# Influence of the Complex Formation between Trypsin and Bovine Basic Trypsin Inhibitor on the Reactivity of Certain Disulfide Bonds*

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ABSTRACT: The chemical reactivity of disulfide bonds toward reducing agents in the absence of denaturing conditions in bovine trypsin, in bovine basic trypsin inhibitor, and in the complex formed by the two proteins has been studied. Treatment of native inhibitor with dithiothreitol or sodium borohydride gave selective reduction of one of the three disulfide bonds. This reactive disulfide which links the half-cystine residues in positions 14 and 38 is known to be at an extremely exposed position at the top of a pear-shaped molecule (Huber, R., Kukla, D., Rühlmann, A., Epp, O., and Formanek, H. (1970), *Naturwissenschaften 57*, 389). Limited reduction of native trypsin is shown to be specific for one single reactive disulfide bond, by (a) retention of high enzymatic activity, and (b) observation of two labeled peptides in peptide maps after S-[$\alpha$-$^{14}$C]carboxymethylation and tryptic digestion. None of the nine disulfide bonds of the complex could be reduced when highly purified complex preparations were treated with reducing agents in the absence of denaturing agents, indicating that both the 14–38 disulfide bond of the inhibitor and the reactive disulfide bond of the enzyme become buried in the interior of the complex and that mutual binding regions are located near these disulfides.

The interaction between bovine trypsin and bovine basic trypsin inhibitor (I)[1] to form a complex devoid of tryptic activity has recently attracted renewed interest. Trypsin contains 223 amino acid residues and possesses 6 disulfide bonds; basic trypsin inhibitor consists of 58 amino acid residues and has three disulfide linkages. Knowledge of the covalent structures of both the enzyme (Walsh and Neurath, 1964; Mikeš *et al.*, 1966a,b) and the inhibitor, Figure 1 (Kassell and Laskowski, 1965), made the complex formation a good model for molecular topology studies of protein–protein interaction. Phenomenologically, protein–protein interactions should be discussed in terms of extended "binding areas" or "binding regions" comprising many amino acid residues rather than in terms of single "binding sites." The hemoglobin structure serves as an excellent illustration for extended binding regions between its individual chains (Perutz, 1969). For the complex between trypsin and basic trypsin inhibitor X-ray diffraction data are not yet available.[2] However, useful information about the location of mutual binding regions might also be obtained from chemical studies. Chauvet and Acher (1967) showed by chemical methods that the lysine residue in position 15 of the inhibitor interacts with trypsin. In the trypsin molecule a binding area for basic trypsin inhibitor was located between amino acid residues[3] 146 and 198 (Dlouhá *et al.*, 1968). This enzyme fragment remained attached to intact inhibitor after exhaustive tryptic and chymotryptic digestion of the complex.

We have observed remarkable changes, associated with complex formation, in the reactivities of certain disulfide bonds of both trypsin and basic trypsin inhibitor toward reducing agents in the absence of denaturing agents. Reduction and alkylation studies on the enzyme, on the inhibitor, and on the complex formed by the two proteins are reported herein. The results indicate that certain disulfide bonds become buried during complex formation.

## Experimental Section

*Reagents.* Sodium borohydride was obtained from Metal Hydrides Inc., Beverly, Mass. Dithiothreitol, benzoyl-DL-arginine-*p*-nitroanilide (Bz-DL-Arg-pNAn), and tris(hydroxymethyl)aminomethane (Tris, 3-times crystallized for enzyme research) were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. *p*-Toluenesulfonyl-L-arginine methyl ester (TAME) was obtained from Calbiochem, Los Angeles, Calif., and was recrystallized from methanol or from acetone, mp 150°, $[\alpha]_D^{20}$ −14.5° (*c* 4, water). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was obtained from Sigma, St. Louis, Mo. Iodoacetamide, iodoacetic acid, and benzamidine hydrochloride

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[1] Bovine (pancreatic) basic polyvalent trypsin inhibitor, also known as Kunitz–Northrop pancreatic trypsin inhibitor or as kallikrein inhibitor; it is called basic trypsin inhibitor in this paper, abbreviated I. Other abbreviations used are: Bz-DL-Arg-pNAn, benzoyl-DL-arginine-*p*-nitroanilide; TAME, *p*-toluenesulfonyl-L-arginine methyl ester; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TPCK, L-(1-toluenesulfonylamido-2-phenyl)ethyl chloromethyl ketone; SE-Sephadex, sulfoethyl Sephadex; Ala-NCA, L-alanine-*N*-carboxyanhydride; CM-Cys, *S*-carboxymethylcysteine; for other abbreviations, see *Biochemistry 5*, 1445 (1966).

[2] During the preparation of this manuscript an X-ray structure study on the molecular conformation of bovine basic trypsin inhibitor has been published (Huber *et al.*, 1970).

[3] Trypsin residue numbers refer to the amino acid sequence of trypsinogen.

