 1 M Tris, pH 8.5, was added in a 5 fold molar excess over the sulfhydryl content. The reaction proceeded at room temperature for 45 min under nitrogen. The sample was dialyzed exhaustively in the cold against 0.1 N acetic acid for 2 days, and the solution was lyophilized.

The disulfides remaining were reduced with 0.25 M mercaptoethanol in 8 M urea at pH 8.6 for 4 hours at room temperature under nitrogen (16). In experiments required for peptide mapping, the reduction was performed with 0.01 M dithioerythritol in 6 M guanidine hydrochloride (17). Sulfhydryl groups were alkylated with a 2-fold molar excess of iodoacetate at pH 8.6 for 30 to 45 min. The procedure was modified to prepare a fully 14C-alkylated sample. The completely reduced protein was precipitated with 9 volumes of cold absolute ethanol containing 2% of 12 N hydrochloric acid. The protein was washed several times with cold ethanol-HCl, redissolved in 6 M guanidine hydrochloride at pH 8.5, and allowed to react with a 10-fold molar excess of 14C-iidoacetate at pH 8.5 for 45 min. The samples of alkylated protein were dialyzed exhaustively against 0.1 M acetic acid and recovered by lyophilization.

Trypsin Digestion of Alkylated Proteins—Protein samples suspended in 0.1 M ammonium carbonate or 0.05 M N-ethylmorpholine buffer, pH 8.5 (5 mg of protein per ml), were digested at room temperature with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone-trypsin at an enzyme to substrate ratio of 1:50. Within a few minutes, the turbid mixture became more soluble and, after 5 hours, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone-trypsin was again added to bring the enzyme to substrate ratio to 1:25. The digestion was terminated at 18 hours by lowering the mixture to pH 3 with 15% acetic acid. The acidified digest was kept at 0° for 3 hours, and an acid-insoluble fraction was centrifuged and washed with a small portion of 0.1 N acetic acid. The soluble fraction and the wash were pooled and lyophilized.

Peptide Maps—The tryptic digest representing 2.5 mg of protein was submitted to a separation in two dimensions on Whatman No. 3MM paper. High voltage electrophoresis was carried out at 2000 volts for 1 to 2 hours with a pyridine-acetate buffer at pH 6.5 (100 ml of pyridine and 4 ml of acetic acid per liter). Chromatography was performed for 18 hours with a solvent system of butanol-pyridine-acetic acid-water (15:10:3:12). Peptides were detected with 0.1% ninhydrin containing 5% collidine in ethanol.

Identification of Disulfide Bond Cleaved with Sodium Borohydride—The soluble peptides from the tryptic digest of 400 mg of carboxymethylated trypsinogen were subjected to gel filtration on a column (2.5 × 95 cm) of Sephadex G-25 (200 to 400 mesh) equilibrated with 15% acetic acid. The sample was applied in 5 ml of 40% acetic acid, and effluent was collected in 5-ml fractions at an initial flow rate of 16.5 ml per hour with 15% acetic acid as solvent. Three 14C-containing fractions were collected, evaporated to dryness, and reconstituted in 5 ml of 15% acetic acid.
Fraction I was not purified further (Peptide I). Fraction II was submitted to preparative chromatography on Whatman No. 3MM paper for 20 hours with the system butanol-pyridine-acetic acid-water (15:10:3:12). The radioactive component was recovered 50 mm from the origin and further purified by electrophoresis at pH 6.5. The radioactivity was associated with an acidic component (Peptide II).

Fraction III was placed on a column (1 × 42 cm) of Bio-Rad 50W-X2 (200 to 400 mesh) equilibrated with 0.2 N pyridine acetate buffer, pH 3.1, containing 5 ml of thioglycolic acid per liter. Thioglycolic acid was added to the buffers as an oxygen scavenger to protect the thioether linkage of the S-carboxymethylcysteine residues (18). The column was eluted with 0.2 pH 3.1, until 200 ml were collected. A gradient elution was started with 350 ml of 0.2 N pyridine acetate buffer, pH 3.1, in the mixing chamber and 350 ml of 2 N pyridine acetate buffer, pH 5.0, in the dropping flask. The flow rate was 30 ml per hour, and 5-ml fractions were collected. Samples of 0.1 ml of every third fraction were spotted on Whatman No. 3MM paper, and 5-ml fractions were collected. Samples of 0.1 ml of every fraction were spotted on Whatman No. 3MM paper for 20 hours and 5-ml fractions were collected. Samples of 0.1 ml of every fraction were spotted on Whatman No. 3MM paper and 5-ml fractions were collected. Samples of 0.1 ml of every fraction were spotted on Whatman No. 3MM paper.

