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Comparative Studies on the Modification of Specific Disulfide Bonds of Trypsinogen and Chymotrypsinogen*

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SUMMARY

Two homologous disulfides of trypsinogen and chymotrypsinogen differ in their reactivity toward specific reducing agents. Disulfide 179 to 203 of trypsinogen was readily reduced with either 0.1 M sodium borohydride or 0.0005 M dithioerythritol. A second disulfide bond, at residues 122 and 189, required 0.01 M dithioerythritol for cleavage to occur. Disulfide 191 to 220 of chymotrypsinogen was reduced with 0.01 M dithioerythritol, while disulfide 136 to 201 was unreactive. The relative reactivity of the bonds provides a way to characterize the behavior of each and to classify the bonds as fully exposed (reactive with sodium borohydride and 0.0005 M dithioerythritol), partly buried (reactive with 0.01 M dithioerythritol), and buried (unreactive in the native state). These observations suggest that the two zymogens differ in conformation in regions of homologous sequences.

***S*(Carboxymethylcysteine)$_4$-(122, 179, 189, 203)-trypsinogen was activated with trypsin at pH 5, and the concentration of active molecules was estimated with *p*-nitrophenyl-*p'*-guanidinobenzoate. The rate of formation of active sites and the yield were similar to the activation of the dicarboxymethyl derivative (LIGHT, A., HARDWICK, B. C., HATFIELD, L. M., AND SONDACK, D. L., *J. Biol. Chem.*, 244, 6289 (1969)). The formation of catalytically active molecules from the modified zymogen shows that the two disulfides are nonessential structural elements.**

Chromatographically purified α- and β-trypsin, and inhibited trypsins, were not reduced in a specific manner with dithioerythritol. Instead, a low yield was observed of randomly reduced disulfides. The apparent reduction of two disulfides of a commercial trypsin sample (LIGHT, A., AND SINHA, N. K., *J. Biol. Chem.*, 242, 1358 (1967)) was caused instead by the reduction of the inert protein component. The difference in reactivity of the disulfides of trypsinogen and trypsin suggests that the conformation change accompanying the activation process includes the region of disulfide 179 to 203.

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‡ The experimental work reported in this paper is taken from the dissertation in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Purdue University (1). Present address, Eli Lilly and Company, Indianapolis, Indiana.

The amino acid sequences of bovine trypsinogen (2, 3) and bovine chymotrypsinogen (4, 5) are closely related, with 40% of the residues occurring in the same positions (6). The homologous proteins would, therefore, be expected to have considerable similarities in their tertiary structures. At the present time, only the structure of tosyl-α-chymotrypsin has been solved by x-ray diffraction techniques, and a molecular model of the inhibited enzyme was described by Matthews *et al.* (7) and Blow (8). However, Keil *et al.* (9) and Hartley (10) constructed a model of trypsin to fit the electron density map of chymotrypsin. The model accommodated the amino acid sequence of trypsin including the two nonhomologous disulfides and other amino acid differences. The reliability of the structure gained further support when a similar model of elastase (10) proved to be consistent with the recent report of its electron density map (11, 12). Thus the tertiary structures of the serine proteinases must be closely similar, and one can assume that this is also true for their zymogens (13).

We have used sodium borohydride to modify a specific disulfide of trypsinogen as a way to evaluate the role of the bond in the zymogen and in the activation of trypsinogen to a functional enzyme molecule (14). The reactive disulfide was identified as the bridge between residues 179 to 203, which bridges the active site serine of trypsin. Disulfide 179 to 203 is located in a homologous sequence in chymotrypsinogen A as well as in other serine proteinases (10). In light of the homology of sequence and conformation existing between trypsinogen and chymotrypsinogen, we were surprised to find a significant difference in the susceptibility of the proteins to reduction (15). The reactivity of the disulfide is high in trypsinogen but low or nonexistent in chymotrypsinogen and chymotrypsin. This finding led us to conclude that a difference in conformation exists in the immediate vicinity of the disulfide (15).

We report here on further studies of the disulfide reactivity of the two zymogens. Dithioerythritol was selected in this investigation as an example of a strong reducing agent, capable of reacting at neutral pH, and over a wide concentration range (16). We show that the reagent selectively cleaved a single disulfide of chymotrypsinogen. Under the same reaction conditions, two disulfides of trypsinogen were modified while chromatographically purified α- and β-trypsin and inhibited trypsins were unreactive.

EXPERIMENTAL PROCEDURE AND RESULTS

Materials and methods were essentially the same as described in an earlier paper (14) with the following additions and modifications. Bovine chymotrypsinogen A was obtained from Worthington. *N*-α-Tosyl-L-lysyl chloromethyl ketone-inhibited trypsin and *p*-guanidinobenzoyltrypsin were prepared following the procedures of Shaw, Mares-Guia, and Cohen (17) and Chase and Shaw (18), respectively. Chromatography of trypsin samples on SE-Sephadex C-50 was by the procedure of Schroeder and Shaw (19).

The active site titration of trypsin and modified trypsin samples were performed spectrophotometrically with *p*-nitrophenyl-*p'*-guanidinobenzoate by modification of the procedure of Chase and Shaw (18) and Hruska, Law and Kezdy (20). Absorbance changes were recorded with a Gilford model 2000 spectrophotometer. A molar extinction coefficient of 8.2×10^3 was used to calculate the molarity of active sites for determinations carried out at pH 5 and 320 nm (21).