TABLE I: Amino Acid Composition of Bovine Trypsin, of Bovine Basic Trypsin Inhibitor, of Their Complex, and of Derivatives.[a]

| | Basic Trypsin Inhibitor | | | | Trypsin | | | | Complex | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | a | b | c | | d | e | f | | |
| Amino Acids | Theory | Purified, Native | 14,38-Bis(*S*-carboxamidomethyl) Derivative | Recovd from Treated Complex | Theory | Purified $\alpha$ Fraction | Purified $\beta$ Fraction | Recovd from Treated Complex | Theory | g Purified |
| Lys | 4 | 4.1 | 4.2 | 4.2 | 14 | 14.3 | 13.6 | 14.0 | 18 | 18.1 |
| His | | | | | 3 | 3.1 | 3.3 | 3.2 | 3 | 2.9 |
| Arg | 6 | 5.9 | 6.2 | 5.6 | 2 | 1.9 | 1.8 | 2.3 | 8 | 7.2 |
| CM-Cys[b] | | | 2.1 | | | | | | | |
| Asp | 5 | 5.2 | 4.9 | 5.2 | 22 | 21.7 | 21.6 | 24.0 | 27 | 27.8 |
| Thr | 3 | 2.9 | 3.0 | 2.9 | 10 | 9.3[c] | 10.0[c] | 9.9[c] | 13 | 12.4[c] |
| Ser | 1 | 1.0 | 1.1 | 1.3 | 34 | 33.1[c] | 31.0[c] | 33.0[c] | 35 | 33.0[c] |
| Glu | 3 | 3.3 | 3.2 | 3.2 | 14 | 14.0 | 14.4 | 14.4 | 17 | 17.6 |
| Pro | 4 | 3.9 | 4.0 | 3.8 | 8 | 8.4 | 8.5 | 8.3 | 12 | 12.5 |
| Gly | 6 | 6.0 | 6.0 | 6.2 | 25 | 24.7 | 25.2 | 26.0 | 31 | 31.6 |
| Ala | 6 | 6 | 6 | 6 | 14 | 14 | 14 | 14 | 20 | 20 |
| $^1/_2$-Cys | 6 | 6.0 | 3.9 | 5.6 | 12 | 11.9 | 10.3 | 11.1 | 18 | 17.1 |
| Val | 1 | 1.1 | 0.9 | 1.2 | 17 | 16.9 | 16.2 | 16.4 | 18 | 17.7 |
| Met | 1 | 1.0 | 1.1 | 1.0 | 2 | 1.8 | 1.8 | 1.8 | 3 | 3.0 |
| Ile | 2 | 1.8 | 1.7 | 1.9 | 15 | 15.0 | 13.0 | 14.0 | 17 | 17.0 |
| Leu | 2 | 2.1 | 2.2 | 2.2 | 14 | 14.8 | 13.6 | 14.5 | 16 | 16.1 |
| Tyr | 4 | 3.9 | 4.0 | 3.8 | 10 | 10.5 | 10.2 | 10.5 | 14 | 13.7 |
| Phe | 4 | 4.0 | 3.9 | 4.0 | 3 | 3.0 | 3.2 | 3.0 | 7 | 7.5 |

[a] Values in columns d, e, f, and g are means of at least four analyses. [b] CM-Cys, *S*-carboxymethylcysteine. [c] Extrapolated to zero time from 15- and 72-hr hydrolysates.

hydrate were obtained from Aldrich Chemical Co., Milwaukee, Wis. Ultrapure water was provided by a tank filter system from Hydro Service and Supplies, Inc., Durham, N. C.

*Basic Trypsin Inhibitor.* Part of the inhibitor used in this work was a gift from Farbenfabriken Bayer AG (Kallikrein inhibitor or Trasylol, lot No. 795996) or it was prepared from pancreatic inhibitor–trypsin compound (PIC, Worthington Biochemical Corp.) by precipitation of trypsin with 2.5% trichloroacetic acid (Kunitz and Northrop, 1936). Both inhibitor preparations were purified by carboxymethylcellulose column chromatography (Sach *et al.*, 1965), followed by desalting on Sephadex G-25. Purified preparations were homogeneous as judged by polyacrylamide gel electrophoresis (at pH 4.3), by analytical chromatography on CM-cellulose, and by amino acid analysis (Table I, column a). They possessed full inhibitory activity, as judged by total inhibition of trypsin in TAME and Bz-DL-Arg-pNAn assays at a 1:1 molar ratio. Basic trypsin inhibitor concentrations were determined spectrophotometrically at 280 nm using $E_{1\text{ cm}}^{0.1\%} = 0.8$, obtained from $\epsilon$ 5234 l. mole$^{-1}$ cm$^{-1}$ and the molecular weight of 6511.5 (calcd for $C_{284}H_{432}N_{84}O_{79}S_7$); lit. (Laskowski, 1961) $E_{1\text{ cm}}^{0.1\%}$ 0.8 at 280 nm.