Partial Acid Hydrolysis of Peptide I—Approximately 0.5 μmole of Peptide I was hydrolyzed with 5 ml of 0.25 M acetic acid under reflux conditions for 10 min (19). The hydrolysate was submitted to electrophoresis at pH 6.5 for 2 hours at 2200 volts. The separation yielded a neutral peptide (Peptide I-N) and two radioactively labeled peptides which migrated 160 mm (Peptide I-A-1) and 280 mm (Peptide I-A-2).

RESULTS

Sodium Borohydride Reduction of Trypsinogen—Native trypsinogen was rapidly converted to a partially reduced molecule on treatment with a buffered solution of sodium borohydride. The reaction produced two sulfhydryl groups in 90 min, and increased reaction times did not change the value appreciably (Fig. 1). Essentially, the same results were found (a) if the initial concentration of sodium borohydride was varied from 0.05 M to 0.25 M; (b) if the mixture was buffered with Tris, borate, or sodium barbital; and (c) if the pH was varied between 8.0 and 10.0. Foaming was appreciable at the lower pH, and limited denaturation may occur. We selected 0.1 M sodium borohydride and pH 9.1 in borate or Tris buffer as standard conditions. The reaction was limited to the formation of two sulfhydryl groups, which differs from our earlier work with an unbuffered system in which the reaction produced approximately four sulfhydryl groups (11).

The rate constants for the reduction of trypsinogen and several model compounds were determined. The reduction of trypsinogen was followed until 95% of a single disulfide bond was modified, and the reaction proceeded by first order kinetics (Fig. 2). Other compounds also followed first order kinetics, and the rate constants are given in the legend to Fig. 2. Although the study was limited to a few structures, the results clearly show differences in the reactivity of the disulfide. A disulfide bond of

\[ \text{Trypsinogen-N} \rightarrow \text{Trypsinogen} \]

Fig. 1. The reduction of trypsinogen by sodium borohydride in a buffered system at 0°. Sulfhydryl groups were determined by the Ellman reagent, and protein was estimated spectrophotometrically. Δ–Δ, reduction with 0.1 M NaBH₄ in 0.1 M Tris buffer, pH 8; •–•, reduction with 0.1 M NaBH₄ in 0.1 M Tris buffer, pH 9.0; □–□, reduction with 0.05 M NaBH₄, 0.1 M borate buffer, pH 9.0.

Fig. 2. The first order rate dependency for the loss of disulfides on reduction with 0.1 M sodium borohydride in 0.1 M borate buffer, pH 9.1, at 0°; △–△, reduction of dithioerythritol (k = 0.50 × 10⁻⁴ sec⁻¹); ○–○, oxytocin (k = 0.59 × 10⁻⁴ sec⁻¹); •–•, cystine (k = 1.23 × 10⁻⁴ sec⁻¹); □–□, oxidized glutathione (k = 2.05 × 10⁻⁴ sec⁻¹); Δ–Δ, trypsinogen (k = 9.45 × 10⁻⁴ sec⁻¹) (see text for further details).
The formation of two sulfhydryl groups in reduced trypsinogen was achieved by the method of Kawashima and Whitmore (19) and was characteristic of the sequence of residues from 193 to 206 (Fig. 5). The single disulfide bond 179 to 203 (21).

The use of sodium borohydride to reduce protein disulfide bonds may produce peptide bond cleavage under certain experimental conditions (18). We examined the reduced and alkylated trypsinogen (1.7 S-carboxymethylcysteine residues per molecule) for end groups by the cyanate procedure of Stark and Smyth (20). The relative rates for the series of compounds are trypsinogen, 100; oxidized glutathione, 22; cystine, 14; oxytocin, 6; and oxidized dithioerythritol, 5.

