The Basic Trypsin Inhibitor of Bovine Pancreas

VIII. CHANGES IN ACTIVITY FOLLOWING SUBSTITUTION OF REDUCED HALF-CYSTINE RESIDUES 14 AND 38 WITH SULFHYDRYL REAGENTS*

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SUMMARY

Basic pancreatic trypsin inhibitor with disulfide bond 14-38 reduced by borohydride was reacted with sulfhydryl group reagents. The reduced inhibitor and the dithiobis (2-nitrobenzoic acid) derivatives retained inhibitory activity toward both trypsin and \( \alpha \)-chymotrypsin. The carboxamidomethyl and aminoethyl derivatives were active toward trypsin but inactive with \( \alpha \)-chymotrypsin. The \( p \)-mercuribenzoate and \( p \)-mercuribenzenesulfonate derivatives were partially active toward trypsin and inactive toward \( \alpha \)-chymotrypsin. The carboxymethyl and \( N \)-ethylnaleimide derivatives were inactive toward both enzymes.

The carboxamidomethyl and aminoethyl derivatives inhibited trypsin temporarily and were gradually digested by the enzyme.

It has previously been shown (1) that the disulfide bond linking half-cystine residues 14 and 38 in the basic pancreatic trypsin inhibitor could be selectively reduced without loss of inhibitory activity. During the above study, several derivatives of the reduced inhibitor were prepared. The reduced dithiobis (2-nitrobenzoic acid) inhibitor was found to be active and resistant to tryptic digestion, but the reduced carboxamethyl inhibitor was inactive and could be digested by trypsin. Because of the striking difference in inhibitory activity of these two derivatives, it became of interest to study in greater detail the effects of modifying half-cystine residues 14 and 38 with other common sulfhydryl reagents.

Moreover, Wu and Laskowski (2) had reported that the intact pancreatic inhibitor is active toward trypsin and both chymotrypsin \( \alpha \) and B. It was thought that modification of half-cystine residues 14 and 38 might also result in inhibitor derivatives with altered activity toward chymotrypsin. Such derivatives would be of use in delineating the differences between the mechanisms of inhibition of trypsin and chymotrypsin by the pancreatic inhibitor.

It was found, during these studies, that certain derivatives of the reduced inhibitor gradually lost their inhibitory activity when incubated with an excess of trypsin. This was in contrast to the intact inhibitor which is stable indefinitely in the presence of trypsin (3). Qualitatively, the loss of activity resembled the phenomenon of temporary inhibition reported by Laskowski and Wu (4) on acidic pancreatic inhibitor and observed independently by Gorini and Audrain (5) on ovomucoid. Recently the phenomenon has been discussed by Greene, Fackre, and Rigby (6), Burck et al. (7), and Fritz et al. (8).

The present paper describes the formation of various derivatives of the reduced inhibitor prepared by reaction of half-cystine residues 14 and 38 with sulfhydryl group reagents. The activity of these derivatives toward trypsin and \( \alpha \)-chymotrypsin is reported and discussed. The gradual loss of inhibitory activity by the reduced carboxamidomethyl and reduced aminoethyl derivatives when incubated with trypsin is described.

EXPERIMENTAL PROCEDURE

Materials—Kallikrein inactivator (Trasylol) which is identical with basic pancreatic trypsin inhibitor (9) was used throughout. It was kindly provided by Dr. G. Haberland of Farbenfabriken Bayer AG (Leverkusen-Bayerwerk, West Germany) as Trasylol Lot 55633c. Trypsin (twice crystallized, 50% MgSO\(_4\)) and \( \alpha \)-chymotrypsin (three times crystallized) were obtained from Worthington. Benzoyl-L-arginine ethyl ester hydrochloride and benzoyl-L-tyrosine ethyl ester were from Mann. DTNB (Aldrich) was prepared fresh as a 0.01 M solution in 0.05 M Tris. Sodium \( p \) hydroxymercuribenzoate and \( p \)-chloromercuribenzenesulfonate were obtained from Sigma. Iodoacetic acid was obtained from Aldrich and recrystallized from ether-hexane prior to use by the method of Noltmann, Mahowald, and Kuby (10).