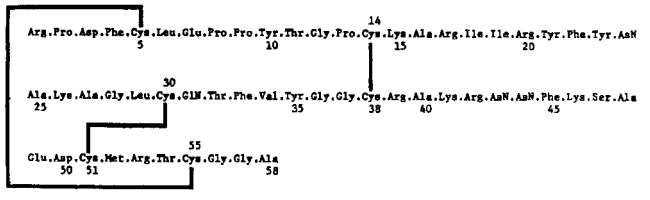


FIGURE 1: Structure of bovine basic trypsin inhibitor (Kassell and Laskowski, 1965).

*Trypsin* (twice crystallized, TRL, from Worthington Biochemical Corp., Freehold, N. J., possessing an activity of 160–250 units/mg, TAME assay) was fractionated by chromatography on sulfoethyl (SE) Sephadex C-50 (beaded) according to Schroeder and Shaw (1968). The procedure was scaled up. Trypsin (1.75 g) was dissolved in 100 ml of 0.1 M Tris-HCl buffer at pH 7.1 which was 0.02 M in $Ca^{2+}$ and $10^{-3}$ M in benzamidine. The solution was placed on a 5 × 94 cm column which had been equilibrated with the same buffer at 4°. Elution was carried out at a flow rate of 40 ml/hr. Fractions (20-ml) were collected. Good separation of $\alpha$ and $\beta$ peaks (Schroeder and Shaw, 1968) was observed (as monitored by the optical density at 280 nm). Pooled fractions were 10 times concentrated by passing them, after acidification to pH 3, through a SE-Sephadex column (7 × 8 cm) equilibrated with 0.05 M Tris-HCOOH buffer at pH 3.0. Bound trypsin was eluted from the column (flow rate 70 ml/hr) with 0.5 M Tris-HCl buffer (pH 7.6), containing $Ca^{2+}$ and benzamidine. Dialysis in the Diaflo ultrafilter using $10^{-3}$ N HCl and lyophilization gave purified $\alpha$-trypsin (350 mg, yield 20%) and $\beta$-trypsin (510 mg, yield 29%). Homogeneity of both fractions was ascertained by correct amino acid analyses (Table I) and by polyacrylamide gel electrophoresis at pH 4.3. Both gave single bands, the $\beta$ fraction migrated further to the cath-

ode. An equal mixture gave two well-separated bands corresponding to the two main bands of TRL trypsin. Enzymatic activities were: α-trypsin, 440–470 units/mg by TAME assay, 50% more potent by Bz-DL-Arg-pNAn assay than TRL trypsin (Worthington, possessing approximately 235 TAME units/mg); β-trypsin, 390–440 units/mg by TAME assay, 75% more potent than TRL trypsin by Bz-DL-Arg-pNAn assay. β-Trypsin which is native, single-chain trypsin (Schroeder and Shaw, 1968), was used in most of this work. The activity decreased somewhat during storage at 0° in lyophilized form, reaching a constant level at about 300–360 TAME units/mg after 2–3 weeks. This material gave a correct amino acid analysis (Table I, column e) and was still homogeneous in acrylamide gel electrophoresis. Trypsin concentrations were determined spectrophotometrically at 280 nm using $\epsilon = 35{,}533$ l. mole$^{-1}$ cm$^{-1}$ to give $E_{1\,cm}^{0.1\%}$ 1.53, based on the molecular weight of 23290.3 (calcd for $C_{1012}H_{1588}N_{282}O_{321}S_{14}$); lit. (Laskowski, 1961) $E_{1\,cm}^{0.1\%}$ 1.49 at 280 nm. The protein content of lyophilized samples was also determined by amino acid analysis results and dry weight determinations to be 89–92%.

*Trypsin activity* was determined by the rate of hydrolysis of *p*-toluenesulfonyl-L-arginine methyl ester (TAME) as measured by the increase in absorbancy at 247 nm according to Hummel (1959). Proteolytic activity was determined by the rate of cleavage of benzoyl-DL-arginine-*p*-nitranilide according to Erlanger *et al.* (1961).

*Trypsin inhibitor activity* was determined by the degree of inhibition of tryptic activity in TAME and Bz-DL-Arg-pNAn assays.

*Spectrophotometric measurements* were made with a Zeiss Model PMQ II or with a Beckman DU spectrophotometer.

*Amino acid analyses* (Spackman *et al.*, 1958) were performed on a Phoenix analyzer M-6800, after hydrolysis in constant-boiling HCl for 20 hr at 110° in glass tubes sealed under high vacuum.

*DTNB Analyses.* In order to determine the extent of reduction samples reduced by sodium borohydride were assayed directly for sulfhydryl content using 5,5′-dithiobis(2-nitrobenzoic acid) as described by Ellman (1959). From samples reduced by dithiothreitol the excess reagent was first removed by Diaflo dialysis. All solutions for DTNB analyses were prepared with 0.001 M EDTA, and were deoxygenated prior to use to prevent significant reoxidation of samples. Deoxygenation of the solvent was carried out by boiling for several minutes and then bubbling nitrogen through the cooled solvent.