Partial Reduction of Proteins with Dithioerythritol—The reaction mixture contained 5 mg of protein per ml, 0.1 M Tris, pH 8.5, 1 mg of EDTA per ml, and dithioerythritol at the specified concentration. The solution was maintained under a nitrogen atmosphere, at 0°, and stirred at slow speed with a magnetic stirrer. Samples of the mixture were removed at time intervals and added to an equal volume of 0.2 M Tris, pH 8.5, containing iodoacetate in a 5-fold molar excess over the concentration of dithioerythritol. After a reaction time of 1 hour at room temperature, the alkylated protein was precipitated with 9 volumes of cold absolute ethanol containing 2% of 12 N hydrochloric acid, and the precipitate was washed three times with the solvent. The *S*-carboxymethylcysteine content of the protein provided an estimate of the extent of the reduction.

Tryptic Digestion of Alkylated Trypsinogen and Trypsin—The protein samples were suspended in 0.1 M ammonium carbonate, pH 8.5, at a concentration of 5 mg of protein per ml. L-(1-Tosylamido-2-phenyl) ethyl chloromethyl ketone-trypsin was added at an enzyme to substrate ratio of 1:50. After 5 hours, the enzyme level was increased to 1:25. The digest was maintained at room temperature for a total of 18 hours and terminated by lowering the pH to 3 with 15% acetic acid. The soluble fraction was evaporated to dryness by lyophilization.

Chymotryptic Digestion of Alkylated Chymotrypsinogen—*S*-carboxymethyl chymotrypsinogen was suspended in 0.1 M ammonium carbonate, pH 8.5, at a concentration of 5 mg of protein per ml. Chymotrypsin was added initially at an enzyme to substrate ratio of 1:50 and, after 4 hours, the enzyme level was increased to 1:25. The digest was maintained at room temperature for a total of 8 hours and the reaction terminated by lowering the pH to 3 with acetic acid. The sample was evaporated to dryness by lyophilization.

Purification of Peptides—Chromatography on columns of Sephadex G-25 was performed as described earlier (14). Additional purification of peptides was carried out on paper systems. Paper electrophoresis was performed at pH 6.4 with System I (100 ml of pyridine and 4 ml acetic acid per liter) and at pH 1.9 with System II (20 ml of formic acid and 80 ml of acetic acid per liter). Chromatography was performed with Solvent System III (1-butanol-pyridine-acetic acid-water in a ratio of 15:10:3:12). Peptides were detected on paper with 0.1% ninhydrin in ethanol containing 5% collidine. The Ehrlich reagent was used to detect tryptophan in peptides, the Pauly reagent for tyrosine and histidine, and the Sakaguchi reagent for arginine.

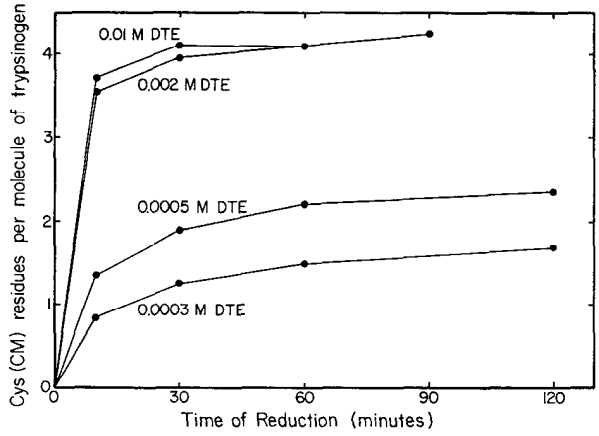

FIG. 1. The reduction of trypsinogen by dithioerythritol (*DTE*). Conditions for the reduction were 5 mg of protein per ml, 0.1 M Tris, pH 8.5, at 0°. Sulfhydryl groups were determined as the *S*-carboxymethyl derivative. Samples of the mixture were added to an equal volume of 0.2 M Tris, pH 8.5, containing iodoacetate in a 5-fold molar excess, and the alkylation proceeded for 1 hour at room temperature.

Amino Acid Analyses—Peptides and proteins were hydrolyzed with 6 N HCl under reduced pressure at 110° for 20 hours. Amino acid analyses were determined with a Spinco model 120B automatic amino acid analyzer with an accelerated system. Single analyses are reported for peptides and the results are not corrected for partial destruction or incomplete liberation of amino acids.

Reduction of Trypsinogen

Cleavage of Disulfide 179 to 203—The time course for the reduction of trypsinogen at a fixed pH and temperature but with different initial concentrations of dithioerythritol is shown in Fig. 1. At 0.0005 M dithioerythritol, the reaction produced two sulfhydryls per protein molecule in 30 min, and the value did not change significantly with longer reaction times. At a lower concentration of dithioerythritol, the reduction was incomplete after 2 hours. The extent of the reduction with 0.0005 M dithioerythritol was the same as with 0.1 M sodium borohydride, and the rate was slightly faster although the dithioerythritol was present in only a 2-fold molar excess. The ease of the reduction of the disulfide is in accord with the low oxidation-reduction potential of dithioerythritol which makes the reagent a very effective reducing agent (16). The specificity of the reduction with the two reducing agents should be the same, and the following experiments show that the same disulfide was modified by each.

The disulfide cleaved with 0.0005 M dithioerythritol was converted to the *S*-^{14}C-carboxymethyl derivative, and both amino acid analysis and radioactivity measurements showed that two carboxymethylcysteine residues were introduced per protein molecule. The partially alkylated protein was fully reduced with 0.01 M dithioerythritol in 6 M guanidine hydrochloride and alkylated with a 2-fold molar excess of nonradioactive iodoacetate. A tryptic digest of the sample was submitted to peptide mapping, and the positions of the radioactively-labeled peptides were compared to maps of the previously characterized digest of the sodium borohydride reduced and ^{14}C-alkylated trypsinogen (14).

