

Studies on Human Plasma C $\bar{1}$ Inactivator-Enzyme Interactions

I. MECHANISMS OF INTERACTION WITH C $\bar{1}$ s, PLASMIN, AND TRYPSIN

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ABSTRACT This study has explored the nature of the molecular events which occur when C $\bar{1}$ inactivator, a human plasma inhibitor of the complement, kinin-forming, coagulation, and fibrinolytic enzyme systems, interacts with C $\bar{1}$ s, plasmin, and trypsin. Purified inhibitor preparations demonstrated two bands, when examined by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). The molecular weights of the major and minor bands were 105,000 and 96,000 daltons, respectively. The minor component appeared to be immunologically and functionally identical to the main C $\bar{1}$ inactivator component. Loss of C $\bar{1}$ s and plasmin functional activity was associated with the formation of a 1:1 molar complex between the inhibitor and each enzyme. These complexes were stable in the presence of SDS and urea. The light chain of both these enzymes provided the binding site for C $\bar{1}$ inactivator. Complex formation and enzyme inhibition occurred only with native and not with an inhibitor preparation denatured by acid treatment, thereby demonstrating the importance of conformational factors in the enzyme-inhibitor reaction. Although peptide bond cleavage of the C $\bar{1}$ inactivator molecule by C $\bar{1}$ s was not documented, plasmin was found to degrade the

inhibitor with the production of several characteristic derivatives. At least one of these products retained the ability to complex with C $\bar{1}$ s and plasmin. Trypsin, which failed to form a complex with C $\bar{1}$ inactivator, degraded the inhibitor in a limited and sequential manner with the production of nonfunctional derivatives one of which appeared structurally similar to a plasmin-induced product. These studies therefore, provide new information concerning the molecular interactions between C $\bar{1}$ inactivator and several of the proteases which it inhibits.

INTRODUCTION

Plasma contains a family of proteolytic enzyme inhibitors which regulate and modulate, in an incompletely understood manner, the participation of several interrelated enzyme systems in various inflammatory and hemostatic reactions. One of these inhibitor's α_2 -macroglobulin, may regulate several different proteolytic effector pathways, as it has been shown to inhibit enzymes of the kinin-forming, coagulation, and fibrinolytic systems. These include kallikrein (1, 2), thrombin (3), and plasmin (4-6) as well as a group of proteases including trypsin, chymotrypsin, elastase, papain, and other enzymes (7). Recent studies have indicated that plasma α_2 -macroglobulin is proteolytically cleaved by the enzymes which it inhibits and that the altered inhibitor molecule is incorporated in a protein-protein complex with the inhibited enzyme (8).

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Another plasma protein inhibitor, C \bar{I} inactivator,¹ inhibits enzymes of the complement, kinin-forming, coagulation, and fibrinolytic systems. These include C \bar{I} and its subcomponents C \bar{I} s and C \bar{I} r (9-13), kallikrein, PF/dil (permeability factor of dilution) (13-16), plasmin (13, 17), Hageman factor (18) and its active fragments (17), and plasma thromboplastin antecedent (2, 18). C \bar{I} inactivator, however, has been found to be a relatively ineffective inhibitor of trypsin and chymotrypsin (10, 19). Although the inhibitory spectrum of C \bar{I} inactivator has been defined, the molecular basis of enzyme inhibition by C \bar{I} inactivator has remained obscure.

In the present study, we have investigated molecular interactions which occur between C \bar{I} inactivator and C \bar{I} s, plasmin, and trypsin. C \bar{I} inactivator was found to interact with each enzyme in a different manner. Incubation with C \bar{I} s led to rapid formation of a stable bimolecular complex with associated loss of C \bar{I} s functional activity. Proteolytic degradation of C \bar{I} inactivator, as judged by the production of lower molecular weight C \bar{I} inactivator products was not detected. Similar complex formation with concomitant functional inactivation occurred on adding plasmin to C \bar{I} inactivator. In addition, however, plasmin degraded C \bar{I} inactivator and produced several characteristic derivatives, at least one of which retained the ability to complex with plasmin and with C \bar{I} s. Trypsin did not form a complex with C \bar{I} inactivator under the analytical conditions used, although it did partially degrade and inactivate the inhibitor. These derivatives, one of which appeared to be structurally similar to that generated by plasmin, were unable to complex with C \bar{I} s or plasmin. Other studies revealed that the light chain of both C \bar{I} s and plasmin provided the binding site for C \bar{I} inactivator. In addition, complex formation and enzyme inhibition occurred only with native and not denatured C \bar{I} inactivator, suggesting a major role for conformational factors in complex formation. These studies thus provide new data on the mechanisms involved in enzyme inhibition and regulation by C \bar{I} inactivator.

METHODS

All chemicals² used were reagent grade and were obtained from the following sources: *N*- α -acetyl-L-lysine methyl ester HCl and *p*-nitrophenyl-*p*'-guanidinobenzoate HCl from Cyclo Chemical, Division Travenol Laboratories, Inc., Los Angeles, Calif.; ϵ -aminocaproic acid from Fluka AG., Basel, Switzerland; Polybrene (hexadimethrine bromide) and urokinase, 200,000 U/vial, from Abbott Laboratories, North Chicago,

¹ The complement terminology used conforms to the recommendations of the World Health Organization Committee on Complement Nomenclature (1968. *Bull. W. H. O.* 39: 935-938).

Ill.; soybean trypsin inhibitor (salt free) (SBTI)² and bovine trypsin (three times crystallized, salt free) from Worthington Biochemical Corp., Freehold, N. J.; dithiothreitol (DTT) and methyl- α -D-mannopyranoside from Calbiochem, San Diego, Calif.; sodium dodecyl sulfate (SDS) from Sigma Chemical Co., St. Louis, Mo.; polyethylene glycol (Carbowax 4000) from Union Carbide Corp., Clifton, N. J.