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Iodoacetamide (Sigma) was recrystallized prior to use from hot water. Ethyleneimine was obtained from K and K Laboratories and N-ethylmaleimide was from Pierce Chemical Company.

Determination of Inhibitor Activity—Trypsin inhibitor activities were determined by the method of Schwert and Takenaka (11), as modified by Kassell et al. (12).

Chymotrypsin activity and inhibitor activity against chymotrypsin were determined by a modification of the method of Hummel (13). Solutions of chymotrypsin and inhibitor buffered at pH 8.0 with 0.05 M Tris plus 0.01 M CaCl₂ were mixed so that a final concentration of chymotrypsin of approximately 31 μg per ml and the desired concentration of inhibitor were obtained. After incubation for 5 min at 25°, 0.1 ml of the mixture was added to a substrate solution which consisted of 1.5 ml of 0.05 M Tris plus 0.01 M CaCl₂, pH 8.0, and 1.4 ml of 1.07 mM buffered at pH 8.0 with 0.05 M Tris plus 0.01 M CaCl₂. The amount of inhibitor was such that, after 30 min the reaction was terminated by titration to pH 2 with 1 N HCl, and excess reagent was removed by gel filtration on Sephadex G-10. The main protein peak showed that the excess DTNB and thiophenolate did not interfere with any of the assay systems used.

Reduced AE inhibitor was prepared by adding 0.5 ml of ethyleneimine (9.6 μmoles) to 20 mg of reduced inhibitor (3.1 μmoles) in 7 ml of 0.5 M Tris-HCl plus 0.02% EDTA, pH 8.7. After 30 min the reaction mixture was adjusted to pH 2 with concentrated HCl and excess reagent was removed by gel filtration on Sephadex G-10. The main protein peak showed only a trace of free sulfhydryl by the DTNB reaction. To determine the amount of AE-cysteine present, the column, 0.9 × 50 cm, of the Beckman/Spinco model 120B analyzer was equilibrated with pH 5.28 buffer. A hydrolyzed sample of reduced AE inhibitor was placed on the column and eluted with the pH 5.28 buffer. AE-cysteine emerged after 220 min.²

² Although this method gives good separation of AE-cysteine from the other basic amino acids, it is wasteful of buffer and ninhydrin. We resorted to it only because we did not have sufficient resin to increase the short column to a height of 15 cm as recommended by Raftery and Cole (15).

<table>
<thead>
<tr>
<th>Inhibitor derivative</th>
<th>Trypsin (100%) inhibition μg trypsin/μg inhibitor</th>
<th>α-Chymotrypsin (50%) inhibition μg α-chymotrypsin/μg inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>3.1</td>
<td>1.9</td>
</tr>
<tr>
<td>Reduced</td>
<td>2.9</td>
<td>2.1</td>
</tr>
<tr>
<td>Dithiobis (2-nitrobenzoic acid)</td>
<td>2.7</td>
<td>1.6</td>
</tr>
<tr>
<td>Carboxymethyl</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Carboxamidomethyl</td>
<td>2.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Aminoethyl</td>
<td>1.9</td>
<td>0.2</td>
</tr>
<tr>
<td>p-Hydroxymercurationoate</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>p-Mercuribenzenesulfonate</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* Since the inhibition of chymotrypsin is nonstoichiometric (2) the 50% inhibition values are reported. To determine these values a constant amount of 3.1 μg of chymotrypsin was used whereas the amount of inhibitor was varied.

Calculations, assuming a color value for AE-cysteine equal to 91% of that of lysine (15), showed that the inhibitor derivative contained 1.91 moles of AE-cysteine per mole of inhibitor.

