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ON THE INTERACTION OF α_2-PLASMIN INHIBITOR AND PROTEASES

EVIDENCE FOR THE FORMATION OF A COVALENT CROSSLINKAGE AND NON-COVALENT WEAK BONDINGS BETWEEN THE INHIBITOR AND PROTEASES

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Summary

α_2-plasmin inhibitor is a proteinase inhibitor in plasma which efficiently inhibits the lysis of fibrin clots induced by plasminogen activator. The nature of the binding of the inhibitor to trypsin or plasmin was studied by the chemical treatment of the enzyme-inhibitor complex with 7.5 M hydrazine at pH 10.0. With the hydrazine treatment, the complexes were degraded to proteins corresponding to the respective enzyme and inhibitor moieties. These results indicate that the covalent bond between the inhibitor and the enzymes is a carboxylic ester.

The binding reaction of the inhibitor to active site-modified trypsin was also studied. The inhibitor formed complexes with anhydrotrypsin and carboxyamidomethylated trypsin. The complexes were dissociated in the presence of 1% sodium dodecyl sulfate, to the individual components: the respective enzyme and inhibitor moieties. The inhibitor, however, did not form a complex with diisopropylphosphorylated trypsin regardless of the presence or absence of the denaturing reagent. These results suggest the contribution of non-covalent interactions to the complex formation between the inhibitor and native enzymes.

Introduction

α_2-plasmin inhibitot is a potent plasmin inhibitor which was recently purified from human plasma [1]. The inhibitor had been referred to as antiactiva-

Abbreviations: TosLysCH$_2$Cl, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; iPr$_2$*P*-F, diisopropylphosphorofluoridate; iPr$_2$*P*-trypsin, diisopropylphosphorylated trypsin.

tor [2] because of its strong inhibitory activity on activator-induced clot lysis. However, using the purified inhibitor, the inhibitor was found to exert its inhibitory effect on activator-induced clot lysis mainly through a mechanism of instantaneous inhibition of plasmin formed and not through the inhibition of urokinase [1]. The inhibitor is the most rapidly functioning inhibitor of plasmin in plasma [3] and is considered to play an important role in the regulation of fibrinolysis. Collen and coworkers [4] and Müllertz [5]reported the complex formation of plasmin with a hitherto unidentified antiplasmin in addition to plasmin-α_2-macroglobulin complex. The unidentified antiplasmin might be identical with α_2-plasmin inhibitor.

There have been some reports concerning a chemical nature characterizing the reaction of proteases and plasma inhibitors. α_1-antitrypsin and trypsin [6] or antithrombin III and thrombin [7,8] form a complex which cannot be dissociated by treatment with denaturing and reducing agents. Moreover, both the complexes were dissociated to the respective enzyme and inhibitor moieties with the treatment of nucleophilic agents. These findings suggest that an acyl bond was formed between the inhibitor and the enzyme.

In the present study, the reaction between α_2-plasmin inhibitor and modified trypsin, and the effect of a nucleophilic agent on the enzyme-inhibitor complex were investigated. The result showed the similarity as well as some dissimilarity of α_2-plasmin inhibitor and other plasma inhibitors, such as α_1-antitrypsin or antithrombin III, with regard to their reactions with proteinase.

Materials

Human α_2-plasmin inhibitor was prepared from human plasma by the method previously described [1]. Human plasminogen was purified from plasma by affinity chromatography using L-lysine-coupled Sepharose 4B [9] and activated with urokinase-coupled Sepharose as previously described [1]. Thrice crystallized bovine trypsin was obtained from Worthington Biochemical Corp.

Anhydrotrypsin was prepared by the method of Ako et al. [10,11] as follows. Phenylmethanesulfonyl trypsin was prepared following the method of Gold [12] and further treated with 0.05 M KOH at 0° C for 20 min. After inactivation of regenerated trypsin by treatment of *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TosLysCH$_2$Cl) (Sigma Chemical Co.), anhydrotrypsin formed was adsorbed to a soybean trypsin inhibitor-coupled Sepharose 4B column and eluted with 0.1 M acetic acid [13]. The agarose derivative of anhydrotrypsin was prepared by the CNBr procedure [14] as previously described [13].

The alkylphosphorylated trypsin (iPr_2*P*-trypsin) was prepared by incubating trypsin (20 mg) and iPr_2*P*-F (10 μl) in 0.05 M Tris · HCl/0.15 M NaCl/10 mM $CaCl_2$, pH 7.4, at 28° C. After 40 min of incubation, the esterolytic activity of the mixture was decreased to less than 0.5%. Subsequently, the solution was dialyzed against 0.01 M Tris · HCl, pH 7.4, to remove the residual reactants.