Human plasma C \bar{I} inactivator was isolated by a methodology not previously described. All procedures were performed with plastic containers or siliconized glassware and in the presence of inhibitors to minimize activation of plasma proteases (8, 20). The materials, including resins used for chromatographic separation, were prewashed in buffer containing Polybrene (50 μ g/ml). All buffers contained sodium azide, 0.02%, to inhibit bacterial contamination. Plasma (200 ml) was harvested from freshly obtained acid citrate dextrose blood by centrifugation at 2,000 *g* for 15 min at 4°C. After separation, SBTI (275 μ g/ml plasma), Polybrene (140 μ g/ml plasma), and sodium EDTA (0.02 M final concentration) were added to the plasma. Barium chloride was added (1.0 M to a final concentration of 0.1 M) and mixed with the plasma 10 min at 25°C to reduce the concentration of the prothrombin complex. After centrifugation, the adsorbed plasma was diluted with 2 vol of 0.02 M phosphate buffer, pH 7.4, containing 0.1 M NaCl, and polyethylene glycol (50% wt/wt in H₂O) was added at 25°C to a final concentration of 12% (vol/vol). After removal of the precipitate by centrifugation (30 min, 2,000 *g*), polyethylene glycol was added to a final concentration of 22% at 25°C and the solution adjusted to pH 5.8. The pellet from the 12-22% polyethylene glycol fraction was obtained by centrifugation (60 min, 2,000 *g*), and was resuspended in buffer (0.05 M Tris-HCl, pH 8.0, containing 0.02 M NaCl, 50 μ g/ml SBTI, and 25 μ g/ml Polybrene) and the C \bar{I} inactivator further purified on a 5.0 \times 40-cm DEAE-cellulose column at 4°C (DE-52, H. Reeve Angel & Co., Inc., Clifton, N. J.) with a linear sodium chloride gradient at constant pH (starting buffer, 1,500 ml 0.05 M Tris-HCl, pH 8.0, and the limit buffer, 1,500 ml, containing 0.4 M NaCl). C \bar{I} inactivator was identified in the second portion of the main protein peak in fractions having a conductivity of 13.5-15.5 mmho/cm (27°C) by using a type CDM 2e conductivity meter with a CDC 104 probe (Radiometer Co., Copenhagen, Denmark) by double diffusion analysis with rabbit anti-C \bar{I} inactivator. The fractions containing the peak of C \bar{I} inactivator were concentrated by ultrafiltration (Diaflo membrane PM-10, Amicon Corp., Lexington, Mass.), and applied to a 5.0 \times 100-cm column of Bio-Gel A-5m, at 4°C (Bio-Rad Laboratories, Richmond, Calif.). The fractions containing C \bar{I} inhibitor were then pooled, concentrated, and subjected to a preparative electrophoresis in Pevikon (Kema Nord, Stockholm, Sweden) (21). After elution, the major contaminating protein, albumin, was removed by passing the C \bar{I} inactivator preparation through a 20-ml column of concanavalin A-Sepharose (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). The inhibitor was eluted from the affinity resin with 5% methyl- α -D-mannopyranoside in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl (22). The average yield of C \bar{I} inactivator by this method was in the range of 10-15% (plasma concentration being 18 mg/100 ml). The final product formed a single precipitin arc in the α_2 region with rabbit anti-human serum antibody by immunoelectrophoresis (23). SDS-acrylamide (5% gel) electrophoretic analysis of the C \bar{I} inactivator preparation demon-

² Abbreviations used in this paper: DTT, dithiothreitol; PAS, periodic acid-Schiff; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate.

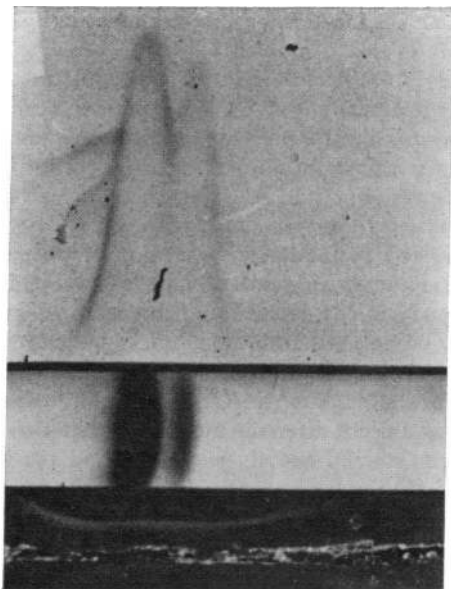


FIGURE 1 Antigen-antibody crossed electrophoresis and immunoelectrophoresis of purified human plasma C $\bar{1}$ inactivator. After electrophoresis of a C $\bar{1}$ inactivator preparation on an acrylamide gel (5%) containing SDS (electrophoresis in stained gel was from left to right) the gel was sliced longitudinally and embedded in agarose for immunodiffusion using rabbit anti C $\bar{1}$ inactivator antibody (beneath stained gel). Other gel slices were overlaid with agarose containing the antibody and electrophoresed at right angles to the gel (top panel). The two peaks which appeared after crossed electrophoresis and which corresponded to the two C $\bar{1}$ inactivator bands are designated "I" and "II."

strated a major and minor protein band (Fig. 1 and Fig. 2, gel b). Rabbits were immunized with inhibitor preparations and produced antibody reactive with the inhibitor. Several of the C $\bar{1}$ inactivator preparations stimulated the production of low concentrations of antibody to IgA, indicating trace contamination of these products with IgA. The specific activity of the C $\bar{1}$ inactivator, measured with acetyl-L-lysine methyl ester as a substrate for C $\bar{1}$ s as previously described (24), was 217 inhibitor U/mg C $\bar{1}$ inactivator.

C $\bar{1}$ s was isolated by a slight modification of the method described for isolation of the C $\bar{1}$ s proenzyme (25). The euglobulin precipitate was dissolved in 0.047 M sodium phosphate buffer, pH 7.5, containing 0.04 M NaCl and incubated with 100 mg of heat-aggregated (63°C, 10 min) human IgG for 30 min at 37°C to activate C $\bar{1}$ s before addition of EDTA and application to the first column. On electrophoresis in SDS-acrylamide gels, (5 and 7.5% gels), the C $\bar{1}$ s preparations exhibited one band, in addition, after reduction with DTT, the C $\bar{1}$ s dissociated into its heavy and light chains.

Plasminogen was purified by a minor modification of the method of Deutsch and Mertz (26) with freshly obtained human plasma as the starting material. After elution of the plasminogen peak with ϵ -aminocaproic acid from a lysine-Bio-Gel A-5m affinity column, the material, concentrated by ultrafiltration, was further purified by chromatography on Sephadex G-200 (Pharmacia Fine Chemicals, Inc.). The final

product possessed less than 1% spontaneous plasmin activity as assayed by a standard caseinolytic assay (27) and displayed one band by SDS-acrylamide (5% gel) electrophoresis. Plasminogen was activated for each experiment in glycerol (25% glycerol in 0.04 M Tris-HCl-0.01 M lysine-0.08 M NaCl, pH 9.0) (28) with urokinase (20 Abbott U urokinase/100 μ g plasminogen) at 25°C for 18 h in plastic tubes. Full activation of the plasminogen was documented by SDS-acrylamide gel electrophoretic analysis of reduced samples of the incubation mixture which demonstrated complete conversion of the plasminogen band to the heavy and light chain of plasmin. Freshly activated plasmin was utilized in incubation studies with C $\bar{1}$ inactivator since our preliminary studies using plasmin spontaneously activated in 50% glycerol (obtained from the Michigan Department of Public Health, Lansing, Mich.) found that this plasmin failed to form a higher molecular weight complex with the inhibitor as analyzed by electrophoresis in SDS-acrylamide gels.