Two radioactive peptides were detected in the correct positions for the peptides containing the disulfide bridge between residues 179 and 203. It is clear that both reducing agents cleaved the same disulfide, and the high specificity of dithioerythritol toward disulfides makes it the preferred reagent.

Cleavage of Disulfides 179 to 203 and 122 to 189—At higher concentrations of dithioerythritol, such as 0.01 M, a maximum of four sulfhydryls per molecule were produced (Fig. 1). The extent of the reduction suggested that two disulfides were cleaved. In a large scale reaction performed for 60 min, the partially reduced protein was converted to the *S*-^{14}C-carboxymethyl derivative. The sample was then reduced completely with 0.01 M dithioerythritol in 6 M guanidine hydrochloride, and the sulfhydryl groups were alkylated with nonradioactive iodoacetate. The amino acid composition and radioactivity of the partially reduced protein showed the presence of 3.9 residues of carboxymethylcysteine per protein molecule. The fully reduced and alkylated protein contained 12.4 residues of carboxymethylcysteine per protein molecule. In both analyses, all other residues were recovered in good agreement with the known composition of trypsinogen.

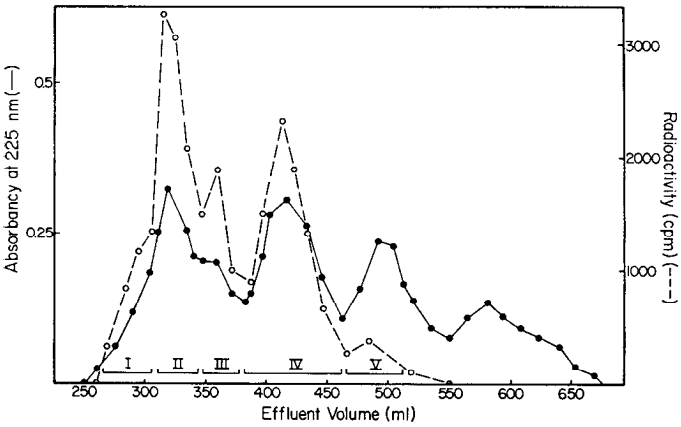

FIG. 2. Elution pattern of peptides from a tryptic digest of carboxymethyl trypsinogen. Trypsinogen reduced with 0.01 M dithioerythritol was treated with ^{14}C-iodoacetate and the remaining disulfides were reduced and carboxymethylated. Sephadex G-25 (fine bead form) chromatography (2.5 × 160 cm) developed at room temperature with 15% acetic acid. Fractions of 5 ml were collected and the absorbance of every third tube was determined at 225 nm (●). Samples of 0.1 ml were mixed with 15 ml of scintillator and the radioactivity was determined (○).

The alkylated protein was hydrolyzed with trypsin, and the radioactivity was associated with the soluble peptides. A peptide map of the digest showed that only three peptides were radioactively labeled in a ratio close to 2:1:1. The restricted number of radioactively labeled peptides suggested that the reduction of the protein proceeded in a specific manner with the cleavage of two disulfides. It was also evident that one peptide contained 2 radioactive residues and the other two contained one each.

The identification of the disulfides cleaved with 0.01 M dithioerythritol followed the same strategy employed earlier with the sodium borohydride-reduced protein (14). The soluble peptide fraction of the digest was resolved on a column, 2.5 × 150 cm, of Sephadex G-25 into five radioactively labeled fractions (Fig. 2). Each fraction was examined by peptide mapping, and the resulting pattern provided a rationale for further purification. The level of radioactivity of Fraction V was below the level of detec-

TABLE I

Amino acid compositions of ^{14}C-labeled tryptic peptides from ^{14}C-carboxymethylated trypsinogen

Amino acid	Extent of electrophoretic or chromatographic movement				
	Peptide T-1 System I: +4 cm (P)[a] System II: 15 cm (P) System III: 5 cm (P)	Peptide T-2 +2 cm (E) 12 cm (E) 28 cm (E)	Peptide T-3 +7 cm 17 cm 4 cm	Peptide T-1a +7 cm	Peptide T-4 0 (P, E) 16 cm (E) 23 cm (P, E)
	Residues/molecule				
Lysine	1.9 (2)[b]	1.0 (1)	0.9 (1)	1.1 (1)	0.9 (1)
Carboxymethylcysteine	2.6 (3)	1.6 (2)	1.6 (2)	1.4 (2)	0.8 (1)
^{14}C-Carboxymethylcysteine	1.5	0.9	1.5	1.6	0.9
Aspartic acid	2.8 (3)	1.2 (1)	1.9 (2)	2.1 (2)	
Threonine	0.7 (1)	2.8 (3)			
Serine	4.4 (5)	4.2 (5)	2.7 (3)	2.9 (3)	1.9 (2)
Glutamic acid	2.9 (3)	1.1 (1)	1.3 (1)	1.0 (1)	1.7 (2)
Proline	1.7 (2)	0.9 (1)	1.2 (1)	0.8 (1)	
Glycine	7.9 (7)	3.1 (3)	4.3 (4)	4.2 (4)	2.6 (3)
Alanine	1.8 (2)	3.1 (3)			0.9 (1)
Valine	1.7 (2)	1.2 (1)	1.9 (2)	1.8 (2)	0.7 (1)
Methionine	0.8 (1)				
Isoleucine	0.8 (1)	2.0 (2)			0.8 (1)
Leucine	0.9 (1)	2.0 (2)			0.8 (1)
Tyrosine	1.7 (2)				
Phenylalanine	0.8 (1)				
Tryptophan[c]	(0)	(1)			(1)
Yield	24%	10%	14%		23%

[a] Letters P and E refer to Pauly- and Ehrlich-positive tests, respectively.

