The Basic Trypsin Inhibitor of Bovine Pancreas

VII. REDUCTION WITH BOROHYDRIDE OF DISULFIDE BOND LINKING HALF-CYSTINE RESIDUES 14 AND 38*

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SUMMARY

Partial reduction of the basic pancreatic trypsin inhibitor with sodium borohydride results in the selective cleavage of the disulfide bond linking half-cystine residues 14 and 38.

The partially reduced inhibitor is fully active, whereas its carboxymethylated derivative is completely inactive and is susceptible to tryptic digestion.

The reduced inhibitor slowly reoxidizes when incubated alone at pH 8. In contrast, when the reduced inhibitor is first allowed to form a complex with trypsin, no reoxidation occurs at pH 8.0 for at least 25 min.

Although many of the trypsin inhibitors of animal origin with molecular weights of about 6500 differ in their properties, chemical composition, and reactivity with trypsin and other enzymes (2), they have as a common feature the presence of three disulfide bonds (3, 4). The linear sequence of amino acids in the basic pancreatic trypsin inhibitor (Kunitz and Northrop's inhibitor (5)) and the location of its disulfide bonds have been described by Kassell et al. (6) and by Kassell and Laskowski (7), and independently by Anderer and Hörnle (8). The presence of three disulfide bridges in a molecule with a molecular weight of 6500 and the unusual resistance of the basic pancreatic inhibitor to enzymatic digestion (7, 9) suggest a compact structure for the molecule.

The effects of chemical modifications on activity of the basic pancreatic inhibitor have been studied in several laboratories. Modifications of lysyl residues were the most common. Succinylation resulted in a totally inactive inhibitor (6). Avineri, Blauer, and Rigby (10) found that acetylation of the inhibitor produced a 75% loss of activity. Guanidination or amidination did not affect the activity (11), but Anderer found that gradual deamination of lysyl residues correlated with the loss of activity (12).

Studies in our laboratory have dealt with the effects of disulfide bond cleavage upon inhibitory activity. Since it is known that complete reduction of the inhibitor results in a complete loss of activity and that reoxidation restores up to 90% of the original activity (8), it was decided to attempt a partial reduction of the inhibitor.

The present work was stimulated by the finding of Light and Sinha (13) that borohydride selectively reduces two $S-S$ bonds in trypsin without causing any loss in enzymatic activity. In this paper we present evidence that, with the basic pancreatic inhibitor, borohydride selectively reduces a single disulfide bond without the loss of inhibitory activity. The identification of the reduced bond as linking half-cystine residues 14 and 38 is also presented.

EXPERIMENTAL PROCEDURE

Materials—Pancreatic trypsin inhibitor was prepared according to the method of Kassell et al. (14). Kallikrein inactivator (Trasylol) was a gift from Dr. G. Haberland of Farbenfabriken Bayer AG, Lot 55633C. Trypsin used for assay purposes was obtained from Worthington (two times crystallized, 50% MgSO4). Salt-free trypsin used for enzymatic digests was purified by the method of Liener (15). Trypsin and inhibitor activities were determined by the method of Schwert and Takenaka (16), as modified by Kassell et al. (14). Benzoyl-L-arginine ethyl ester hydrochloride was obtained from Mann. DTNB (Aldrich) was prepared fresh as a 0.01 M solution in 0.05 M Tris-HCl, pH 8.0. The sodium borohydride used was either from K and K Laboratories (Brooklyn, New York) or from Metal Hydrides (Beverly, Massachusetts; 98% + %). No differences were noted in the reactivity of the borohydride from these two sources. IA was obtained from Aldrich and recrystallized from ether-hexane just prior to use as described by Noltmann, Mahowald, and Kuby (17). Amberlite IRC-50 (XE64) was cycled according to Hirs, Moore, and Stein (18) and equilibrated with 5% acetic acid.

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1 The abbreviations used are: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); IA, iodoacetic acid; reduced CM inhibitor, inhibitor in which one disulfide bond has been reduced and the resultant sulfhydryl groups carboxymethylated with IA.
CM-cellulose (Selectacel) was purchased from Schleicher and Schuell. Dialysis tubing (Visking) was acetylated with 5% acetic anhydride in pyridine as described by Craig (19). All other chemicals were reagent grade, and glass-distilled water was used throughout. Buffers were adjusted to the desired pH at the temperature at which they were to be used.