Functional C $\bar{1}$ inactivator activity was assayed by quantitatively its ability to inhibit C $\bar{1}$ s-mediated inactivation of C2 or

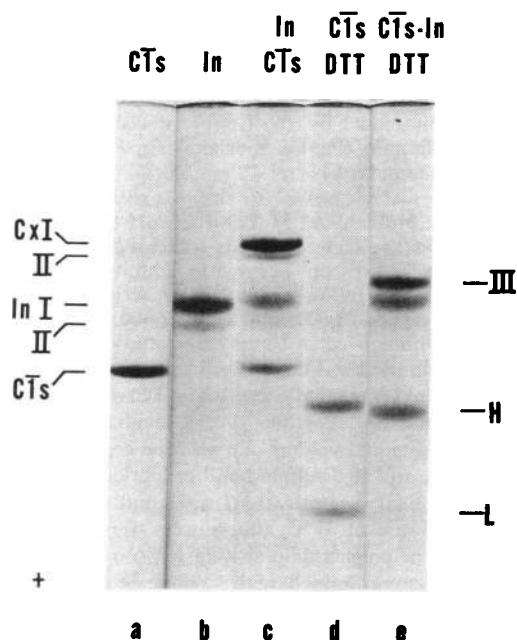


FIGURE 2 SDS gel (5% acrylamide) electrophoretic analysis of C $\bar{1}$ s, C $\bar{1}$ inactivator (In), and an incubation mixture of the two. C $\bar{1}$ s (0.9 mg/ml) was incubated 10 min at 37°C with 0.1 M sodium phosphate buffer, pH 7.6, or with C $\bar{1}$ inactivator (1.5 mg/ml). The samples were then incubated in a solution of 10 M urea, 2% SDS, either with or without 14 mM DTT for 45 min at 37°C and applied to the gels as follows: gel a, C $\bar{1}$ s; gel b, C $\bar{1}$ inactivator; gel c, C $\bar{1}$ inactivator-C $\bar{1}$ s incubation mixture (10 min, 37°C); gel d, C $\bar{1}$ s, reduced with DTT; gel e, reduced C $\bar{1}$ inactivator-C $\bar{1}$ s mixture. "Cxl" and "II" indicate the positions occupied by the complex formed between C $\bar{1}$ s and C $\bar{1}$ inactivator bands I and II. "In I" and "II" are designations for the major and minor C $\bar{1}$ inactivator proteins bands. The complex formed by the light chain of C $\bar{1}$ s and C $\bar{1}$ inactivator band I observed after reduction (gel e) is designated "III." The heavy (H) and light (L) chains of C $\bar{1}$ s are indicated. The direction of electrophoresis is toward the anode indicated by a "+."

C4. The amount of C \bar{I} s required to inactivate approximately 80% of the hemolytic activity of 1 μ g of isolated human C4 (29) or oxidized human C2 (30, 31) in 30 min at 37°C in a total volume of 0.6 ml was first determined. In various experiments this averaged 10–30 ng of C \bar{I} s to inactivate C2 and 1–10 ng of C \bar{I} s to inactivate C4. The concentration of C \bar{I} s selected from these studies was then incubated with 60–300 ng of C \bar{I} inactivator or enzyme-treated C \bar{I} inactivator for 5 min at 37°C in a total volume of 0.4 ml before addition of 0.2 ml of C2 or C4. After 30 min at 37°C, the remaining active C2 or C4 was assayed. In all experiments inactivation of C2 and C4 was assessed by measurement of residual hemolytic activity (32).

The plasmin-inhibiting activity of C \bar{I} inactivator was assessed in a fibrinolytic assay (33). Varying concentrations of C \bar{I} inactivator were preincubated (5 min, 37°C) with urokinase-activated plasmin, and the residual fibrinolytic activity was determined.

Antisera against C \bar{I} inactivator, plasminogen, whole human serum, IgG, IgA, and IgM were obtained from Behring Diagnostics, Inc., subsidiary of the American Hoechst Corp., Woodbury, N. Y. Immunochemical quantitation of C \bar{I} inactivator preparations was performed by single diffusion analysis (34).

Radio-labeling of C \bar{I} inactivator preparations with 125 I was performed by the technique of McConahey and Dixon (35).

Protein was measured by the Lowry procedure (36) with bovine serum albumin (Pentex Biochemicals, Kankakee, Ill.) as the reference standard.

C \bar{I} inactivator and the proteolytic enzymes studied were incubated at 37°C in buffer (0.05 M Tris-HCl, pH 8.0, containing 0.1 M NaCl) in the concentrations indicated in the figure legends. The molar ratios of enzyme to inhibitor were calculated by using the following molecular weights: trypsin, 23,300 (37); plasmin, 73,500; and C \bar{I} s, 72,000 as determined for the preparations used in the present study. The concentration of trypsin used was determined by active site titration with *p*-nitrophenyl *p*'-guanidinobenzoate HCl as described by Chase and Shaw (38). Portions of the incubation mixtures were added to an equal volume of a solution containing 10 M urea, 2% SDS, and if disulfide bond reduction was desired, DTT (14 mM final concentration) was employed. After incubation for 45 min at 37°C, the samples were analyzed by electrophoresis in polyacrylamide gels (5%) containing SDS (39). The gels were stained with Coomassie Brilliant Blue. Selected gels were stained with periodic-acid Schiff (PAS) reagent (40). Densitometric scans of gels were carried out in a Gilford Model 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio), equipped with a linear transport device.

The molecular weight of C \bar{I} inactivator and its reaction products was determined by SDS-acrylamide gel electrophoresis as described (37, 39). The proteins used for molecular weight markers were reduced before electrophoresis. They included phosphorylase A, 94,000 (39) from Sigma Chemical Co.; ovalbumin, 43,000 (39) from Pharmacia Fine Chemicals, Inc.; chymotrypsin, 25,000 (39) from Calbiochem; α_2 -macroglobulin subunit chain, 185,000 and its trypsin-induced proteolytic derivative, 85,000 were prepared as described (8). Human fibrinogen (A α -chain, 70,900; B β -chain, 60,400; and γ -chain, 49,400) (41) was provided by Dr. Michael Mosesson and the heavy chain of myosin, 212,000 (42) by Dr. Paul Dreizen, both of the State University of New York, Downstate Medical Center, Brooklyn, N. Y.

RESULTS

All purified C \bar{I} inactivator preparations derived from individual donors demonstrated two bands with approximate molecular weights of 105,000 and 96,000 daltons, respectively, on analysis by acrylamide gel electrophoresis in the presence of SDS. Although different preparations exhibited variable proportions of the two bands (I and II), the slower migrating band (I) was generally the major component. The use of measures designed to minimize activation of plasma proteolytic enzymes during isolation, including the drawing of the blood directly into the anticoagulant containing both Polybrene and SBTI, did not significantly alter the proportion of bands I and II obtained in the final preparations. Reduction of C \bar{I} inactivator before electrophoresis did not alter the mobility of the two bands. Both bands stained heavily with PAS and the concentration of PAS-staining material in each band relative to duplicate SDS-acrylamide gels stained with either amido black or Coomassie blue stain was constant. This indicated that band II possessed PAS-reactive material in a concentration similar to that found for the major C \bar{I} inactivator band (I).