[b] Residue numbers in parentheses are the actual number of residues expected from the known amino acid sequences.

[c] Value assumed based on a positive Ehrlich reaction with the intact peptide.

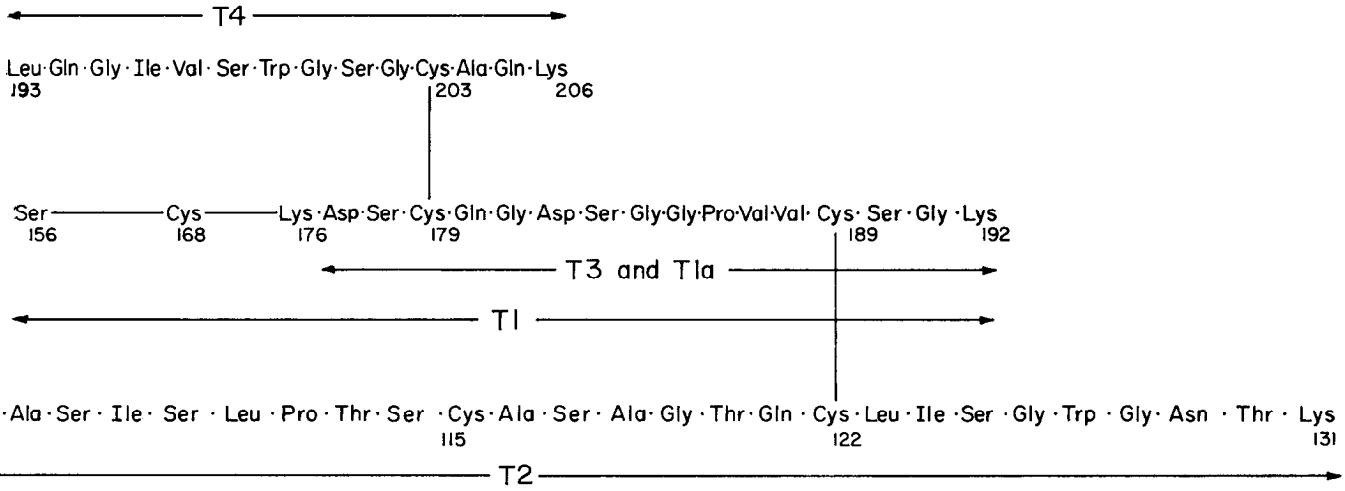

FIG. 3. Partial amino acid sequence of trypsinogen in the region around disulfides 179 to 203 and 122 to 189 (22). The designations *T1*, *T2*, *T3*, *T4*, and *T1a* represent tryptic peptides of carboxymethylated trypsinogen.

tion for the peptides present, and the fraction was not characterized further.

The remaining fractions were purified by paper electrophoresis and chromatography. Table I gives the amino acid composition of the purified peptides, their ^{14}C-content, yield, and behavior on purification.

Peptide T-1 was present in both Fractions I and II, and its amino acid composition agreed with the sequence of residues from 156 to 192. The proposed assignment contains 3 carboxymethylcysteine residues but only two were radioactive. Further tryptic hydrolysis of the peptide and purification by electrophoresis (14) gave two fractions (T-1a and T-1b). Peptide T-1a was nonradioactive and its composition agreed with the sequence of residues 156 to 176. Peptide T-1b (residues 177 to 192), Cys(cm)[1] 1.4 (2), ^{14}C-Cys(cm) 1.6, Asp 2.1 (2), Ser 2.9 (3), Glu 1.0 (1), Pro 0.8 (1), Gly 4.2 (4), Val 1.8 (2), Lys 1.1 (1).

The amino acid composition of Peptide T-1b and its electrophoretic and chromatographic properties agreed with the sequence of residues 177 to 192. The peptide contained the 2 ^{14}C-labeled residues which indicates that half-cystines 179 and 189 were derived from the two reactive disulfides. Peptide T-3 (isolated from Fractions II and III) had an amino acid composition and ^{14}C content identical with Peptide T-1b, and it represented the authentic tryptic peptide for this region of the structure.

Peptide T-4, obtained from Fraction IV, gave a positive test with the Ehrlich reagent showing that tryptophan was present. The amino acid composition of the peptide agreed with the sequence of residues from 193 to 206. The half-cystine at residue 203 is derived from the disulfide 179 to 203, and residue 179 was already found as a radioactively labeled derivative in Peptides T-1b and T-3.

Peptide T-2 was present in both Fractions II and III. Tryptophan was present in the peptide based on a positive test with the Ehrlich reagent. The composition of the peptide agreed with the sequence of residues from 106 to 131. The peptide contains 2 residues of carboxymethylcysteine, but only one was radioactive. Half-cystine residue 122 is derived from disulfide

[1] The abbreviation used is: Cys(Cm), *S*-carboxymethylcysteine.