Reduction with Borohydride—The procedures employed were essentially those described by Light and Sinha (13). For the batch preparation of reduced inhibitor used in the alkylation experiments described below, 289 mg of inhibitor were dissolved in 10.2 ml of cold water containing 0.02% EDTA. The solution was adjusted to pH 8.5 with 0.4 ml of 0.2 M CaCl₂ and maintained at 2°C with a circulating water bath. A stream of nitrogen gas was passed over the reaction vessel. After a 5-min equilibrium period, 10.6 ml of a freshly prepared aqueous solution of 0.2 M sodium borohydride were added slowly and with gentle stirring. The reaction was allowed to proceed at the pH of aqueous borohydride (approximately 9.8). At appropriate intervals, 0.1 ml-aliquots were withdrawn and titrated to pH 2 by the addition of 0.5 ml HCl, with care being taken to avoid excessive foaming. The aliquots were kept at 2°C for 15 min and analyzed for free sulfhydryl group content according to the method of Ellman (20). At 60 min the reaction was ended by the slow addition of 4 ml of 0.5 M HCl. The solution was kept at 2°C for 45 min and a final aliquot was taken for assay purposes and sulfhydryl determination.

Carboxymethylation of Reduced Inhibitor—The temperature of the reduced inhibitor solution was raised to 25°C and 1 ml of a solution of IA (150 mg per ml) in 1.0 M NaOH was added. The pH was maintained at 8.2 by the addition of 2 N NaOH, and a nitrogen barrier was present throughout the reaction. After 30 min the pH was lowered to 3.3 by the addition of 0.5 ml acetic acid, and the solution was immediately placed on a column of IRC-50, 2.2 × 11 cm. Excess reagents were removed by elution with 5% acetic acid as described by Neumann, Moore, and Stein (21). (Approximately 40% of the reduced CM inhibitor also emerged with this peak.) The remaining of the reduced CM inhibitor was eluted with 50% acetic acid, pooled, transferred to an acetylated dialysis bag, and dialyzed 12 hours against 6 liters of water. The solution was lyophilized and the resultant white powder was stored in the freezer.

Digestion of Reduced CM Inhibitor with Trypsin—Reduced CM inhibitor (15 mg) was dissolved in 2.5 ml of water containing 0.01 M CaCl₂ and the pH was adjusted to 8.0 with 1.0 N NaOH. Of a trypsin solution (2.8 mg per ml), 250 μl were added. After 24 hours digestion, another 125 μl of the trypsin solution were added. After 5 hours the reaction was stopped by titration to pH 1.7 with 2 N HCl. The solution was placed on a column of Sephadex G-50, 1.8 × 34 cm, and eluted with 0.02 M HCl. The eluted peaks were pooled, taken to dryness in a rotary evaporator, and hydrolyzed in 6 N HCl at 110°C for 20 hours as described by Moore and Stein (22). Amino acid analyses were performed on a Beckman/Spinco model 120 B analyzer.

RESULTS

Reduction of Inhibitor—The rate of reduction of the inhibitor in the presence of 0.1 M sodium borohydride is shown in Fig. 1. It can be seen that, under the conditions described, two sulfhydryl groups are generated quite rapidly, and the reaction stabilizes at this level of reduction. No additional sulfhydryl appeared after 24-hour reduction with 0.1 M borohydride. When the borohydride concentration was increased to 0.2 M, the sulfhydryl concentration increased to 2.3 moles after 96 hours of reduction. The kinetics of the reaction suggest that a single exposed disulfide bond is selectively cleaved, and that more drastic conditions would be required for further cleavage. No significant differences were noted between the rate of reduction of the pancreatic inhibitor and that of the kallikrein inhibitor (not shown). Fig. 1 also shows that the partially reduced inhibitor retains 90 to 100% of its original inhibitory activity (the specific activity values are averages of several different experiments). When stored at pH 2.0 and 4°C, the reduced inhibitor showed no reoxidation over a period of 5 months.

Location of Disulfide Bond Cleaved—Since the results shown in Fig. 1 suggested that only one of the three disulfide bonds had been readily reduced, it became of interest to determine the location of the bond cleaved. Fig. 2 shows the sequence of the intact inhibitor and the cleavages which occur during tryptic digestion of the completely reduced and carboxymethylated inhibitor (1). It can be seen that if only one disulfide bond has been reduced, and the resultant sulfhydryl groups suitably protected, different "core peptides" and sets of smaller peptides will result from tryptic digestion, depending upon which of the three disulfide bonds has been cleaved. Since intact inhibitor, core peptide, and the small peptides differ in molecular weight, they should be separable on Sephadex. Accordingly, partially reduced inhibitor was allowed to react with IA. The reduced CM inhibitor, which possessed no inhibitory activity, was then digested with trypsin and the reaction products were separated on Sephadex G-50. The results of the elution are shown in Fig. 3. The compositions of the peaks eluted from the Sephadex G-50 column are discussed in terms of the amino acid analyses shown in Table I.