*Chromatography.* All buffers were sterilized for 25 min at 120°. When necessary, the pH was subsequently readjusted. Sephadex G-25 and G-50 (obtained from Pharmacia, Fine Chemicals, Inc., Piscataway, N. J.) were sterilized at 120° for 25 minutes while suspended in water or 0.1 M acetic acid. Sephadex C-50 (beaded) and carboxymethylcellulose cannot be heat sterilized. They were passed through alkaline and acidic cycles prior to use.

*Dialysis* was done with a Diaflo ultrafilter (Amicon Corp., Lexington, Mass.) using membrane type UM-2, which is reported by the manufacturer (Amicon Technical Bulletin No. 201) to retain solutes of molecular weights above 1000. This membrane provides for a high flow rate of water and permeable solutes. A nitrogen pressure of 50 psi was maintained. Dialyses were carried out at 4° using 0.1 N acetic acid.

*Lyophilization.* Solutions for lyophilization were shell frozen at rigorously controlled mild temperatures, not lower than −30°. Lower freezing temperatures were found to have denaturing effects on peptides and proteins.

*Polyacrylamide gel disc electrophoresis* was performed on an apparatus obtained from Metaloglass, Inc., Boston, Mass., using 7.5% gels, 0.278 M β-alanine–acetate buffer (pH 4.3) for 20 min at 8 mA/tube according to Reisfeld *et al.* (1962) or Tris-HCl buffer (pH 8.1) according to Jovin *et al.* (1964).

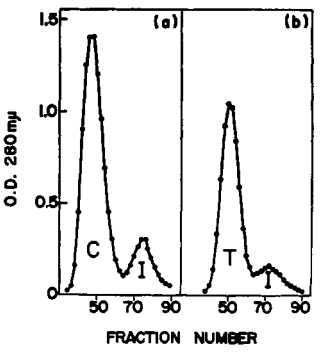


FIGURE 2: (a) Purification of the complex of bovine trypsin with bovine basic trypsin inhibitor on Sephadex G-50. Column size, 2.5 × 90 cm; eluent, 0.046 M Tris-HCl buffer (pH 8.1) containing 0.0115 M $Ca^{2+}$; flow rate, 53 ml/hr; volume per fraction, 3.5 ml; C, complex; I, excess inhibitor. (b) Typical pattern of separations of trypsin and basic trypsin inhibitor from complex that had been treated with reducing and, in some experiments, with alkylating agents. Sephadex G-50 column, 2.2 × 115 cm; eluent, 50% acetic acid; flow rate, 42 ml/hr; volume per fraction, 5 ml; T, trypsin; I, inhibitor.

*Preparation of Complex.* Trypsin (50 mg, 2.1 μmoles) and excess inhibitor (20 mg, 3.1 μmoles) were dissolved in 0.046 M Tris-HCl buffer at pH 8.1 (10 ml). Complex formation was completed within 10 min at room temperature as indicated by complete disappearance of tryptic activity (TAME assay). Purification and removal of excess inhibitor was carried out by chromatography at 4° on a Sephadex G-50 column (2.5 × 90 cm) in 0.046 M Tris-HCl buffer (pH 8.1), 0.0115 M in $Ca^{2+}$ (flow rate, 53 ml/hr). Desalting by passing through a Sephadex G-25 column (2.5 × 69 cm) with 0.1 M ammonium bicarbonate (pH 7.9) as an eluent and lyophilization gave a colorless powder, 50 mg, 80%. Homogeneity was tested by gel electrophoresis, by analytical column chromatography on Sephadex G-50 giving a single symmetrical peak, and by amino acid analysis (Table I, column g). *Anal.* S calcd 2.0, found 2.1. Complex concentrations were determined spectrophotometrically at 280 nm using $\epsilon = 35{,}006$ l. mole$^{-1}$ cm$^{-1}$ giving a conversion factor of about 0.85; molecular weight 29801.8.

*Dissociation of complex* was carried out according to Kassell *et al.* (1963). To a solution of complex (1.5 μmoles) in 0.046 M Tris-HCl buffer (50 ml), at pH 8.1 and at room temperature, was added glacial acetic acid (50 ml) to bring the pH to 2.0. After concentration to a small volume (10 ml, by limited lyophilization), the solution was passed through a column of Sephadex G-50 (2.2 × 115 cm) which had been equilibrated with 50% acetic acid. A typical separation of trypsin (first peak, T) and of inhibitor (second peak, I) is shown in Figure 2b. Lyophilization gave trypsin with 75–95% recovery (in several experiments) possessing esterolytic activity of 190–225 units/mg (75–87%, based on an activity of 260 units/mg of starting trypsin, TAME assay). Recovery of inhibitor possessing 93–99% inhibitory activity (based on untreated material) was quantitative. Amino acid analyses are shown in Table I, columns c and f. Polyacrylamide gel electrophoresis at pH 4.3 showed both components which were recovered from the complex to be indistinguishable from starting components.