In the C \bar{I} inactivator preparation shown in Fig. 1, the concentration of band II relative to band I was approximately 15% as assessed by densitometric scans of the acrylamide gels. The following studies demonstrated the immunological identity of bands I and II. After electrophoresis of C \bar{I} inactivator in SDS-acrylamide gels, the gels were cut longitudinally and embedded in an agarose plate and analyzed by immunodiffusion against rabbit antiserum to C \bar{I} inactivator placed in a parallel slot in the agarose (Fig. 1). Alternatively, the sectioned gels were electrophoresed at right angles to their longitudinal axes into agarose containing the antibody (43) (Fig. 1). Immunodiffusion revealed two arcs which joined without spur formation in positions corresponding to bands I and II of the C \bar{I} inactivator preparation. On crossed electrophoresis larger and smaller peaks of precipitation corresponding to bands I and II, respectively, were apparent (Fig. 1).

These studies document the existence of two immunologically identical forms of C \bar{I} inactivator differing from each other by approximately 9,000 daltons in molecular weight. Both forms apparently represent a single polypeptide chain and both contain large amounts of carbohydrate. Functional studies of bands I and II are presented below.

Studies on the interaction between C \bar{I} inactivator and C \bar{I} s (Fig. 2). Mixtures of C \bar{I} inactivator and C \bar{I} s were prepared and subjected to electrophoresis in SDS-acrylamide gels (Fig. 2). In the absence of reducing agents, two new slower moving bands (complex I and II) were apparent (Fig. 2, gel c). In association with

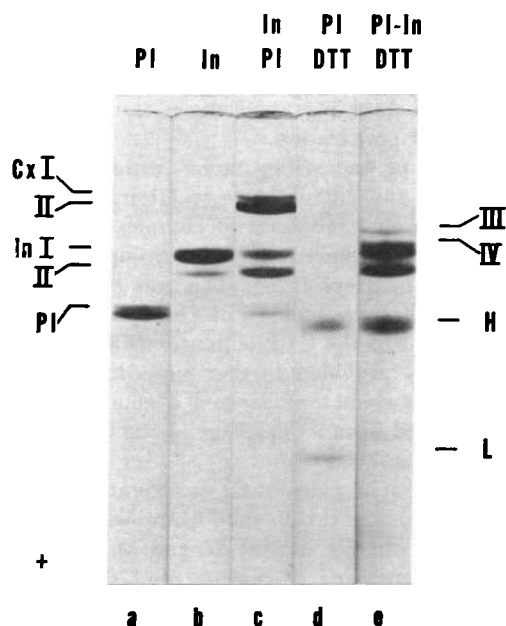


FIGURE 3 SDS gel (5% acrylamide) electrophoretic analysis of a plasmin (PI), C \bar{I} inactivator (In) mixture. Equal volumes of plasmin (1.1 mg/ml) and C \bar{I} inactivator (1.5 mg/ml) were incubated with 0.1 M sodium phosphate buffer or with each other 30 min at 37°C. The samples were then incubated with an SDS-urea solution. DTT was included in the samples which were to be reduced. Gel a, plasmin; gel b, C \bar{I} inactivator; gel c, C \bar{I} inactivator incubated with plasmin; gel d, plasmin reduced with DTT; gel e, C \bar{I} inactivator incubated with plasmin, reduced with DTT. "PI" indicates the position occupied by the plasmin bands.³ The complexes formed between the light chain of plasmin and bands I and II of the C \bar{I} inactivator observed after reduction (gel e), are designated "III" and "IV" in this figure. The heavy (H) and light (L) chains of plasmin are indicated.

this, the density of the C \bar{I} inactivator bands I and II was reduced as was the density of the C \bar{I} s band. The two new bands had approximate molecular weights of 180,000 and 170,000 daltons, respectively, well within the range expected for stoichiometric complex formation between C \bar{I} inactivator bands I and II (Fig. 2, gel b, 105,000 and 96,000 daltons, respectively) and C \bar{I} s (Fig. 2, gel a, 72,000 daltons). The relative concentrations of complex bands I and II (approximately 90% band I) were similar to that found for C \bar{I} inactivator bands I and II (Fig. 2, gels b and c). These studies indicated that both bands of the inhibitor preparation formed a 1:1 molar complex with C \bar{I} s.

Major alterations in the band pattern of C \bar{I} s-C \bar{I} inactivator mixtures were apparent on reduction of the sample before electrophoresis (Fig. 2, gel e). As compared to the unreduced sample, high molecular weight C \bar{I} s-C \bar{I} inactivator complex bands I and II were absent and a new band (complex band III), with an

approximate molecular weight of 125,000 daltons, was identified. In the representative experiment shown, a widening of C \bar{I} inactivator band I was also noted, however, in other experiments an additional band migrating slightly slower than the C \bar{I} inactivator band I and having a molecular weight of approximately 115,000 daltons could be appreciated. In addition, the heavy chain of C \bar{I} s reappeared in a concentration similar to that of the reduced C \bar{I} s preparation (Fig. 2, gel d). In sharp contrast, no C \bar{I} s light chain could be identified in the reduced C \bar{I} s-C \bar{I} inactivator mixture (Fig. 2, gel e). These findings indicate that the two bands formed with apparent molecular weights greater than the C \bar{I} inactivator represent complexes between C \bar{I} inactivator bands I and II and the light chain of C \bar{I} s.

Studies on the interaction between C \bar{I} inactivator and plasmin (Figs. 3, 4). Analysis of the band patterns produced after electrophoresis in SDS-acrylamide gels of plasmin-C \bar{I} inactivator incubation mixtures showed similarities and also differences from that observed between C \bar{I} s and the inhibitor. In analogy to the pattern observed with C \bar{I} s, two higher molecular weight bands (180,000 and 170,000 daltons, respectively) were ob-

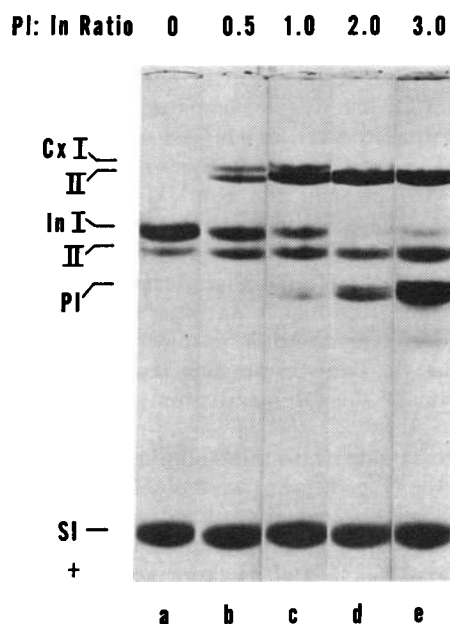


FIGURE 4 SDS-acrylamide gel electrophoretic analysis of increasing plasmin (PI)-C \bar{I} inactivator (In) ratios. Equal volumes of increasing concentrations of plasmin (0.55, 1.1, 2.2, and 3.1 mg/ml) were incubated with C \bar{I} inactivator (1.5 mg/ml) for 30 min at 37°C. After the addition of an equal volume of SBTI ("SI," 2.0 mg/ml) in order to inhibit plasmin, either buffer (gels a-e) or C \bar{I} s (1.0 mg/ml) (gels not shown) were added and incubated for an additional 10 min. The mixtures were analyzed in the absence of reduction. The molar ratio of plasmin to C \bar{I} inactivator used in each sample is indicated.