Reduced N-ethylmaleimide inhibitor was formed by reacting 4.1 mg of reduced inhibitor in 0.1 M phosphate, pH 6.8, with 0.001 N N-ethylmaleimide. Spectrophotometric determination according to Alexander (16) showed that the reaction was completed immediately, and gave the expected amount of the addition product assuming 2.0 moles of sulfhydryl per mole reduced inhibitor.

Reduced p-hydroxymercurationoate inhibitor and reduced p-mercurationoate sulfonate inhibitor were prepared by allowing 0.1 μmole of reduced inhibitor to react with 0.22 μmole of reagent in 0.33 M acetate, pH 4.6. The reaction was immediate, and spectrophotometric calculations according to Boyer (17) showed 2.1 moles of mercaptide per mole of inhibitor.

Temporary Inhibition—For studies with reduced AE, reduced carboxamidomethyl, and reduced DTNB inhibitors, trypsin (80 μg per ml) in 0.05 M Tris + 0.01 M CaCl₂, pH 8.0, was mixed with an equal volume of the desired inhibitor solution in 0.05 M Tris + 0.01 M CaCl₂. The amount of inhibitor was such that 13 to 26% of the trypsin remained active. The mixtures were incubated at 25°, and aliquots were periodically withdrawn and assayed for tryptic activity. Controls which contained trypsin alone were run under identical conditions.

RESULTS

Relative Inhibitory Activities—Table 1 shows the specific activities of the inhibitor derivatives toward trypsin and chymotrypsin. It can be seen that the reduced inhibitor and the carboxamidomethyl and DTNB inhibitor derivatives showed activities toward trypsin which were only slightly less than that shown by the intact inhibitor. The AE derivative was approximately 65% as active as the intact inhibitor. This lower activity was due to the heterogeneity of the preparation. The derivative was separated from excess reagent on Sephadex (see "Experimental Procedure") and two protein peaks were eluted from the column. The main peak (84% of the total protein) consisted of the AE inhibitor. However, the presence of a second peak which consisted of smaller peptide fragments indicated that a slight
FIG. 1. Temporary inhibition of trypsin. Trypsin (80 μg per ml) in 0.05 M Tris-HCl-0.01 M CaCl₂, pH 8.0, was incubated with an equal volume of the desired inhibitor derivative at 25°. Amounts of inhibitor were chosen so as to inhibit initially 75 to 85% of the trypsin. Aliquots were assayed for trypsin activity at the indicated times. ●—●, AE inhibitor; △—△, carboxamidomethyl inhibitor; ▲—▲, DTNB inhibitor; ○—○, trypsin control.

FIG. 2. Digestion of inhibitor derivatives by α-chymotrypsin. Solutions of α-chymotrypsin (0.08 μmole in all cases) were incubated at 25° in 0.1 M sodium borate-0.01 M CaCl₂, pH 8.0, with the following amounts of inhibitor derivatives: △—△, CM inhibitor (0.11 μmole); ●—●, DTNB inhibitor (0.03 μmole); ▲—▲, AE inhibitor (0.10 μmole); ○—○, α-chymotrypsin. The rate of digestion was followed by the ninhydrin method, and results are expressed as micromoles of a standard leucine solution which gave an absorbance at 570 mµ of 2.19 per μmole.

FIG. 3. Digestion of inhibitor derivatives by trypsin. The conditions were the same as those shown in Fig. 2. △—△, CM inhibitor; ●—●, DTNB inhibitor; ▲—▲, AE inhibitor; ○—○, trypsin (0.09 μmole).