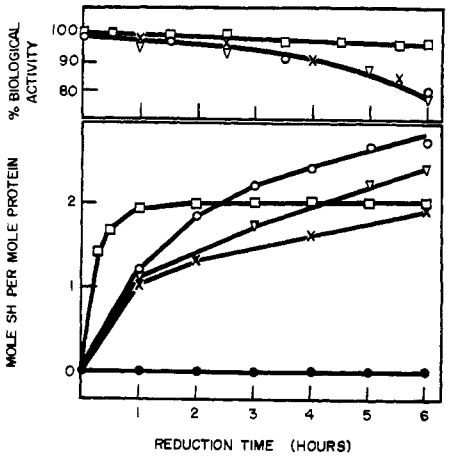


FIGURE 3: Chronological studies of reductions by sodium borohydride in the absence of denaturing agents. Lower part, results of sulfhydryl titrations with 5,5′-dithiobis(2-nitrobenzoic acid); upper part, enzymatic or inhibitory potency in per cent of starting potency, by TAME assay. (□) Bovine basic trypsin inhibitor, Tris-HCl buffer (pH 9); (×) purified β-trypsin, Tris-HCl buffer (pH 8); (▽) purified β-trypsin, Tris-HCl buffer (pH 9); (○) TRL trypsin (Worthington), Tris-HCl buffer (pH 9); (●) purified complex between trypsin and inhibitor, Tris-HCl buffer (pH 9).

*Reductions and Alkylations.* (A) BY DITHIOTHREITOL WITH pH-STAT CONTROL ACCORDING TO BEWLEY AND LI (1969). A Radiometer Model TTT 11a-PHM 28 automatic titrator equipped with an all-glass thermostated reaction vessel was used in combination with TTA 31 titration assembly and $SBR_2$ titrograph recorder. In a typical experiment the protein was dissolved in deoxygenated 0.1 M KCl to a concentration of 0.25%. The solution was kept under a nitrogen atmosphere and stirred with a magnetic stirrer. The pH was adjusted to 8.1 by adding 0.1 N KOH and maintained at that pH by the pH-Stat through addition of 0.1 N KOH. A 20-molar excess of dithiothreitol over the protein disulfide content was added and the reaction allowed to proceed at room temperature for 90 min. The pH was then raised to 8.4 and a tenfold excess of alkylating agent over dithiothreitol was added. The alkylation was allowed to proceed for 15 min. Excess reagents were then removed by desalting on Sephadex G-25, or, after acidification to pH 3 with glacial acetic acid, by dialysis in the Diaflo ultrafilter. After desalting, the products were isolated by lyophilization.

(B) BY SODIUM BOROHYDRIDE IN BUFFERED SOLUTION. The protein was dissolved in deoxygenated 0.1 M Tris-HCl buffer (pH 8.0 or pH 9.0) (containing 0.05 M $CaCl_2$ for trypsin reductions), to a concentration of 0.4%. The solution was stirred at 0° and kept under a nitrogen atmosphere. An equal volume of 0.1 M $NaBH_4$ in ice-cold deoxygenated 0.1 M Tris-HCl buffer (pH 9.0) was added. Samples (0.5 ml) were withdrawn periodically for sulfhydryl determinations. Alkylations were carried out as described above. Foaming during final acidification with 0.1 N HCl was controlled by saturating the nitrogen stream with propanol–octanol by bubbling it through a 3:1 mixture of the alcohols. Work-up was as above.

*Alkylation of Partially Reduced Trypsin with [α-$^{14}$C]Iodoacetic Acid and Peptide Mapping.* Partial reduction of trypsin (360 units/mg, TAME) by $NaBH_4$ was carried out as described above. When the sulfhydryl titration gave a value of about 1.8 moles/mole of trypsin a 50-fold molar excess of [α-$^{14}$C]-iodoacetic acid (sp act. 0.125 μCi/μmole) was added. Work-up was as described above. The bis(S-[α-$^{14}$C]carboxymethylated) sample (amino acid analysis: CM-Cys, 1.75 based on Ala, 14.0; enzymatic activity: 199 units/mg of TAME) was treated with performic acid, prepared according to Li (1957) at 4° for 1 hr. Digestion by TPCK-treated trypsin (Kostka and Carpenter, 1964) was done in 0.1 M $NH_4HCO_3$ at 37° for 5 hr (E:S = 1:50, w/w); 2 mg of the lyophilized product was subjected to two-dimensional paper chromatography and paper electrophoresis (Katz *et al.*, 1959); chromatographic solvent system, pyridine–acetic acid–water (100:4:900, v/v); electrophoresis buffer (pH 2.1), formic acid–acetic acid–water (25:87:888, v/v). Autoradiography was done with X-ray film which was exposed for 14 days and developed to show two main radioactive spots.[4]

*Improved Preparation of 14,38-Bis(S-carboxamidomethyl)-(Basic Trypsin Inhibitor) (Liu and Meienhofer, 1968).* Basic trypsin inhibitor (30 mg) was dissolved in 0.1 M KCl (12 ml). The pH was adjusted to 8.1 by adding 0.1 N KOH and it was then maintained at pH 8.1 by the pH-Stat. Dithiothreitol (50 mg) was added and the reaction was allowed to proceed for 90 min at room temperature under nitrogen atmosphere. The pH was then adjusted to 8.4 and α-iodoacetamide (600 mg) was added. After 10 min, glacial acetic acid was added and the pH was adjusted to 3.0. Dialysis in the Diaflo ultrafilter using 0.1 N acetic acid (24 hr at 4°) and subsequent lyophilization gave a colorless powder, 30.55 mg, 100%, homogeneous in polyacrylamide gel electrophoresis at pH 4.3. It gave a correct amino acid analysis (Table I, column b) and its biological activity was identical with that of native trypsin inhibitor in TAME and Bz-DL-Arg-pNAn assays.