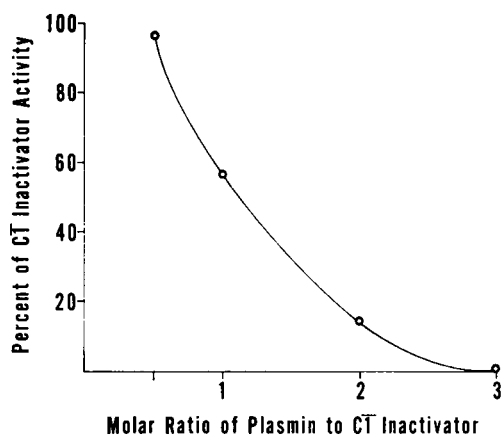


FIGURE 5 Functional C \bar{I} inactivator activity was assessed after incubation with varying molar ratios of plasmin with the same concentration of reactants detailed in the legend to Fig. 4. After neutralization of plasmin with excess SBTI, portions of the mixtures were analyzed on SDS-acrylamide gels (Fig. 4), and were tested as well for their ability to inhibit C \bar{I} s-mediated inactivation of C4 as described in Methods.

served on SDS-acrylamide gel analysis, in the absence of reducing agents, of equimolar mixtures of C \bar{I} inactivator and plasmin (Fig. 3, gel c). Thus, plasmin also formed a 1:1 molar complex with the C \bar{I} inactivator. Functional studies of the fibrinolytic inhibitory activity of C \bar{I} inactivator confirmed this stoichiometric relationship. However, in contrast to the predominance of band I in the C \bar{I} s-C \bar{I} inactivator complex (cf Fig. 2, gel c), with plasmin the band II complex was predominant. These findings suggested that plasmin had produced a lower molecular weight derivative of the C \bar{I} inactivator molecule that formed a complex with the enzyme.³ Residual, unincorporated C \bar{I} inactivator was also converted to a pattern in which material occupying the band II position represented the major proportion of the C \bar{I} inactivator preparation (Fig. 3, gel c).

Results analogous to those obtained with C \bar{I} s and C \bar{I} inactivator (Fig. 2, gel e) were observed after reduction of the plasmin-C \bar{I} inactivator mixtures (Fig. 3, gel e). As compared to the unreduced sample, high molecular weight complex I and II were absent and two new bands III and IV, with molecular weights of 125,000 and 115,000 daltons were observed. Since the heavy chain but not light chain of plasmin appeared after re-

³ As is apparent from inspection of Fig. 3, gel a, the plasmin preparation contained two bands of molecular weights 75,000 and 72,000 daltons, and the loss of these bands in the mixture of plasmin and inhibitor (Fig. 3, gel c) indicated that both of these molecular weight forms of plasmin were bound in the enzyme-inhibitor complex. The 9,000-dalton difference between bands I and II of the plasmin-inhibitor complex ruled out the possibility that these bands were due solely to the binding of the two forms of plasmin.

duction (Fig. 3, gel e), bands III and IV apparently represented the formation of complexes between the light chain of plasmin and band I of the C \bar{I} inactivator preparation and the plasmin-induced derivative of the C \bar{I} inactivator preparation.

Incubation of increasing molar ratios of plasmin (0.5, 1.0, 2.0, and 3.0) relative to C \bar{I} inactivator produced proportional increases in complex band II and of residual uncomplexed material occupying the C \bar{I} inactivator band II positions. This was coupled with a parallel loss of both band I of the complex and the residual band I of the C \bar{I} inactivator (Fig. 4, gels a-e). These findings provided further support for the conclusion that plasmin had formed a complex with the product derived from its cleavage of the C \bar{I} inactivator molecule.

Portions from a plasmin-C \bar{I} inactivator incubation mixture (0.5 molar ratio) were examined sequentially to determine whether a time-dependent conversion of complex I (consisting of plasmin and C \bar{I} inactivator band I) to complex II might occur. No such conversion occurred as the reaction was found to be complete at the time of the first sample (1 min), and the SDS-acrylamide gel pattern remained unchanged and similar to that shown in Fig. 4, gel b, during an additional 3-h incubation period at 37°C.

An additional feature of the plasmin-C \bar{I} inactivator interaction was apparent at plasmin concentrations greater than equimolar (Fig. 4, gels d and e). Even in the presence of a molar ratio of 3, there was a substantial amount of material in the position of the C \bar{I} inactivator band II, indicating that a C \bar{I} inactivator derivative had been produced by the action of plasmin which did not form a complex with this enzyme. Thus, it can be inferred that plasmin cleaved the C \bar{I} inhibitor molecule (band I) yielding at least two derivatives with similar molecular weights. One of these derivatives was functionally active as it formed a complex with plasmin, whereas the other appeared to be functionally inactive.

Studies of the interaction between plasmin, C \bar{I} inactivator, and C \bar{I} s (Fig. 5). The residual C \bar{I} s binding and inhibiting activity of C \bar{I} inactivator in the plasmin incubation mixture was assessed in order to correlate the observed changes in the acrylamide gel pattern with functional alterations since inactivation of the inhibitor's esterolytic inhibiting activity by plasmin has previously been reported (44). After the addition of SBTI to inhibit the residual proteolytic activity in the plasmin-C \bar{I} inactivator mixtures, concentrations of C \bar{I} s equimolar to the C \bar{I} inactivator were added. Acrylamide gel electrophoretic analyses of these mixtures (not shown) demonstrated that the addition of C \bar{I} s was associated with a loss of material from C \bar{I} inactivator bands I and II of the samples of C \bar{I} inactivator previously treated with 0.5 and 1.0 molar ratios of plasmin.

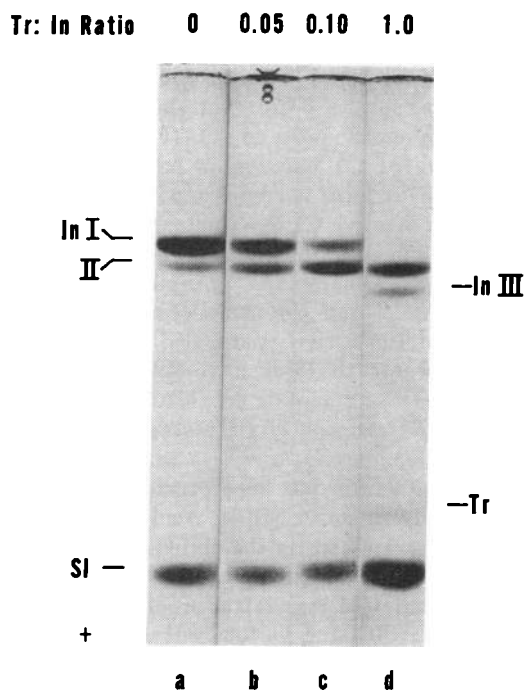


FIGURE 6 SDS-acrylamide gel electrophoretic analysis of the interaction between trypsin and C \bar{I} inactivator. Equal volumes of increasing molar ratios of trypsin (16, 32, and 320 $\mu\text{g}/\text{ml}$; gels b-d) were incubated with C \bar{I} inactivator (1.5 mg/ml) for 10 min at 37°C. After the addition of an equal volume of SBTI (2.0 mg/ml) the mixtures were analyzed in the absence of reduction. The molar ratios of trypsin to C \bar{I} inactivator used in each sample are indicated. "In III" designates the new C \bar{I} inactivator band which appeared in the C \bar{I} inactivator trypsin incubation mixture (gel d). "Tr" indicates the position of the trypsin band, and "SI" the position of the SBTI band.