The amino acid composition of Peptide I (Table II, Column 2) and the 14C incorporation (1.8 residues). The remaining disulfides were reduced with dithioerythritol in guanidine hydrochloride, and the fully reduced protein was treated with unlabeled iodoacetate.

The insoluble core separated after tryptic digestion was not radioactive, and peptide mapping of the radioactively labeled soluble fraction showed that only two of the peptides contained 14C; one of the peptides was neutral and gave a positive test with the Ehrlich reagent, and the second was acidic (Fig. 3).1 The radioactive spots were excised from the map, counted in a liquid scintillation counter, and represented a 72% yield of the radioactivity placed on the maps. No other radioactive components were detected although the limit of detection was 10% of the radioactivity observed.

Identification of Disulfide Reduced by Sodium Borohydride—The amino acid composition and 14C content of partly reduced and 14C-S-carboxymethylated trypsinogen are given in Table I, Column 2. Excellent agreement was found for the sulfhydryl content (1.8 residues), the S-carboxymethylcysteine (1.8 residues) and the 14C incorporation (1.8 residues). The remaining disulfides were reduced with mercaptoethanol in 8 M urea and converted to the unlabeled carboxymethyl derivative. The amino acid composition of the fully alkylated trypsinogen is presented in Table I, Column 3, and the molar ratios again agree with the predicted number.

We selected proteolysis with trypsin to take advantage of its known specificity and to obtain a mixture of peptides in which the S-carboxymethyl derivatives would be present in only 8 peptides. The tryptic digest of the fully alkylated substrate was separated into a soluble and an acid-insoluble fraction. The insoluble fraction contained only 3% of the total radioactivity, and the fraction was not examined further.

The soluble tryptic peptides were separated into three radioactive fractions on Sephadex G-25 chromatography (Fig. 4). Purified Peptide III gave a positive test for tryptophan with the Ehrlich reagent, and the amino acid composition is presented in Table II, Column 5. The composition of the peptide agrees with the sequence of residues from 193 to 206 (Fig. 5). The single carboxymethylcysteine at Residue 203 is derived from the dithioerythritol derivative, the 14C-carboxymethyl groups in the tryptic digest of the fully reduced and labeled protein provided a reference for the study of the partially reduced trypsinogen.

Partially reduced trypsinogen obtained from the reaction of the protein with sodium borohydride was converted to the 14C-carboxymethyl derivative. The remaining disulfides were reduced with dithioerythritol in guanidine hydrochloride, and the fully reduced protein was treated with unlabeled iodoacetate.

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The composition of W-carboxymethylcysteine 0.6 0.8 0.8 0.8
Threonine 1.3 [1]
Methionine 0.2 [1]a
Isoleucine 1.2 [1]
Leucine 1.4 [1] 1.0 [1]
Tyrosine 1.0 [2]
Phenylalanine 0.7 [1]
Tryptophan

* Residue numbers shown in brackets are the actual number of residues expected from the known amino acid sequences.

b Not determined.

c Sum of carboxymethylcysteine (1.8) and half-cystine (0.6).
d These values only are from 72-hour hydrolysates of these peptides.

* Value assumed based on a positive Ehrlich reaction with the intact peptide.

with the sequence of residues from 1/7 to 192 (Fig. 5). Purified Peptide II was isolated in low yield with an amino acid composition which also agrees with the sequence of residues from 177 to 192 (Table II). Peptide I was derived from an incomplete hydrolysis of Lys-Asn at position 176, and Peptide II is the product of the small degree of hydrolysis which did occur at lysine 176.