## Results

*Preparation of Purified Complex.* Chromatographically and electrophoretically pure complex of trypsin with basic trypsin inhibitor was obtained by treating the enzyme at pH 8.1 for 30–60 min with a 50% excess of the inhibitor followed by gel filtration on Sephadex G-50 with 0.1 M ammonium bicarbonate, pH 7.9. The excess inhibitor was completely separated from the complex (Figure 2a). The first peak appearing with the hold-up volume contained the complex in yields ranging from 80 to 90% (based on trypsin) in several experiments. The homogeneity was corrobroated by analytical chromatography on Sephadex G-50 and by gel electrophoresis at pH 8.1. Amino acid analysis (Table I, column g) and elemental sulfur analysis gave the expected values. Tryptic activity was zero by TAME assay.

*Partial Reduction of Basic Trypsin Inhibitor.* Treatment of the inhibitor with sodium borohydride or dithiothreitol at pH 8.2–9.0 in the absence of denaturing agents gave a completely selective reduction (Figure 3) of the disulfide bond connecting half-cystine residues 14 and 38 (compare Figure 1) leaving the other two disulfide linkages fully intact, as described before (Kress and Laskowski, 1967; Liu and Meienhofer, 1968; Kress *et al.*, 1968). The selectively reduced inhibitor and its bis(*S*-carboxamidomethyl) derivative retained full inhibitor activity. In contrast, the bis(*S*-carboxymethyl)

[4] When large amounts of tryptic digest were applied to the origin of the peptide map, three additional very weakly radioactive spots were detected indicating very slight (<3%) reduction of other disulfide bonds. The two main spots were excised, eluted, and subjected to amino acid analysis. From these data four disulfide bonds (13–143, 31–47, 122–189, and 154–168) could be dismissed from consideration, leaving disulfide bonds 115–216 and 179–203. Due to heavy overlapping a conclusive identification was not possible.

derivative was totally inactive indicating the importance of the nature of S-alkylating agents for obtaining biologically active derivatives. The previously described procedure (Liu and Meienhofer, 1968) for the preparation of 14,38-bis(*S*-carboxamidomethyl)-(basic trypsin inhibitor) has been improved to give now essentially quantitative yields by replacing Sephadex G-25 gel filtration for desalting and removal of excess reagents and by-products with dialysis using the Diaflo UM-2 membrane.

*Partial Reduction of Trypsin.* For this study it was desirable to use homogeneous and well-characterized trypsin. The chromatographic procedure of Schroeder and Shaw (1968), developed recently for the separation of native single-chain trypsin ($\beta$-trypsin) from an enzymatically active contaminant ($\alpha$-trypsin) in which the 131-lysyl peptide bond is cleaved, was adapted to a larger scale, allowing the application of 1.5–2 g of commercial trypsin to a single SE-Sephadex column. Highly active $\beta$-trypsin (390–440 units/mg, TAME assay) was obtained in 27–31% recoveries based on the starting TRL trypsin (Worthington, 235 units/mg, TAME assay). This purified native trypsin migrated as a single band in polyacrylamide gel electrophoresis at pH 4.3 or pH 8.1 and was homogeneous by amino acid analysis (Table I, column e).

Treatment of $\beta$-trypsin with reducing agents in the absence of denaturing agents gave partial disulfide reduction but the reaction was slower and not as selectively specific as that observed with basic trypsin inhibitor. Reduction by sodium borohydride proceeded best at pH 9 and 0°. Chronological studies of partial reductions are shown in Figure 3. After 3–4 hr, averages of 1.6–2 moles of sulfhydryl/mole of enzyme were determined by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959), Figure 3 (lower part). The enzymatic activity (TAME assay) remained high, approximately 80–90%, Figure 3 (upper part). After 5 hr, 2.2–2.5 moles of sulfhydryl was titrated. After 22 hr, sulfhydryl contents rose to 3.5–4.5 moles with a concomitant drop of enzymatic activity to between 30 and 50% by TAME assay. Reductions below pH 8 were very slow; reductions above pH 9.5 were random and accompanied by rapid decrease of enzymatic activity. Treatment of $\beta$-trypsin with dithiothreitol at pH 8.1 in the absence of denaturing agents similarly resulted in limited reduction. In a typical experiment 1.9 moles of sulfhydryl was obtained within 1.5 hr. Ellman titration was performed after complete removal of dithiothreitol by careful dialysis (acidic solution, low temperature, $N_2$ atmosphere). Results of such titrations were found by Bewley and Li (1969) to correspond to amounts of *S*-alkylcysteine obtained by thiol alkylation immediately after reduction.