The control sample of reduced CM inhibitor possessed 2.23 residues of CM-cysteine per mole of inhibitor. This value is in
close agreement with the value of 2.07 moles of sulfhydryl per mole of inhibitor determined with DTNB for this preparation. All of the other amino acids are present in the expected amounts, an indication that no side reactions have occurred with either borohydride or IA.

Peak I in Fig. 3 emerged with the void volume and corresponded in amino acid composition (not shown) to trypsin, as expected.

Peak II corresponds to the composition expected for undigested reduced CM inhibitor (Table I). The absence of 1 arginine and 1 glutamine residue and the low values for alanine, valine, methionine, and tyrosine might indicate a slight amount of tryptic cleavage of susceptible bonds. Judging from the magnitude of Peak II, approximately 35% of the partially reduced carboxymethylated inhibitor has not been digested by trypsin under the conditions employed. By contrast, the completely reduced and carboxymethylated inhibitor is totally digested by trypsin under the same conditions (1). On the other hand, intact inhibitor is completely resistant to enzymatic attack (9).

The location and composition of Peak III correspond to those of the two peptides of 20 residues each, which would be expected if the disulfide bond linking residues 14 and 38 had been cleaved (Fig. 2C). This is the only cleavage for which 2 residues of

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**Fig. 2.** Peptides expected from tryptic digestion of reduced CM inhibitor. The sequence marked A is that of the intact inhibitor. The peptides shown in B, C, and D are those expected if one of the three disulfides in the inhibitor has been reduced and subsequently carboxymethylated.

**Fig. 3.** Gel filtration of tryptic peptides of reduced CM inhibitor on Sephadex G-50. Reduced CM inhibitor (15 mg) was digested with trypsin and the reaction products were placed on a column of Sephadex G-50, 1.8 × 34 cm, and eluted with 0.02 M HCl at room temperature. The flow rate was 55 ml per hour, and 4.0-ml fractions were collected. The solid bars indicate those tubes in each peak which were pooled for amino acid analysis (see Table I).
CM-cysteine would be obtained in the core peptides. The possibility of splitting the disulfide bond between residues 5 and 55 is eliminated because of the presence of 3 residues of threonine; cleavage at the disulfide bond between residues 30 and 51 is excluded by the presence of serine (see Fig. 2, B and D).

The fact that borohydride cleaved the disulfide bond between residues 14 and 38 is strengthened by the composition of the small peptides which were obtained (Peak IV, Fig. 3 and Table I). The absence of threonine and serine from this peak excludes cleavage of the disulfide bonds between residues 5 and 55 and 30 and 51, respectively. The presence of a small amount of CM-cysteine and some glycine indicates that slight cleavage of the bond between residues 5 and 55 probably occurred. This fits well with the DTNB and carboxymethylation values, which showed that cleavage of a second disulfide bond in the inhibitor had occurred to a slight extent. The high values for the phenylalanine and tyrosine in Peak IV are probably due to incomplete acid hydrolysis of the peptide Ile-Ile-Arg (1).

As a result of the above analyses it was concluded that reduction of the basic inhibitor with 0.1 M borohydride had resulted in the selective cleavage of the disulfide bond connecting cysteine residues 14 and 38.

Reoxidation of Partially Reduced Inhibitor—It was shown in Fig. 1 that the inhibitor retained full inhibitory activity even after cleavage of the disulfide bond between residues 14 and 38. Since the inhibitor molecule is presumably quite compact, the activity noted could have been due to a rapid reoxidation of the sulphydryl groups during the assay to regenerate intact inhibitor. (A 5-min prior incubation of inhibitor with trypsin at pH 8.0 is required for full inhibitor activity.) The reoxidation of the partially reduced inhibitor was therefore studied. Fig. 4 shows that when reduced inhibitor is incubated alone at 25° and pH 8 only 22% reoxidation occurs within 5 min, and the rate of reoxidation gradually decreases, so that after 25 min of incubation 38% of the sulphydryls has been reoxidized. Since the inhibitor with one S-S bond reduced retains at least 90% of its original activity, the observed values for activity cannot be due to rapid reoxidation. This is substantiated by the results of incubation of reduced inhibitor with trypsin, shown in the upper line of Fig. 4. It can be seen that when reduced inhibitor forms a complex with trypsin the sulphydryl groups are stabilized and there is no tendency for them to reoxidize for at least 25 min. Therefore, the activity of the partially reduced inhibitor cannot be ascribed to reoxidation, and it was concluded that the inhibitor retains essentially all its activity even after cleavage of the disulfide bond between residues 14 and 38.