Peptide I (Residues 156 to 192) was degraded further in order to identify a partial sequence containing the 14C-carboxymethyl residue. Partial acid hydrolysis was used to obtain hydrolysis at aspartic acid and asparagine residues. The limited amount of Peptide I restricted the isolation to the recovery of a major radioactive peptide (Peptide I-A-1) with acidic properties. The composition of the fragment was S-carboxymethylcysteine 0.69 (1), Asp 0.26 (0), Ser 0.88 (1), Glu 0.06 (1), Gly 1.16 (1), Val 0.21 (0). The composition agrees with a partial sequence around Residue 179 which establishes the residue as the second peptide sequence derived from disulfide 179 to 203 (Fig. 5). Thus, the isolation of two unique peptide sequences containing the radioactive label provides a conclusive identification of the disulfide modified by sodium borohydride. Moreover, a neutral nonradioactive peptide (Peptide I-N) was also isolated from the partial acid hydrolysate with the composition S-carboxymethylcysteine 0.86 (1), Ser 2.10 (2), Pro 1.28 (1), Gly 3.54 (3), Val 1.21 (2), Lys not determined (1). Peptide I-N agrees with the sequence from Residues 182 to 192 (Fig. 5), and the absence of a radioactive label eliminates disulfide 122 to 189 from further consideration.

Activation of Carboxymethyl Trypsinogen—Partially reduced and carboxymethylated trypsinogen may contain unmodified
Modification of Trypsinogen

Trypsinogen in low amounts if the sodium borohydride reduction was not quantitative. Residual trypsinogen was separated from carboxymethyl trypsinogen by chromatography on a column (1.5 x 85 cm) of DEAE-Sephadex A-50. In a typical experiment, 130 mg of sample in 6 ml of 0.03 M Tris buffer (pH 9.1), 0.01 M CaCl₂, and 0.001 M benzamidine were applied to the column which was equilibrated and developed with the same buffer mixture. The column purified carboxymethyl trypsinogen was used as substrate in the activation mixture.

The conversion of zymogen to active enzyme was examined with trypsin as catalyst and with the physiological activator enterokinase. The active molecules were detected with the trypsin active site titrant p-nitrophenyl-p'guanidinobenzoate (NPGB). The following observations support this conclusion:

(a) Reduction of the protein with sodium borohydride produced 2 sulfhydryl groups per molecule which is the expected value if 1

 Attempts to activate carboxymethyl trypsinogen at pH 8 were not successful, possibly reflecting rapid proteolysis of the derivative at the higher pH.

Table III presents a comparison of the esterolytic behavior of trypsin and carboxymethyl trypsin toward the two substrates N-a-tosyl-L-arginine methyl ester and N-a-benzoyl-L-arginine ethyl ester. Carboxymethyl trypsin catalyzed the hydrolysis of both substrates, but the specific activities were lower than normal. Activity with N-a-tosyl-L-arginine methyl ester was 14% of the comparable value found for trypsin, and with N-a-benzoyl-L-arginine ethyl ester it was 17%. Although carboxymethyl trypsin was 86% active toward p-nitrophenyl-p'-guanidinobenzoate, it showed altered properties in its kinetic behavior.

Discussion

Of the six disulfides present in native trypsinogen, only one is rapidly reduced by sodium borohydride under mild reaction conditions. The following observations support this conclusion:

(a) Reduction of the protein with sodium borohydride produced 2 sulfhydryl groups per molecule which is the expected value if 1

(b) Activation of trypsinogen by trypsin at pH 5 is slower than the rate usually observed at pH 8, but the yield of enzyme was the same. Trypsin converted carboxymethyl trypsinogen to active molecules in a yield close to 80%.

(c) Activation of the trypsinogen derivative with enterokinase also followed the same progress curve (Fig. 6, Curve D). In contrast to the autocatalytic activation of trypsinogen, carboxymethyl trypsinogen was converted to active molecules in accord with first order kinetics.
The reduction followed first order kinetics over the entire reaction for the disappearance of a single disulfide (Fig. 2). (b) Peptide maps of tryptic peptides of 14C-carboxymethyl tryosinogen (1.8 14C-carboxymethylcysteine residues) contained two radioactively labeled peptides (Fig. 3). Only two labeled peptides would be produced if a single disulfide bond was reduced and if the two 14C-carboxymethylcysteine residues appeared in separate peptide sequences. Furthermore, the two peptides were recovered from the peptide map in equal amounts, and they represented a 72% yield of radioactivity based on the sample size. (c) A large scale separation of the tryptic peptides provided further confirmation that only two unique amino acid sequences were present which contained 14C-carboxymethyl derivatives. Peptides were also found which contained unlabeled carboxymethylcysteine. The two labeled peptides provided direct evidence for the specificity of the reaction and the unlabeled peptides provided indirect evidence.