In addition there was a concomitant increase in the concentrations of both bands I and II of the complex. In contrast, in the incubation mixtures containing plasmin-C \bar{I} inactivator molar ratios of 2 and 3, no loss of material in the C \bar{I} inactivator band II position was observed after the addition of C \bar{I} s, nor was there an increase in band II position of the complex as compared to the samples without C \bar{I} s. Therefore, the C \bar{I} inactivator band II derivative generated with large amounts of plasmin that failed to complex with plasmin was similarly unable to bind C \bar{I} s.

The impairment of the functional ability of C \bar{I} inactivator after incubation with plasmin was also assessed (Fig. 5). Plasmin-C \bar{I} inactivator mixtures after addition of excess SBTI were incubated with C \bar{I} s, and residual C \bar{I} s function was tested by examining the ability of the mixture to inactivate C4 (Fig. 5). Loss of functional C \bar{I} inhibitory activity paralleled the loss of the ability of plasmin-treated C \bar{I} inactivator to complex with C \bar{I} s as demonstrated by the acrylamide gel elec-

trophoretic pattern. As shown in Fig. 5, C \bar{I} inactivator incubated with the higher plasmin concentrations had lost all ability to inhibit C \bar{I} s. Identical results were obtained on testing the ability of plasmin-treated C \bar{I} inactivator to inhibit C \bar{I} s-mediated inactivation of C2.

Studies of the interaction between trypsin, C \bar{I} inactivator, and C \bar{I} s (Fig. 6). In partial analogy to the results obtained with plasmin, increasing concentrations of trypsin caused a progressive depletion of C \bar{I} inactivator band I with a proportional increase in a band which occupied the band II position of the inhibitor (Fig. 6, gels a-d). At higher molar ratios of trypsin (1.0) an additional band with an approximate molecular weight of 86,000 daltons was observed (C \bar{I} In band III, Fig. 6, gel d). This band appeared only after the C \bar{I} inactivator band I had disappeared, suggesting that this product was derived from the band II material. No evidence for complex formation between trypsin and C \bar{I} inactivator

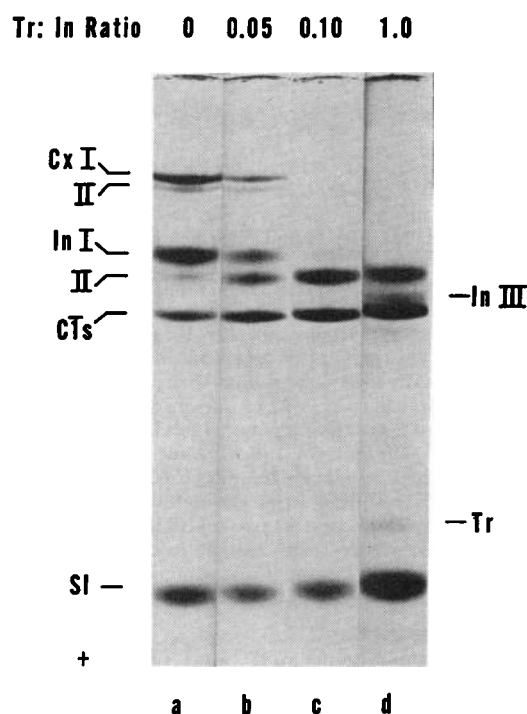


FIGURE 7 SDS-acrylamide gel electrophoretic analysis of the interaction between trypsin, C \bar{I} inactivator, and C \bar{I} s. Equal volumes of increasing concentrations of trypsin (16, 32, and 320 $\mu\text{g}/\text{ml}$; gels b-d) were incubated with C \bar{I} inactivator (1.5 mg/ml) for 10 min at 37°C. After the addition of an equal volume of SBTI (2.0 mg/ml), C \bar{I} s (1 mg/ml) was added and incubated an additional 10 min. The mixtures were analyzed in the absence of reduction. The molar ratios of trypsin to C \bar{I} inactivator used in each sample are indicated. "In III" designates the new C \bar{I} inactivator band which appeared in the C \bar{I} inactivator trypsin incubation mixtures (gel d). "Tr" indicates the position of the trypsin band, and "SI" the position of the SBTI band.

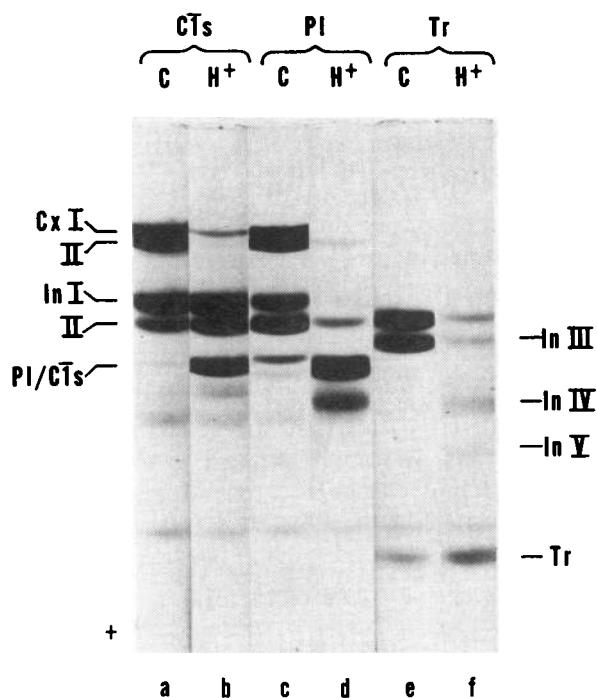


FIGURE 8 SDS gel (5% acrylamide) electrophoretic analysis of the interaction of acid-treated C \bar{I} inactivator with C \bar{I} s, plasmin (PI), and trypsin (Tr). C \bar{I} inactivator (5 mg/ml) was acidified to pH 2.0 with equal volumes of 1/6 N HCl for 30 min at 37°C and neutralized with 1/6 N NaOH. The untreated (C) and acid-treated (H⁺) inhibitors (1.5 mg/ml) were then incubated with C \bar{I} s (0.9 mg/ml), plasmin (1.1 mg/ml), or trypsin (320 μ g/ml) for 10 min at 37°C. The mixtures were analyzed after the addition of an SDS-urea solution. The native C \bar{I} inactivator with buffer, plasmin, or trypsin was applied to gels a, c, and e; and the denatured inhibitor with buffer or the enzymes to gels b, d, and f. "In III" indicates the 86,000 dalton C \bar{I} inactivator trypsin derivative. "In IV" and "In V" indicate the new C \bar{I} inactivator proteolytic derivatives resulting from the action of plasmin (IV) and trypsin (IV, V) on the acid-treated inhibitor (gels d and f). The gels were overloaded with sample in order to render the bands in gel f visible at comparable protein concentrations.

band I or trypsin-generated band II or III was observed under any of the concentrations examined.⁴ Further studies showed that the carbohydrate portion of the C \bar{I} inactivator molecule was retained in the trypsin derivative bands II and III as well as in the plasmin-derived band II material as documented by densitometric scans of PAS-stained SDS-acrylamide gels.