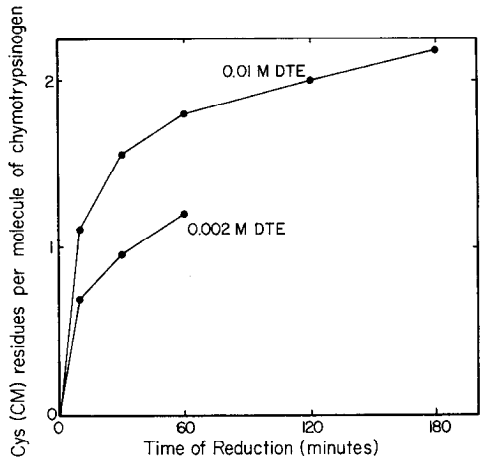

FIG. 4. The reduction of chymotrypsinogen by dithioerythritol. Conditions were the same as given in Fig. 1. *DTE*, dithioerythritol.

122 to 189 (22) and residue 189 was also found as a ^{14}C-labeled derivative in Peptide T-1b.

The identification of the radioactively labeled peptides (Fig. 3) and the known disulfide bridges present in trypsinogen (22) led to an unequivocal assignment of the reactive disulfides. All ^{14}C-labeled half-cystine residues were completely accounted for and identified as residues 122, 179, 189, and 203. The recovery of ^{14}C-labeled peptides and the absence of radioactivity in other fractions provided further support for the specificity of the dithioerythritol reduction and subsequent alkylation.

Reduction of Chymotrypsinogen

Cleavage of Disulfide 191 to 220—The time course for the reduction of chymotrypsinogen at two concentrations of dithioerythritol is shown in Fig. 4. At 0.01 M dithioerythritol, two sulfhydryls per protein molecule were produced while nonstoichiometric values were found at a lower concentration of dithioerythritol (0.002 M). The requirement of a high concentration of dithioerythritol is in sharp contrast to the behavior of trypsinogen where a concentration of 0.0005 M dithioerythritol was sufficient to cleave a single disulfide. The differential reactivity of the di-

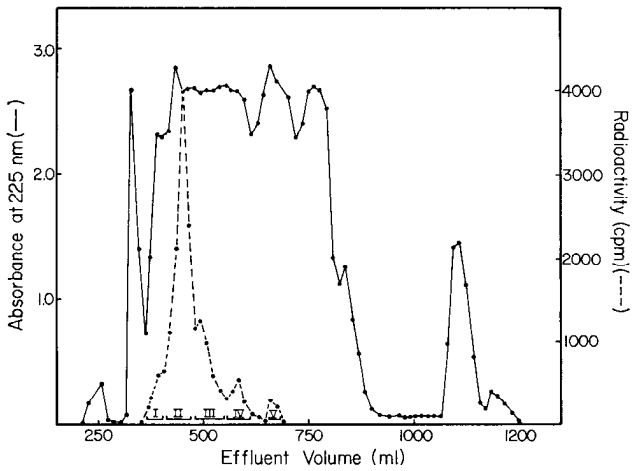

FIG. 5. Elution pattern of peptides from a chymotryptic digest of carboxymethyl chymotrypsinogen. Chymotrypsinogen reduced with 0.01 M dithioerythritol was treated with ^{14}C-iodoacetate and the remaining disulfides were reduced and carboxymethylated. Sephadex G-25 chromatography (2.5 × 150 cm) developed at room temperature with 0.05 M ammonium bicarbonate. Other conditions were similar to those given in Fig. 2.

TABLE II

Amino acid compositions of ^{14}C-labeled chymotrypsin peptides from ^{14}C-carboxymethylated chymotrypsinogen

Amino acid	Extent of electrophoretic or chromatographic movement		
	Peptide C-1	Peptide C-2	Peptide C-3
	System I: +2 cm (P, Y)[a]	+7 cm	+5 cm (Y)
	System II: 7 cm (P, Y)	8 cm	9 cm (Y)
	System III: 19 cm (P, Y)	23 cm	0 cm (Y)
	Residues/molecule		
Carboxymethylcysteine	0.9 (1)[b]	1.0 (1)	1.0 (1)
^{14}C-Carboxymethylcysteine	0.8	1.0	0.9
Aspartic acid		1.0 (1)	0.3 (0)
Threonine	3.0 (3)		0.9 (1)
Serine	3.4 (4)	2.1 (3)	2.7 (3)
Proline	0.9 (1)	0.6 (1)	
Glycine	2.2 (2)	3.1 (3)	1.0 (1)
Alanine	0.5 (0)	0.4 (0)	
Valine	1.1 (1)		
Methionine		0.8 (1)	
Leucine		0.9 (1)	
Tyrosine	0.7 (1)		
Yield	29%	29%	9%

[a] Letters P and Y refer to Pauly-positive test and yellow color with ninhydrin, respectively.

[b] Residue numbers in parentheses are the actual number of residues expected from the known amino acid sequences.

sulfides of chymotrypsinogen and trypsinogen toward dithioerythritol was similar to the results found earlier with 0.1 M sodium borohydride (14).

The reactive disulfide of chymotrypsinogen was identified from a study of the chymotryptic peptides of the *S*-carboxymethyl derivative of the reduced protein. The amino acid composition and radioactivity of the partially reduced protein contained 2.4 residues of ^{14}C-carboxymethylcysteine per protein molecule.

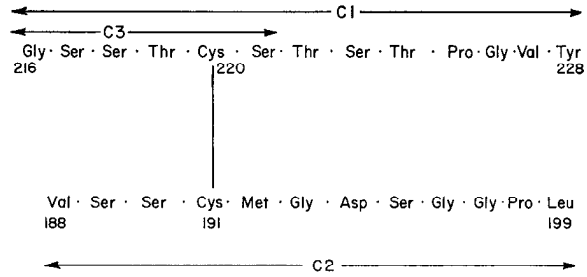

FIG. 6. Partial amino acid sequence of chymotrypsinogen in the region around disulfide 191 to 220.