In the amino acid sequence of tryosinogen, each half-cystine residue is present in a unique sequence of neighboring residues (21). Thus, it is necessary only to match the radioactively labeled peptides with the sequence of the known peptides in order to identify the reactive disulfide of trypsinogen. Peptides II and III are the tryptic peptides which account for the half-cystine residues produced on reduction with sodium borohydride. The amino acid compositions of the radioactively labeled peptides agreed with the sequence of residues around the disulfide bond 179 to 203 (Fig. 5). Disulfide 179 to 203 is present in the trypsin structure as the disulfide loop which contains the active site serine at residue 183 (23-25).

In the experiments described above, a single disulfide was reduced while in our earlier work, with an unbuffered system and at a higher pH, two disulfides were modified (11). We tentatively identified one of the bonds as the methionine loop of disulfide 154 to 168 (11). It is now clear that the reduction is sensitive to reaction conditions, and the use of a high pH may increase the instability of the protein. Other possibilities can be considered, but studies presently underway may help to clarify the system further.

The limitation of the reduction to a single disulfide provided indirect evidence that the reduced protein remained in the native state. If partial or complete unfolding of the molecule occurred on cleavage of the 179 to 203 bond, additional disulfides would be reduced. Thus, in the extreme case in which the protein was denatured in 8 M urea, sodium borohydride reduction of trypsinogen produced 11 sulfhydryl groups in 1 hour (11). The limited reduction of native trypsinogen suggests that Disulfide 179 to 203 represents a bond which is nonessential as a structural element. Presumably, side chain interactions of residues in the immediate vicinity of the disulfide must be sufficient to maintain the reduced protein in a conformation close to that of trypsinogen.

If carboxymethyl trypsinogen has the structure of the native molecule, then the synagen should be activated to an active enzyme. The process of limited proteolysis which accompanies activation was used as a probe of structural similarities, and the appearance of activity was used to evaluate the reaction. Approximately 60% active enzyme molecules were formed on activation of carboxymethyl trypsinogen with either trypsin or enterokinase. The high conversion to active enzyme provided a direct demonstration that carboxymethyl trypsinogen resembled the native structure and that carboxymethyl trypsin was a functional molecule.

The rate of activation of carboxymethyl trypsinogen was slower than normal. The slow conversion to active molecules is a consequence of the first order behavior observed with carboxymethyl trypsinogen in the activation process. However, the calculated rate constant for the activation of carboxymethyl trypsinogen is identical with the one found for trypsinogen if the treatment of the kinetics corrects for autoactivation. Thus, it is clear that carboxymethyl trypsinogen was subject to limited proteolysis by a mechanism identical with the one normally observed.

The titration of carboxymethyl trypsin with p-nitrophenyl-p'-guanidinobenzoate produced a "burst" of p-nitrophenol and formed the stable p-guanidinobenzoate ester. Thus, alyation of the enzyme derivative with the active site titrant appeared to be identical with that of trypsin itself (14). In contrast, carboxymethyl trypsin showed altered properties toward synthetic ester substrates such as N-a-tosyl-L-arginine methyl ester and N-a-benzoyl-L-arginine ethyl ester. Hydrolysis of the substrates was observed, but the specific activity of the enzyme was no greater than one-fifth the normal value.

The activity of trypsin in catalysis involves substrate binding, acylation of the enzyme, and deacylation of the enzyme-substrate compound (26). If an enzyme contains an active site but altered catalytic behavior, it is reasonable to assume that one or more steps of the mechanism of action is different from normal. The disulfide reduced by sodium borohydride is part of the sequence at the active site serine. The half-cystine at Residue 179 is only three residues removed from the serine at position 183. The cleavage of the disulfide and the introduction of carboxymethyl groups close to the serine may sufficiently alter the orientation of the active site to cause the observed enzymic behavior. An evaluation of the mechanism of action of carboxymethyl trypsin is required to test these possibilities. Studies are now underway to gather this information.

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