To further corroborate that the nature of the partial disulfide reduction was specific and not random, the homogeneity of bis(carboxymethyl)ated material was checked by peptide mapping after proteolytic digestion using TPCK-trypsin. To facilitate chromatographic detection, [$\alpha$-$^{14}$C]iodoacetic acid was employed for alkylation. Two main radioactive spots[4] were obtained on the autoradiograph of the peptide map, as expected from a specific reduction of one single disulfide bridge.

*Treatment of Complex with Reducing Agents.* Purified complex was treated with sodium borohydride at pH 9 and 0° for 6 hr under conditions which closely resembled those used in partial reductions of trypsin and of basic trypsin inhibitor. Aliquots were titrated at regular time intervals with 5,5′-dithiobis(2-nitrobenzoic acid). The sulfhydryl content remained zero during the entire period, see Figure 3. Similarly, treatment of complex with dithiothreitol at pH 8.1 and 20° for 1.5 hr showed a negative Ellman reaction after careful dialysis.

Initially variable amounts of sulfhydryl (0.5–1.5 moles per mole of complex) were obtained during reduction of complex preparations prepared in the usual manner of mixing supposedly stoichiometric amounts (by weight) of the components. It was soon realized that these preparations contained invariably slight excesses of one or the other component and that complex preparations containing precisely equimolar amounts of enzyme and inhibitor were required for these studies. Highly purified homogeneous 1:1 complex was readily obtained by adding a large excess of inhibitor to the enzyme followed by Sephadex G-50 chromatography at pH 7.9 as described above.

*Treatment of Complex with Reducing and Alkylating Agents.* Purified complex was treated with reducing agents as described above, followed in the usual manner by addition of excess of iodoacetic acid. Amino acid analyses of total hydrolysates of complex isolated after such treatment showed no *S*-carboxymethylcysteine.

*Dissociation of Treated Complex.* To further confirm the complete resistance of native complex to disulfide reduction, preparations treated with reducing agents alone as well as preparations treated with both reducing and alkylating agents were subjected to acid dissociation followed by isolation and examination of the two components.

Dissociation after acidification of solutions to pH 2 was followed with time by the reappearance of tryptic activity. TAME assays at regular time intervals showed recovery of 75–87% of enzymatic activity (based on the potency of starting trypsin) in several experiments. Separation of the two components was effected by column chromatography on Sephadex G-50 with 50% acetic acid as an eluent. Complete separation of the components was always obtained as shown in the typical pattern in Figure 2b. Trypsin, retaining 75–87% of the starting esterolytic activity by TAME assay was recovered with 75-95% yields; inhibitor possessing 93–99% of inhibitory potency was recovered quantitatively. Both the recovered inhibitor and the recovered enzyme were in all experiments indistinguishable from starting native products in their amino acid analyses (Table I, columns c and f) and in their behavior in polyacrylamide gel electrophoresis at pH 4.3 or 8.1.

*Summary of Results.* (1) Reduction of basic trypsin inhibitor at pH 8–9 in the absence of denaturing agents gave a rapid specific cleavage of one of the three disulfide bonds while full biological activity was retained. The reduced disulfide bridge was that connecting half-cystine residues in positions 14 and 38. (2) Reduction of trypsin under identical conditions gave a slower specific reduction of one of the six disulfide bonds with retention of high enzymatic activity. (3) None of the nine disulfide bonds of the complex was reduced under identical conditions. Unchanged native trypsin and unchanged native basic trypsin inhibitor were recovered after dissociation of complex which was treated with reducing and alkylating agents.

## Discussion

The observed complete protection from chemical reduction of one reactive disulfide in each component of the complex between bovine trypsin and bovine basic trypsin inhibitor is interpreted to indicate that the interaction between the two proteins occurs in regions surrounding these disulfide bonds. It is necessary for this interpretation to discuss first the results of studies on disulfide reactivity in each of the two components alone.

*Specific Reduction of the 14–38 Disulfide Bond in Bovine Basic Trypsin Inhibitor.* When native inhibitor was treated with dithiothreitol (Liu and Meienhofer, 1968), sodium borohydride (Kress and Laskowski, 1967), or mercaptoethanol (Meloun *et al.*, 1968; Kress *et al.*, 1968), the disulfide bond linking half-cystine residues 14 and 38 was selectively reduced while the other two disulfide bonds, connecting half-cystine residues 5 with 55 and 30 with 51 (Figure 1), remained entirely intact (*cf.* Figure 3). The partially reduced inhibitor retained its biological activity. Alkylation with iodoacetamide gave fully active 14,38-bis(*S*-carboxamidomethyl)-(basic trypsin inhibitor)[5] (Kress and Laskowski, 1967; Liu and Meienhofer, 1968; Kress *et al.*, 1968), while carboxymethylation resulted in complete loss of inhibitory potency. The easy access for several reducing agents to the 14–38 disulfide bond indicated that it might be at an exposed position near the surface of the inhibitor molecule. The recent X-ray structure analysis (Huber *et al.*, 1970) revealed indeed a striking exposure of this disulfide linkage. It is located at the extreme top of the narrow end of a pear-shaped molecule while the other two disulfides reside in the interior of the thick half. In this comparatively small polypeptide consisting of only 58 amino acid residues, 2 of the disulfides are completely shielded from the chemical attack of such small-sized reagents as mercaptoethanol or borohydride merely by residing in the interior of the molecule. This is a remarkable example of the very strong influence which the conformation of a protein molecule can exert on the chemical reactivity of its functional groups.