His-46 of trypsin was carboxyamidomethylated with iodoacetamide in the presence of methylguanidine according to the method of Kasai and Ishii [15]. Trypsin (3 mg/ml) was incubated in 0.4 M methylguanidine hydrochloride/0.6 M iodoacetamide/0.1 M $CaCl_2$ solution (pH 7.0) at room temperature for 48 h. After dialysis against 1 mM HCl, unmodified active trypsin remaining was inac-

tivated with $TosLysCH_2Cl$ at pH 7.5. The $TosLysCH_2Cl$-treated solution was applied to a column of soybean trypsin inhibitor-coupled Sepharose equilibrated with 0.05 M Tris · HCl/0.15 M NaCl/10 mM $CaCl_2$, pH 7.4. The column was washed with the same buffer. Carboxyamidomethyl trypsin bound to the column was then eluted with 0.1 M acetic acid. The eluate was concentrated by lyophilization. The material was dissolved in 0.05 M Tris · HCl/0.15 M NaCl/10 mM $CaCl_2$, pH 7.4, to obtain a proper concentration and dialyzed against the same buffered saline. The carboxyamidomethyl trypsin preparation thus obtained (the activity was less than 1% of that of native trypsin) was further treated with $TosLysCH_2Cl$ to inactivate any native trypsin remaining.

Methods

Polyacrylamide gel disc electrophoresis was carried out at pH 8.9 with 7.5% gels as described by Davis [16] and sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed with 5% polyacrylamide gels according to the method of Weber and Osborn [17].

α_2-plasmin inhibitor was assayed by the clot-lysis method described previously [1]. The inhibitor activity of each effluent fraction of chromatography was expressed simply as the lysis time obtained with a fixed amount of the test sample.

Protein concentrations were calculated using the following $E^{1\%}_{1\,cm}$ (280 nm) values: trypsin, 15.4 [18]; plasmin, 17.0 [19]; α_2-plasmin inhibitor, 7.03 [1].

Procedures for hydrazine treatment

The complex of the inhibitor and enzyme was allowed to react with the nucleophilic reagent, hydrazine, by the method previously described [6]. The inhibitor (61 μg) was mixed with β-trypsin (21 μg) or plasmin (59 μg) in an ice bath to form the complex, followed by addition of equal volume of 2% sodium dodecyl sulfate solution. After incubation at 55°C for 30 min, 10 M hydrazine hydrochloride solution, pH 10.0, was added to yield a final hydrazine concentration of 7.5 M. The mixture was incubated for 1 h at 37°C and then dialyzed against 1% ammonium carbonate/0.05% sodium dodecyl sulfate solution. The dialyzed solution was lyophilized. The lyophilized material was dissolved in deionized water to obtain a proper concentration and subjected to sodium dodecyl sulfate gel electrophoresis after prior reduction with 2% β-mercaptoethanol. As a control experiment, the mixture of the inhibitor and enzyme was treated similarly as above but without the treatment with hydrazine.

Results

Reaction of the complex with hydrazine.

The complex formed between α_2-plasmin inhibitor and trypsin or plasmin was allowed to react with hydrazine. The gel patterns of electrophoresis before and after the hydrazine treatment were compared (Fig. 1). It was found that the bands with a molecular weight 80 000 corresponding to the complexes with plasmin B chain or with trypsin disappeared and bands with molecular weights of 55 000, 40 000 and about 25 000 appeared after the treatment. Bands with

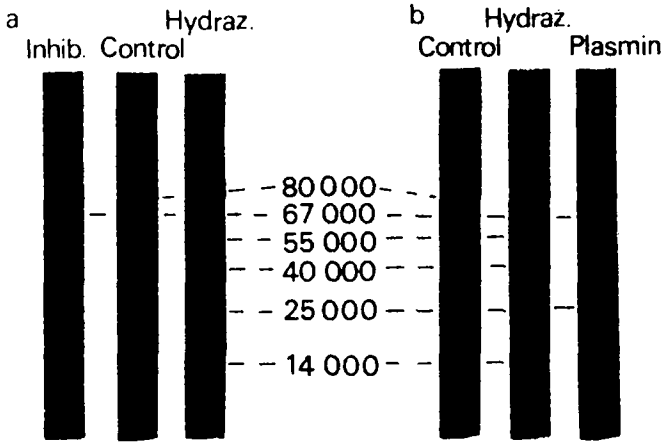

Fig. 1. Effect of hydrazine on the complex of α_2-plasmin inhibitor and trypsin (a) and of the inhibitor and plasmin (b). Hydrazine (Hydraz) and Control indicate the gel patterns of the reaction mixture after the hydrazine treatment and those of the control experiments without the hydrazine treatment, respectively. Plasmin and Inhib. indicate the gel patterns of plasmin and the inhibitor, respectively. See text for details.