The p-hydroxymercuribenzoate and p-mercuribenzenesulfonate derivatives possessed little inhibitory activity toward trypsin. The loss of activity could not be due to the presence of a slight excess of mercuribenzoate, since control experiments showed that trypsin activity and complex formation were not affected by an excess of these reagents, nor could the activity be attributed to the presence of unreacted reduced inhibitor, since mercaptide formation occurred in the amounts expected (assuming two free sulphydryls on the reduced inhibitor). When increasing amounts of p-mercuribenzoate inhibitor were allowed to react with trypsin, the trypsin activity decreased in a linear fashion. This indicated that the stoichiometry of complex formation was not altered.

The CM-inhibitor showed no activity as previously noted (1). The N-ethylmaleimide derivative also was inactive toward trypsin. No activity was noted for these derivatives with either trypsin or chymotrypsin even when the derivative was present in amounts 15 to 20 times those ordinarily used for assay purposes. There appeared to be no complex formation with these derivatives.

The activities of the derivatives toward α-chymotrypsin generally paralleled those shown with trypsin. The two notable exceptions were the carboxamidomethyl and AE derivatives. The carboxamidomethyl derivative retained 87% of the control activity with trypsin, but was essentially inactive toward chymotrypsin even with a 15-fold excess of inhibitor present. The AE derivative possessed substantial activity toward trypsin, but retained only a trace of activity with chymotrypsin.

Striking differences were also apparent between the CM and carboxamidomethyl inhibitors. The negatively charged carboxymethyl groups abolished all inhibitor activity toward both trypsin and chymotrypsin. The presence of carboxamidomethyl groups, however, did not affect trypsin inhibition, but prevented chymotrypsin inhibition by this derivative.

Temporary Inhibition by Derivatives—Fig. 1 shows the gradual reappearance of enzyme activity during the course of incubation of trypsin with two of the inhibitor derivatives. Trypsin activity reappeared most rapidly in the case of the AE inhibitor, with essentially all of the free enzyme present after 24 hours. The kinetics was somewhat slower in the case of the carboxamidomethyl inhibitor, with all trypsin activity reappearing after 72 hours. No reactivation of trypsin occurred with the DTNB inhibitor. Control solutions of trypsin alone lost approximately 20% of their initial activity during a 72-hour period.

Enzymatic Digestion of Inhibitor Derivatives—The results previously reported (1) suggested that as long as an inhibitor molecule remained active it would be resistant to enzymatic digestion. The data presented in Figs. 2 and 3 indicate that this statement must be qualified. The figures show that the inactive CM inhibitor was digested by both trypsin and α-chymotrypsin as indicated by the progressive increase in ninhydrin color development after incubation for the indicated time periods. However, in the case of the active AE inhibitor incubated with trypsin, there was also an increase in ninhydrin color until at 24 hours the value approximated that for the digestion of CM inhibitor. The digestion apparently was correlated with loss of inhibitor activity and reappearance of free trypsin (cf. Fig. 1).
These results confirmed that the mechanism of temporary inhibition involved tryptic cleavage of the inhibitor molecule. The AE derivative was also rapidly digested by chymotrypsin (Fig. 2). For purposes of comparison, the figures indicate that the DTNB inhibitor showed no significant increase in ninhydrin color when incubated with trypsin or chymotrypsin. The trypsin control showed a slightly greater increase than the DTNB inhibitor-trypsin solution, probably indicating some autolysis in the absence of complex formation. The reduced inhibitor (not shown) also gave no increase in ninhydrin color when incubated with trypsin or chymotrypsin. However, separate experiments not yet reported have shown that over a 24-hour period about 65% of the reduced inhibitor becomes reoxidized under the conditions of the incubation.

**DISCUSSION**

The objective of the present study was to find suitable derivatives of the basic trypsin inhibitor which would enable determination of the reactive center (18, 19). The formation of modified inhibitor which results from tryptic cleavage during complex formation is reversible in the presence of trypsin (20). This makes determination of the reactive center difficult unless the dissociation constants result in the ultimate formation of free modified inhibitor. The mechanism proposed by Ozawa and Laskowski (21) for soybean trypsin inhibitor and chicken ovomucoid was confirmed by Feinstein, Osuga, and Feeney (22) for chicken ovomucoid but not for ovomucoids of turkey and cassowary. Recently Tschesche (23) isolated a Kazal type inhibitor from porcine pancreas which contained a Lys-X bond cleaved during purification. Greene* has shown that the Kazal type inhibitor from bovine pancreas possesses an Arg-X bond at its reactive center.