The fully reduced and alkylated protein contained 9.6 residues of carboxymethylcysteine per protein molecule. In both samples, all other residues were recovered in good agreement with the known composition of chymotrypsinogen.

The peptides of the chymotryptic digest were resolved on a column of Sephadex G-25 with the results shown in Fig. 5. Further purification was performed by electrophoresis and chromatography on paper.

Peptides C-1 and C-2 were obtained from Fraction II on further purification and both were acidic at pH 6.5 (Table II). Peptide C-1 gave a yellow color with the ninhydrin reagent which is characteristic of a terminal glycyl residue. The composition of Peptide C-1 agreed with the sequence of residues from 216 to 228 (Fig. 6), and the single residue of *S*-carboxymethylcysteine contained a stoichiometric amount of ^{14}C. The other radioactively labeled peptide, C-2, agreed with the sequence of residues from 188 to 199. Peptide C-3, recovered in low yield from Fraction III, represented a radioactively labeled fragment of Peptide C-1. Peptide C-3 arose from hydrolysis of the peptide bond between serine 221 and threonine 222 and must represent a chemical rather than a chymotryptic cleavage.

The identification of the purified radioactively labeled peptides accounted for all of the ^{14}C derivative of the chymotryptic digest. It is clear that the composition of the isolated peptides, C-1 and C-2, provided the means to identify the reactive disulfide of chymotrypsinogen as the one linking residues 191 and 220.

Purified peptides were also obtained for the sequence containing the half-cystine of each of the four other disulfides. The ^{14}C content of the *S*-carboxymethylcysteine residues were not stoichiometric and ranged from 10 to 20% of a residue. The low level of radioactivity suggested that these arose from the reduction of a small amount of denatured material present in the zymogen preparation.

Reduction of Trypsin and Inhibited Trypsins

In a preliminary report, we noted that sodium borohydride caused a rapid reduction of a commercial sample of trypsin with the formation of four sulfhydryl groups (15). Since that time, Schroeder and Shaw (9) described a chromatographic procedure capable of resolving a commercial sample into an inert fraction and two active forms of the enzyme which they named α- and β-trypsin. We were prompted to examine again the disulfide reactivity of trypsin with both chromatographically purified and commercial samples. Dr. L. M. Hatfield (23) of this laboratory examined the progress curves for the reduction of the three species with sodium borohydride (Fig. 7). The inert protein (*Curve B*) was rapidly and extensively reduced and its behavior was typical of the rate observed with denatured trypsin (15). The reactivity of the active trypsin species (*Curves C* and *D*) was far slower than

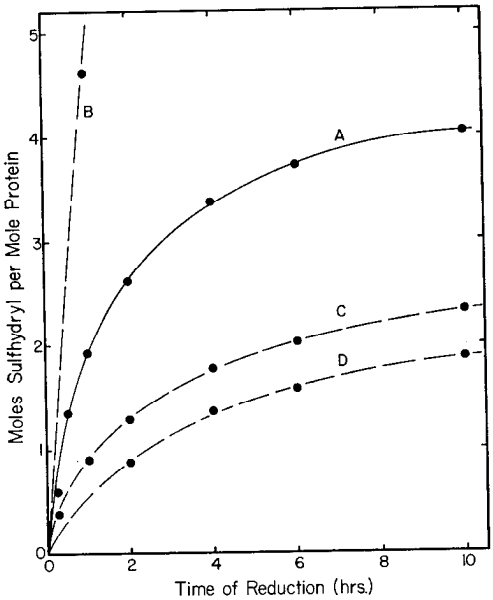

FIG. 7. The reduction of trypsin samples by 0.1 M sodium borohydride in 0.1 M borate buffer, pH 9.1, at 0°. Sulfhydryl groups were determined with the Ellman reagent, and protein was estimated spectrophotometrically. *Curve A*, commercial trypsin; *Curve B*, inert protein; *Curve C*, α-trypsin; *Curve D*, β-trypsin.

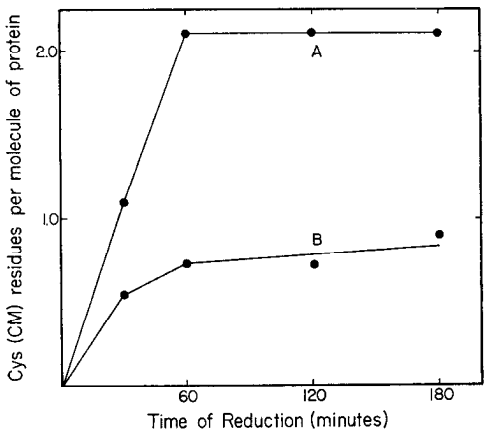

FIG. 8. The reduction of inactive trypsins by 0.001 M dithioerythritol. Conditions were the same as given in Fig. 1. *Curve A*, trypsin inactivated with L-tosyl lysyl chloromethyl ketone; *Curve B*, trypsin inactivated with ethyl-*p*-guanidinobenzoate.

with the commercial sample (*Curve A*) which, in turn, was slower than the inert fraction. It is clear from the progress curves that purified trypsin was only slowly reduced, and the rapid and extensive reaction with the commercial sample primarily represented the reduction of the inert component. Furthermore, samples of partially reduced and alkylated Worthington trypsin were also examined by peptide mapping. In contrast to the specific reduction of trypsinogen, the map of partially reduced trypsin contained low levels of radioactivity associated with each peptide containing carboxymethylcysteine.