⁴ No complex formation between plasmin or trypsin and SBTI was apparent from inspection of SDS-acrylamide gels (Fig. 4, gel e; Figs. 6 and 7, gel d). This suggests that the bonds involved in their interactions are noncovalent as they are disrupted at neutral pH by the denaturing agents used for sample preparations (SDS and urea).

The trypsin-treated C \bar{I} inactivator preparations were also examined for their ability to form a complex with C \bar{I} s. Complex formation was partially inhibited with molar ratios as low as 0.025 (not shown) and totally inhibited at a molar ratio of 0.1 (Fig. 7, gels a-d). Trypsin-generated C \bar{I} inactivator band II was completely unable to form a complex with C \bar{I} s. Thus, it resembled the inactive C \bar{I} inactivator band II material generated by plasmin. Functional studies of ability of trypsin-treated C \bar{I} inactivator to inhibit C \bar{I} s-mediated destruction of C2 and C4 demonstrated that loss of functional inhibitor activity paralleled the loss of ability of C \bar{I} inactivator to form a complex with C \bar{I} s as analyzed by SDS-acrylamide gel electrophoresis.

Effect of acid treatment of C \bar{I} inactivator on its interaction with C \bar{I} s, plasmin, and trypsin (Fig. 8). Acid-treated C \bar{I} inactivator has been previously reported to be functionally inactive (10). Acid-treated C \bar{I} inactivator was examined for the ability to complex with plasmin and C \bar{I} s. After acidification to pH 2.0 for 30 min at 37°C followed by neutralization, C \bar{I} inactivator was incubated with the various enzymes. Acid treatment produced no evident alteration in the apparent molecular weight or proportion of bands I and II as assessed by SDS-polyacrylamide gel electrophoresis. The acidified inhibitor, however, was unable to form a complex with C \bar{I} s or plasmin (Fig. 8, gels a-d). Although C \bar{I} s did not alter the acid-treated C \bar{I} inactivator (as compared to gels containing each component alone) both plasmin and trypsin degraded C \bar{I} inactivator (Fig. 8, gels d and f), and produced a new plasmin derivative (IV) with an apparent molecular weight of 58,000 daltons, and the two new trypsin derivatives (IV and V) with apparent molecular weights of 58,000 and 42,500 daltons. The relative faintness of the bands which were apparent after the reaction of trypsin with acid-treated inhibitor (Fig. 8, gel f) suggested that a considerable portion of the inhibitor molecule had been hydrolyzed to produce lower molecular weight products not identifiable by the method used.

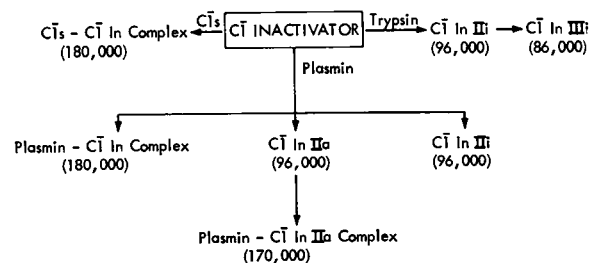


FIGURE 9 Schematic representation of the interactions between C \bar{I} inactivator (C \bar{I} In) and C \bar{I} s, plasmin, and trypsin. The explanation for the nomenclature used is found in footnote 5. The apparent molecular weights of the complexes and of the C \bar{I} inactivator derivatives are indicated in parentheses.

Studies of radiolabeled C \bar{I} inactivator-enzyme interactions were undertaken in an attempt to identify the low molecular weight products cleaved from the inhibitor by plasmin and trypsin. The theoretical molecular weight of these products would represent, for example, the difference between plasmin-derived band II and native band I, or approximately 9,000 daltons. Incubation mixtures of ^{125}I -labeled C \bar{I} inactivator and unlabeled C \bar{I} s, plasmin, or trypsin were analyzed by SDS-acrylamide gel electrophoresis. After the electrophoretic procedure, the gels were sliced and the segments counted or the whole gel was dried and autoradiography performed (45). These studies, although confirming that the inhibitor was incorporated into higher molecular weight complexes, revealed no evidence for radioactive fragments having molecular weights of 9,000 daltons or lower.

DISCUSSION

Acrylamide gel electrophoresis in the presence of SDS has made it possible to define more precisely the structure of C \bar{I} inactivator and the nature of the interactions which occur between C \bar{I} inactivator and several of the plasma enzymes which it inhibits. These reactions are outlined in Fig. 9, and depending on the enzyme, were characterized by the formation of stable stoichiometric complexes as well as by the production of both active and inactive inhibitor derivatives. The native structure of the inhibitor appeared to be required both for complex formation with C \bar{I} s and plasmin as well as for limiting the hydrolytic attack of plasmin and trypsin, since after acid denaturing C \bar{I} inactivator did not bind C \bar{I} s or plasmin and new regions of the molecule became susceptible to plasmin- and trypsin-induced degradation.

All purified C \bar{I} inactivator preparations demonstrated two bands when examined by SDS-acrylamide gel electrophoresis and each of these components appeared to consist of a single polypeptide chain since their apparent molecular weight was not altered by disulfide bond cleavage. The molecular weights of the major and minor bands were 105,000 and 96,000 daltons, respectively, confirming for the major component the molecular weight estimates previously determined for C \bar{I} inactivator by sedimentation equilibrium ultracentrifugation (19). The lower molecular weight, minor component of the purified C \bar{I} inactivator preparation was chemically, immunologically, and functionally similar to the major protein band. Both components stained intensely with PAS, a finding consistent with the 35% carbohydrate content previously reported for the inhibitor (19), and densitometric scans of the stained gels indicated that both bands had a similar carbohydrate to protein ratio. Further, both bands of the inhibitor preparation displayed a reaction of im-

munologic identity and, in addition, formed a stable complex with C \bar{I} s, providing support for their functional similarity as well. Although purification procedures were performed by methods designed to minimize the activation and subsequent action of plasma proteolytic enzymes, the data do not permit us to conclude whether this lower molecular weight C \bar{I} inactivator-related protein represented a circulating form of the inhibitor, a catabolic intermediate, or a proteolytic derivative produced during the isolation procedures.