a molecular weight of about 25 000 in the both gels correspond to B chain of plasmin and β-trypsin which have been cleaved out from the complex by hydrazine. The species of molecular weight 55 000 seems to be an another constituent in the complex and is presumably derived from the inhibitor moiety. The faint band of molecular weight 40 000 appeared after the hydrazine treatment seems to be also derived from the inhibitor moiety of the complex which has been partially degraded by free enzyme. The partially degraded complex with a molecular weight of 67 000 was previously identified [1]. A protein band with a molecular weight 14 000 was observed in the gel patterns of the reaction mixtures before and after the hydrazine treatment. These changes of gel patterns suggest that the complex of the inhibitor and the enzyme was degraded by the treatment with hydrazine to the protein with molecular weight of 55 000 and the constituent enzyme moiety.

Reaction of α_2-plasmin inhibitor with anhydrotrypsin

α_2-plasmin inhibitor was allowed to react with anhydrotrypsin and with native trypsin, and the mixtures were subjected to polyacrylamide disc gel electrophoresis in the presence and absence of sodium dodecyl sulfate. In the absence of sodium dodecyl sulfate, the mixture of the inhibitor and anhydrotrypsin formed a distinct band at the same position as that of a band corresponding to a complex of the inhibitor and native trypsin (Panel a in Fig. 2). On the other hand, no band corresponding to the complex was observed when the mixture of the inhibitor and anhydrotrypsin was examined in the presence of sodium dodecyl sulfate (Panel b in Fig. 2). These results indicate that the inhibitor and anhydrotrypsin formed a dissociable complex, which was also confirmed by the following experiment.

Solution of the inhibitor was applied on a column of anhydrotrypsin-coupled Sepharose 4B. After washing the column with 0.1 M sodium phosphate buffer, pH 7.6, the protein was eluted with the buffer containing 0.2 M ϵ-amino-

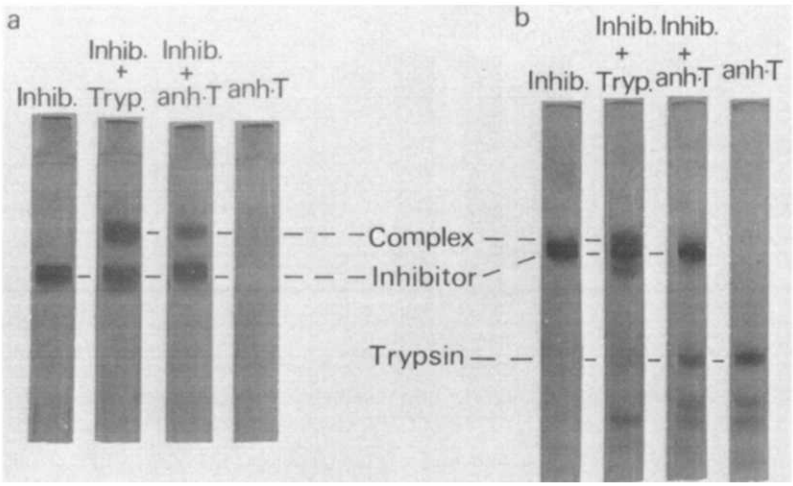

Fig. 2. Gel electrophoretic patterns of α_2-plasmin inhibitor, anhydrotrypsin and their mixture. a. Polyacrylamide disc gel electrophoresis at pH 8.9. b. Sodium dodecyl sulfate gel electrophoresis. α_2-plasmin inhibitor (39.6 μg) and anhydrotrypsin (23.6 μg) were mixed in 0.05 M Tris · HCl/0.15 M NaCl/5 mM $CaCl_2$ (pH 7.4). The reaction mixture was divided into 2 parts. One part was subjected to gel electrophoresis at pH 8.9, and another part was subjected to sodium dodecyl sulfate-containing gel electrophoresis after denaturing by 1% sodium dodecyl sulfate and reduction by 4% β-mercatoethanol. The inhibitor (39.6 μg) and native trypsin (10.5 μg) were treated in the same manner. Inhib., Tryp., and anhy.T indicate the inhibitor, native trypsin and anhydrotrypsin, respectively.

caproic acid (Fig. 3). As the presence of ε-aminocaproic acid interferes the assay of the inhibitor activity, the combined eluate (Fractions 16—20) was dialyzed against 0.01 M sodium phosphate-1 mM β-mercaptoethanol, pH 7.0 to remove ε-aminocaproic acid. Subsequently, the inhibitor activity of the dialyzed eluate was examined. 5, 10 and 20 μl of the eluate inhibited clot lysis and prolonged the lysis time from the control value of 10.5 min to 13, 16 and 25 min, respectively. However, 20 μl of each fraction under the break-through protein peak did not prolong the lysis time at all and fractions after the break-through protein peak prolonged the lysis time only a little, which may indicate the leakage of a small amount of the inhibitor from the column (Fig. 3).