In the previous paper of this series (1) it was reported that partial reduction of the basic pancreatic inhibitor with borohydride selectively cleaved disulfide bond 14-38 in the molecule. Alkylation of both free sulfhydryl groups with iodoacetic acid to form the carboxymethyl derivative not only abolished all inhibitory activity, but also rendered the inhibitor susceptible to tryptic digestion (and also to chymotryptic digestion, as shown in the present paper).

When the reduced inhibitor was reacted with iodoacetamide, however, entirely different results were obtained, as shown in Table I. This derivative retained full initial activity with trypsin, indicating that the presence of the two negatively charged carboxymethyl groups was probably responsible for the inability of the CM inhibitor to complex with trypsin. Elimination of the charge by introducing the amide groups resulted in an active derivative. However, structural changes in the inhibitor molecule must have occurred, because the carboxamidomethyl derivative did not form a stable complex, but instead inhibited trypsin only temporarily (Fig 1). The inhibitor derivative was apparently slowly digested by trypsin (Fig. 3).

Similar results were also noted with the AE derivative, which retained a significant amount of initial inhibitory activity but was gradually digested by trypsin. It should be noted that whereas the negatively charged carboxamethyl derivative was inactive, the presence of positively charged amino ethyl groups in the same position did not interfere with initial inhibitory activity.

* L. J. Greene, personal communication.

Chauvet and Acher (24) have recently implicated the e-amino group of lysine 15 in the binding of the pancreatic inhibitor to trypsin. The derivatives described in the present paper all involve substitution on cysteine residues 14 and 38. The substitution on cysteine 14 occurs in the immediate vicinity of lysine 15, and the observed lack of activity with the CM derivative might be due to interference with the positive charge on lysine 15, thereby preventing complex formation. Presence of a bulky substituent such as N-ethylmaleimide could also conceivably interfere with the postulated binding function of lysine 15. Similar reasoning can be applied to those derivatives which lacked tryptic activity or possessed only slight activity.

Prior to this publication only two naturally occurring inhibitors were known to be temporary inhibitors of trypsin, namely, Kazal type inhibitors from pancreas (4-8) and chicken ovomucoid (5). Both were inactive toward chymotrypsin (2). In the present paper it was shown that the basic pancreatic inhibitor could be converted into a temporary inhibitor by reduction of disulfide bond 14-38 and reaction of the two resultant sulfhydryl groups with either iodoacetamide or ethylenediamine. Like the naturally occurring temporary inhibitors, both the carboxamidomethyl and AE inhibitors also failed to inhibit chymotrypsin. This fact would appear to be more than coincidental. It seems likely that the tryptic cleavage (or cleavages) which resulted in temporary inhibition, as well as the chymotryptic cleavages which prevented inhibition, occurred in an exposed portion of the molecule removed from the reactive center. Such an interpretation agrees with the existence of the transient trypsin-trypsin inhibitor-trypsin complex postulated for temporary inhibitors (4). Alternatively, in the case of temporary inhibitors, the cleavage which occurred with complex formation and that which caused loss of inhibitor activity could have involved one and the same bond. The above possibilities are presently being investigated in detail with the carboxamidomethyl inhibitor, and these results will be published separately.

**REFERENCES**

The Basic Trypsin Inhibitor of Bovine Pancreas: VIII. CHANGES IN ACTIVITY FOLLOWING SUBSTITUTION OF REDUCED HALF-CYSTEINE RESIDUES 14 AND 38 WITH SULFHYDRYL REAGENTS
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