C \bar{I} inactivator formed a complex with both C \bar{I} s and plasmin which remained intact in the presence of the denaturing agents urea and SDS. The molecular weight of the complex reflected the sum of the individual chains providing direct evidence for a 1:1 molar binding ratio between the inhibitor and these enzymes, a finding extending functional (12) and ultracentrifugal studies (46) which indicated a stoichiometric relationship between C \bar{I} s and its inhibitor. Electrophoretic analysis on SDS-acrylamide gels of mixtures containing equimolar concentrations of enzyme and inhibitor, after reductive cleavage of disulfide bonds by DTT, showed that the heavy chain of the enzyme had separated from the complex, however, no band occupying the position of the light chain could be identified. Further, a new band had appeared which possessed a molecular weight equal to the sum of the weights of the inhibitor and the light chain of the enzyme. Thus, the binding site(s) of these enzymes for C \bar{I} inactivator was found to be located on the subunit chain which contained the active site of C \bar{I} s (47) and plasmin (48). Functional studies of the proteolytic activity of these enzymes after incubation with C \bar{I} inactivator confirmed that complex formation, as documented by electrophoretic analysis, was associated with inhibition of enzymic activity.

In addition to complex formation, the interaction of C \bar{I} inactivator with plasmin resulted in proteolytic degradation of the inhibitor (as outlined in the schema shown in Fig. 9). Plasmin produced a lower molecular weight C \bar{I} inactivator derivative (IIa),⁵ which participated in complex formation with plasmin. Increasing concentrations of plasmin led to the formation of an inactive derivative (IIi)⁵ incapable of forming such complexes. The decrease in C \bar{I} s-inhibiting active of plasmin-inhibitor mixtures as documented by a functional assay (Fig. 5) paralleled the development of the

⁵ The 96,000-dalton derivative produced by interaction of plasmin with C \bar{I} inactivator and which forms a complex with plasmin has been designated band "IIa" for the "active" product. The derivative with similar mobility in SDS-acrylamide gels which was produced by plasmin was designated band "IIi" for the "inactive" product. Similarly, the trypsin-induced C \bar{I} inactivator derivative with an apparent molecular weight of 86,000 which was inactive in complex formation was designated band "IIIi."

plasmin-inhibitor complex as well as of the III derivative. Therefore, both complex formation with blockage of C \bar{I} s binding sites and proteolytic degradation of the inhibitor contributed to the previously described destruction of C \bar{I} inactivator's inhibiting activity by plasmin (44).

Trypsin, which did not form a complex with C \bar{I} inactivator, cleaved the inhibitor molecule thereby producing two identifiable derivative chains with apparent molecular weights of 96,000 and 86,000 daltons as indicated in Fig. 9. These products failed to bind C \bar{I} s or inhibit its functional activity. Trypsin derivative III resembled plasmin derivative III by having a similar mobility in SDS-acrylamide gels and in being functionally inactive. Trypsin derivative III, on the other hand, had no plasmin counterpart, indicating that the region of the C \bar{I} inactivator molecule attacked by trypsin to produce this product was not susceptible to the action of plasmin. Its appearance following the total degradation of the C \bar{I} inactivator band I by trypsin suggested that the peptide bond(s) hydrolyzed to yield peptide chain III was protected from the action of trypsin in the intact molecule. Peptides released from C \bar{I} inactivator during the protease-induced conversion of band I to bands II and III were not visualized on the stained gels, nor were they identified in the gel slices in experiments using ^{125}I -labeled inhibitor. Their absence may be explained by the failure of these products to stain or to radiolabel, or, alternatively, by the further degradation of the peptides by the proteases to small peptide fragments not appreciated by the methods used.

These studies have illuminated some of the structural requirements necessary for complex formation between C \bar{I} inactivator and C \bar{I} s. The IIa derivative produced by interaction of plasmin and the inhibitor retained the capacity to form a complex with C \bar{I} s and to inhibit its activity, indicating that the loss of a 9,000-dalton portion of the native molecule did not affect its inhibitory function. In contrast, the trypsin- and plasmin-produced III derivative possessed an apparent molecular weight similar to that of the IIa product, but it had lost its inhibitory activity. It is likely that trypsin as well as the higher concentrations of plasmin used had attacked an additional region in the inhibitor molecule releasing a small peptide, and producing a molecular weight change in the parent chain too small to be appreciated by SDS-acrylamide gel electrophoresis. Although it is possible that the released peptide contained a critical binding site for the enzyme, it is more likely that the additional cleavage altered the conformation of the molecule and thereby destroyed its function. This concept is supported by data which demonstrated that acid treatment

of C \bar{I} inactivator, a procedure which altered its native conformation, rendered it functionally inactive (Fig. 8).

The role of peptide bond cleavage in the interaction between the proteolytic enzymes and their naturally occurring inhibitors has not been established. From his studies of the trypsin-SBTI reaction, as well as of other inhibitors of plant origin, Laskowski (49) has concluded that proteases hydrolyze peptide bonds in their inhibitors as a requirement for complex formation and enzymic inhibition. In the case of the trypsin-SBTI reaction, he postulated that cleavage of the inhibitor by trypsin resulted in the formation of a covalent acyl enzyme intermediate between the enzyme and the inhibitor. In contrast, Feeney (50) suggested that the strength of binding between enzyme and its inhibitor is secondary to the summation of many hydrophobic and electrostatic interactions. In this theory, proteolysis of the inhibitor is a coincidental event. Data supporting this assumption has been provided by Ako, Foster, and Ryan (51) who demonstrated complex formation between an enzymically inactive trypsin derivative and SBTI with an energy of inhibitor binding similar to that established for active trypsin.

Additional evidence suggesting that complex formation between an enzyme and its inhibitor, active site inhibition, and inhibitor hydrolysis may be unrelated events was provided by studies that demonstrated that formation of a complex between α_2 -macroglobulin and trypsin, thrombin, plasmin, and plasma kallikrein was accompanied by limited proteolysis of the α_2 -macroglobulin molecule (8). The active site of the enzyme in the complex, however, retained the capacity to hydrolyze synthetic amino acid esters, and in the case of the α_2 -macroglobulin-plasmin complex, retained fibrinogenolytic activity (52). In the present investigation, derivatives were not identified after the interaction of C \bar{I} s and C \bar{I} inactivator. Furthermore, acid-denatured C \bar{I} inactivator resisted proteolytic attack by this enzyme suggesting that C \bar{I} inactivator was not a substrate for C \bar{I} s and consequently that cleavage was not a requirement for complex formation.

In summary, in this study a variety of molecular interactions occurring between C \bar{I} s, plasmin, trypsin, and C \bar{I} inactivator, a circulating inhibitor of components of the complement, coagulation, fibrinolytic, and kallikrein enzyme systems have been examined. A spectrum of reactions was identified and included the formation of stable complexes, as well as of proteolytic cleavage of the inhibitor yielding functionally active lower molecular weight inhibitor derivatives. In addition, proteolysis also resulted in the production of inactive inhibitor derivatives. These interactions may serve as a model for the study of mechanisms of action of the other plasma enzyme inhibitors.

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