The reactivity of the disulfides was also examined under conditions which would minimize autolysis during the course of the reduction. Active site-directed reagents were used to inhibit tryptic activity. We reasoned that these inhibitors would form a covalent linkage with the active site, and the side chain substituent of the reagent would be buried in the specificity pocket

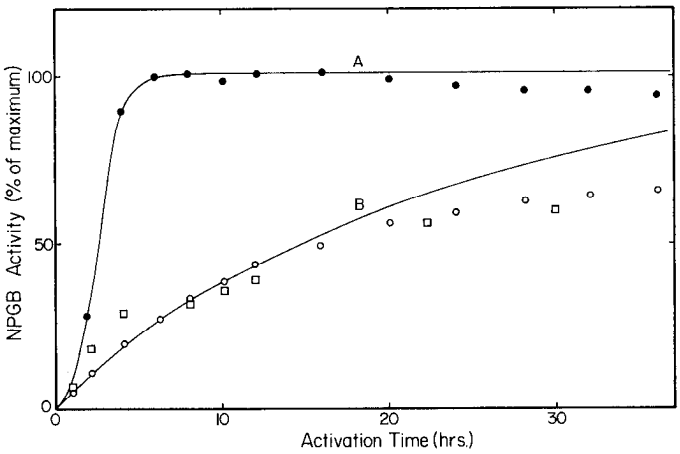

FIG. 9. Activation of trypsinogen and *S*-carboxymethylated trypsinogen derivatives with trypsin at pH 5 and 25°. Activity was measured with *p*-nitrophenyl-*p'*-guanidinobenzoate (*NPGB*) at pH 5. The solid *Curves A* and *B* represent the theoretical activation curves (23). (●——●), trypsinogen; (○——○), *S*-dicarboxymethyl trypsinogen; (□——□), *S*-tetracarboxymethyl trypsinogen.

(8). Thus an inactive enzyme would be produced with minimum steric hindrance of the active center. Trypsin inactivated with N-α-tosyl-L-lysyl chloromethyl ketone and ethyl *p*-guanidinobenzoate were prepared, the former producing a stable derivative with histidine 46 (17) and the latter with serine 183 (18).

On treatment with 0.001 M dithioerythritol, the inhibited trypsins followed the progress curves shown in Fig. 8. N-α-tosyl-L-lysyl chloromethyl ketone-trypsin reacted rapidly and produced two sulfhydryls per protein molecule while *p*-guanidinium benzoyl-trypsin reacted slowly and to a lesser extent. Samples of the partially reduced and ^{14}C-alkylated proteins were digested with trypsin and submitted to peptide mapping. At least five soluble peptides were radioactive and the insoluble fraction also contained an appreciable level of ^{14}C. The radioactivity of the peptides was low suggesting that the reduction was not quantitative for any single disulfide. The peptides derived from disulfide 179 to 203 also contained low levels of radioactivity, and it was clear that the inhibited trypsins were not selectively reduced in the same manner as we found for trypsinogen. Instead it appeared likely that a small quantity of inert protein molecules was present which was completely reduced.

Activation of S-Tetracarboxymethyl Trypsinogen

Our earlier report on the activation of *S*-[Cys(Cm)]$_2$-(179, 203)-trypsinogen showed that the modified zymogen closely resembled the behavior of trypsinogen in the formation of active enzyme molecules (14). Accordingly, a similar study was initiated to determine whether the tetracarboxymethyl zymogen could also be activated.

The zymogen derivative was incubated with trypsin at pH 5 and 25°, and active molecules were determined on titration with *p*-nitrophenyl-*p'*-guanidinobenzoate (Fig. 9). The titration of active sites had to be performed at pH 5 since tetracarboxymethyl trypsin precipitated at pH 8.3, which is the usual pH for the titration. The modified zymogen was converted to functionally active molecules at the same rate and to the same extent as previously found with the dicarboxymethyl derivative. The yield of active molecules on an absolute basis was 32%. It should be

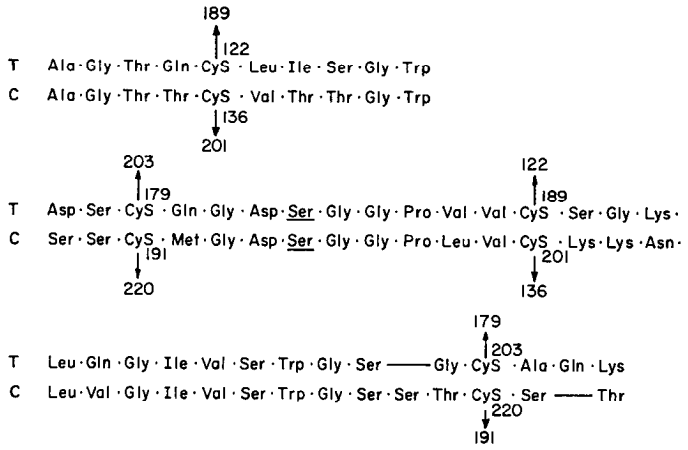

FIG. 10. Partial amino acid sequences of trypsinogen (*T*) and chymotrypsinogen (*C*) in the regions of the reactive disulfides. The active site serine is *underlined*.

noted that the initial rate of activation of tetracarboxymethyl trypsinogen followed first order kinetics and in this respect it behaved in the same way as the dicarboxymethyl derivative (23). With activation times greater than 20 hours for trypsinogen and 10 hours for the modified trypsinogens, a loss of activity occurred presumably from further degradation of the molecules.