**Table I**

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<th>Amino acid</th>
<th>Literature value</th>
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* The values are uncorrected for decomposition or incomplete hydrolysis.

Kassell et al. (13), 16-hour hydrolysis.

* Control sample of partially reduced carboxymethylated pancreatic trypsin inhibitor.

**Fig. 4.** Rate of reoxidation of reduced inhibitor. Plotted is the percent of the original activity remaining at the indicated times. The reduced inhibitor (1.0 x 10⁻⁶ M final concentration) was incubated at 25° in Tris-HCl, pH 8.0, and assayed for sulphydryl content at the times indicated. O---O, reduced inhibitor (0.1 mg) was incubated at 25° in Tris-HCl, pH 8.0, with 0.88 mg of trypsin. At the indicated times, aliquots were assayed for sulphydryl content.

DISCUSSION

The complete reduction and subsequent reoxidation of bovine lung kallikrein inactivator, which is identical with the basic pancreatic trypsin inhibitor, have been studied by Anderer and Hörnle (8). They reported that all inhibitory activity was lost upon complete reduction with mercaptoethanol in urea, but could be almost completely regained by slow reoxidation. Since the objective of studies in our laboratory has been to find conditions under which the inhibitor would be only partially reduced, the milder conditions of reduction with borohydride employed by Light and Sinha (13) proved more suitable. The chief advantages of borohydride reduction are that excess borohydride can be decomposed by lowering the pH of the solution to 3, and that the extent of reduction can then be easily followed by the use of DTNB (20). Moreover, the absence of thiol reagents ordinarily used for complete reduction avoids the complication of possible disulfide interchange reactions which might take place under conditions of limited reduction. Lowering the borohydride concentration and the reaction temperature also eliminates the
possibility of reductive cleavage of peptide bonds mentioned by Crestfield et al. (23). The methods described in this paper should prove applicable to comparative studies of the effects of disulfide bond cleavage in other trypsin inhibitors.

The data presented in this paper indicate that the basic trypsin inhibitor contains one exposed disulfide bond which is readily susceptible to reduction. Cleavage of this bond apparently does not result in a significant change in the conformation of the inhibitor molecule, since it retains essentially all of its activity. Also, the rate with which it reacts with trypsin to form a complex is not slowed down significantly (i.e. a 5-min prior incubation of the inhibitor with trypsin is sufficient for full inhibitor activity). In contrast, it was shown that, when carboxymethyl groups are substituted on the exposed sulfhydryls of the partially reduced inhibitor, all inhibitory activity is lost. Moreover, the reduced CM inhibitor is susceptible to digestion. Apparently carboxymethylation of the partially reduced inhibitor not only prevents complex formation, but it also exposes susceptible bonds so that the inhibitor can be digested by trypsin. This could be due to an unfolding of the inhibitor molecule.

A marked difference was also noted in the tendency of reduced inhibitor to reoxidize when incubated alone, as compared to the slow rate of reoxidation when reduced inhibitor had formed a complex with trypsin. Apparently, binding of the reduced inhibitor to trypsin keeps the sulfhydryls spatially separated or buried within the complex and thereby partly stabilizes the inhibitor in the reduced state.

Ozawa and Laskowski (24) have shown that complex formation between trypsin and soybean inhibitor or chicken ovomucoid involves proteolysis of a specific bond in the inhibitors. Since the reaction is enzymatic, only active trypsin can be expected to form a complex. In our studies an active inhibitor was also necessary for complex formation. As long as the partial reduction did not inactivate the inhibitor, it remained resistant to tryptic digestion. However, carboxymethylation of the two sulfhydryls sufficed not only to inactivate the inhibitor, but also to render it susceptible to tryptic digestion. Whether this is a general phenomenon remains to be seen.

Studies are in progress to determine the effects of further reduction of the inhibitor. Preliminary results indicate that cleavage of more than one disulfide bond results in a loss of inhibitory activity. However, conditions suitable for the determination of which bond or bonds are being cleaved have not as yet been found.

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REFERENCES
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