*Specific Reduction of One Single Disulfide Bond in Bovine Trypsin.* Specific disulfide bond reduction in trypsin was first reported by Light and Sinha (1966, 1967). Light and coworkers (1969), in careful and elegant studies, have recently identified the single reducible disulfide linkage in native bovine trypsinogen as that connecting the half-cystine residues in positions 179 and 203. 179,203-Bis(*S*-carboxymethyl)trypsinogen was also prepared and converted into bis(*S*-carboxymethyl)trypsin possessing high enzymatic activity. The 179–203 disulfide bond is therefore not necessary for maintaining the essential conformation of the trypsin molecule required for enzymatic activity. However, additional reduction of other disulfide bonds resulted in loss of activity.

Our chronological studies on the partial reduction of native trypsin by sodium borohydride (Figure 3) clearly indicated that the reduction proceeded only initially with some specificity and that with time a slow but steady increase beyond the value of 2 moles of SH/mole of enzyme was observed even for the most highly purified preparations. Nevertheless, highly active preparations of partially reduced trypsin were obtained by terminating reductions whenever the sulfhydryl content reached approximately 2 moles/mole of enzyme. Reductions with dithiothreitol at pH 8.1 similarly resulted in the cleavage of approximately one disulfide within 1.5 hr. The experimental evidence in favor of a specific rather than a random reduction was threefold. (a) Partially reduced trypsin still possessed high enzyme activities (about 80–90% by TAME assay, see Figure 3). Only when more than one disulfide became reduced did enzyme activities begin to decrease rapidly with time. (b) The rate of reduction decreased considerably after one disulfide was reduced (Figure 3). (c) Autoradiography of peptide maps obtained from a tryptic digest of *S*-[$\alpha$-$^{14}$C]carboxymethylated, partially reduced enzyme showed two radioactive spots. Two radioactively labeled peptides would be expected from specific reduction of one individual disulfide bond, provided complete tryptic cleavage occurred between its constituent half-cystine residues as well as at all other cleavable points, while random reduction should manifest itself in many approximately equally radioactive spots. In conclusion, the observation of a different chemical reactivity of one individual disulfide in bovine trypsin strongly suggests that this bond[6] resides at a site that is easily accessible to the surrounding solvent and that the other five disulfide bonds are located in the interior of the molecule analogous to the known situation in bovine basic trypsin inhibitor (Huber *et al.*, 1970).

*Resistance to Reduction of All Disulfide Bonds in the Complex.* None of the nine disulfide bonds of the complex between bovine trypsin and bovine basic trypsin inhibitor could be reduced when highly purified complex preparations were treated with various reducing agents in the absence of denaturing conditions. The experimental evidence was threefold. (a) Sulfhydryl titrations remained zero over prolonged periods of time. (b) No *S*-alkylcysteine was detectable in amino acid analyses after successive treatment of native complex preparations with reducing and alkylating agents. (c) Both the enzyme and the inhibitor recovered from chemically treated complex preparations were chemically (amino acid analysis), biologically (enzymatic and inhibitory potency), and physically (electrophoretic mobility) unchanged and indistinguishable from starting native products.

Our observations offer support for the previously proposed interaction (Chauvet and Acher, 1967; Imhoff and Keil-Dlouhá, 1971) of the 15-lysine residue of the inhibitor with the 177-aspartic acid residue of the enzyme which, in addition to the 183-serine residue, has been implicated as active-site participant (Walsh *et al.*, 1964; Mikeš *et al.*, 1966a,b; Kasserra and Laidler, 1969; Tomášek *et al.*, 1970). Such an interaction, enhanced by hydrophobic forces in the regions surrounding these residues (Auhagen, 1967; Dlouhá *et al.*, 1968; Imhoff and Keil-Dlouhá, 1971), would be expected to bury the 14–38 disulfide bridge of the inhibitor and the 179–203 disulfide bridge of the enzyme in the interior of the complex. Chemical studies can thus provide useful interim information on the topology of protein–protein interaction until X-ray structure analysis will provide details of spatial correlations.

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[5] For the preparation of this compound an improved procedure giving essentially quantitative yields is described in the Experimental Section of this paper.

[6] It is likely that the single reactive disulfide bond reducible in native bovine trypsin is the same 179–203 bond[3] which Light *et al.* (1969) identified as the specifically reducible disulfide linkage in native bovine trypsinogen, since enzymatically highly active products were obtained in both cases. It seems to be unlikely that the specific reduction of yet a different disulfide bond (and exclusive of any reduction of the 179–203 bridge) should give us an enzymatically highly active product.

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