DISCUSSION

The differential reactivity of the disulfides of trypsinogen and chymotrypsinogen toward reducing agents, such as sodium borohydride and dithioerythritol, must be related to their accessibility as they occur in the structures of the native proteins. Disulfide 179 to 203 of trypsinogen was rapidly reduced with either sodium borohydride or low concentrations of dithioerythritol. Indeed, the rate of reduction of the protein exceeded the rate observed with model compounds, such as oxytocin and oxidized glutathione (14). The reactivity of the bond is typical of a fully exposed group and, accordingly, the bond will be designated as freely accessible.

On the other hand, the homologous disulfide of chymotrypsinogen, bridging residues 191 to 220, differed in its reactivity, and chymotrypsinogen was refractory toward sodium borohydride (15) or low levels of dithioerythritol. However, at high concentrations of dithioerythritol, namely 0.01 M, the bond was quantitatively reduced. The clear difference in reaction conditions required to reduce the homologous disulfides suggested that they differed in their relative accessibility. In contrast to trypsinogen, the disulfide of chymotrypsinogen behaved as if it was only partly exposed.

A further difference was noted in the reaction of trypsinogen with 0.01 M dithioerythritol. A second disulfide, bridging residues 122 and 189, was cleaved at a rate which was similar to that observed with chymotrypsinogen. It is clear that the two reactive disulfides of trypsinogen differ markedly in their relative accessibility, and the difference in reaction conditions made it possible to modify selectively the first disulfide only, or the two together. Furthermore, the limited reduction of only two of the six disulfides present in the molecule suggested that the modified zymogen remained as a folded structure. The unreactive disulfides must be located as buried residues.

It is noteworthy that disulfide 122 to 189 of trypsinogen is also present in the chymotrypsinogen structure as a homologous bond at residues 136 to 201 (10). The reactivity of the bond in trypsinogen and the lack of reactivity in chymotrypsinogen points to a difference in their environment. Disulfide 122 to 189 behaved as if it was partly exposed, and the resistance to reduction of disulfide 136 to 201 is in accord with its location as a buried residue.

The partial amino acid sequence of both proteins are almost identical in the immediate neighborhood of the disulfides (10), and most, but not all,[2] of the differences that occur represent conservative substitutions (Fig. 10). The high level of homology minimizes the role of neighboring group effects or steric hindrance by adjacent residues as possible explanations for the reactivity of the disulfides. Instead, it is more reasonable to assume that the disulfides differ in their relative accessibility as a consequence of a small difference in the conformation of the two proteins in these regions of the molecule. The homology of the disulfide sequence suggests that the conformational difference must be generated from changes in the tertiary structure of neighboring polypeptide chains. These possibilities will be better defined when additional details become available of the three-dimensional structure of the zymogens (13).

In view of our previous studies on the reactivity of two disulfides of Worthington trypsin (15), we were surprised to find that chromatographically purified samples of α- and β-trypsin (19) were resistant to reduction with sodium borohydride or dithioerythritol. The explanation for this apparent discrepancy is the complete reduction of the inert protein present in the commercial sample and the lack of reactivity of trypsin itself, either as a purified sample or as inhibited trypsin. These results point to a clear difference in the environment of the reactive disulfides as they occur in trypsinogen and in trypsin. This conclusion applies to both of the reactive disulfides since high levels of dithioerythritol were used in the attempted reduction of the trypsin samples.

The change from accessible disulfides in trypsinogen to inaccessible disulfides of trypsin must be related to the structural changes that accompany the activation of the zymogen. On activation, the molecule undergoes a change in conformation sufficient to form the active site (24) and it becomes more stable toward several denaturing conditions (25). Disulfide 179 to 203 is 3 residues removed from the active site serine found in trypsin, and the 122 to 189 bridge is 5 residues removed on the carboxyl-terminal side (Fig. 10). The close proximity of the two disulfides to the active site makes it highly likely that they are involved in the conformational changes accompanying activation. From these studies, it is impossible to decide whether the change in the disulfide location is necessary for the formation of the active center or whether the conformation of the disulfide is a result of the structural changes.

A sample of tetracarboxymethyl trypsinogen was submitted to activation at pH 5 to determine whether a functional enzyme molecule could be produced. Active trypsin molecules were de-

[2] It should be noted that the substitution of an isoleucine at position 124 of trypsin to threonine at position 138 of chymotrypsin is not a conservative change, nor the substitution of the sequence Ser–Gly–Lys at positions 190 to 192 of trypsin for Lys–Lys–Asn in chymotrypsin. In the x-ray crystallography of chymotrypsin, the threonine residue is located in a sequence of buried residues (6), and the change from isoleucine to threonine could conceivably alter the disulfide reactivity. The tripeptide sequence is found in chymotrypsin as exposed residues (6) and these residues may vary widely and not be expected to influence the accessibility of the neighboring disulfide.

tected with the active site directed reagent, *p*-nitrophenyl-*p'*-guanidinobenzoate (18, 20). The sample was activated at the same rate and to almost the same extent as reported earlier for the dicarboxymethyl derivative (14). The similar behavior of the tetra- and dicarboxymethyl derivatives in the activation process showed that the modification of disulfide 122 to 189 apparently did not further alter the conformation of the zymogen. Thus, two disulfides of trypsinogen are nonessential in the activation of the zymogen to active molecules.

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