Complex formation of the inhibitor with iPr_2P-trypsin

The complex formation of the inhibitor and iPr_2P-trypsin was studied. The inhibitor and iPr_2P-trypsin at several concentrations in 0.05 M Tris · HCl/0.15 M NaCl, pH 7.4, were mixed and incubated for 20 min at 37°C. The mixtures were subsequently subjected to polyacrylamide disc gel electrophoresis at pH 8.9. A mixture of the inhibitor and native trypsin was treated similarly, as a control. Fig. 4a shows the gel patterns obtained. The mixture of the inhibitor and trypsin gave an additional distinct band which corresponds to the enzyme-inhibitor complex. However, the mixture of the inhibitor and iPr_2P-trypsin did not give any additional band, indicating that the inhibitor and iPr_2P-trypsin does not form the complex.

Complex formation of the inhibitor with carboxyamidomethyl trypsin

Complex formation of α_2-plasmin inhibitor with carboxyamidomethyl tryp-

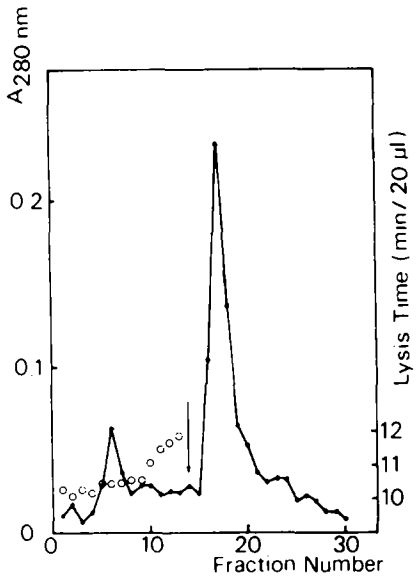

Fig. 3. Affinity chromatography of α_2-plasmin inhibitor on an anhydrotrypsin-Sepharose column. The inhibitor (1.4 mg) in 0.05 M Tris · HCl/0.15 M NaCl/1 mM β-mercatoethanol, pH 7.4, was applied to the column (1.4 × 3.2 cm) equilibrated with 0.1 M sodium phosphate, pH 7.6. The column was washed with the same phosphate buffer and, at the point indicated by the arrow, elution was started with the phosphate buffer containing 0.2 M ϵ-aminocaproic acid. Fractions of 1.1 ml were collected. Inhibitor activity and absorbance were represented by ○ and ●, respectively. The inhibitor activity was expressed directly as the clot lysis time obtained with 20 µl of the test sample.

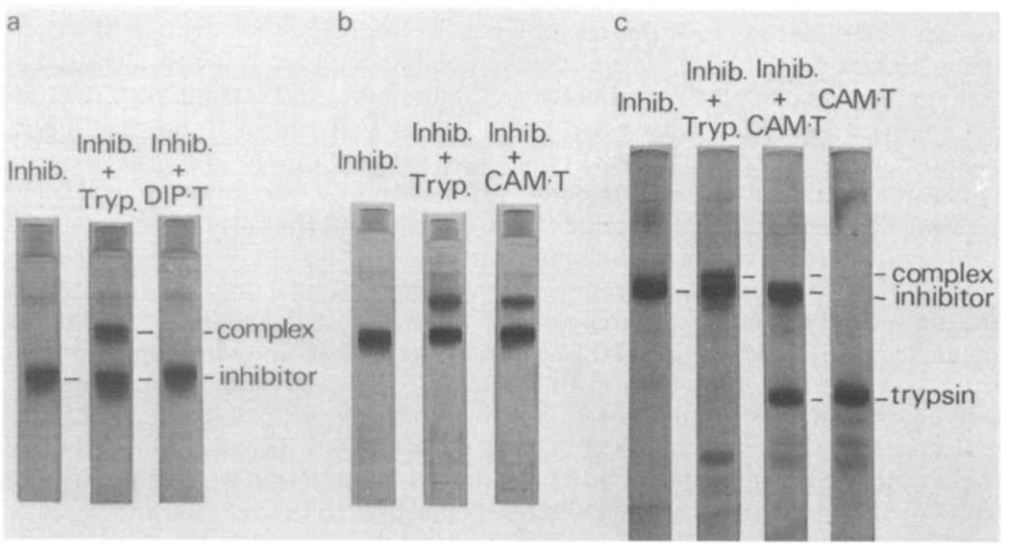

Fig. 4. a; Disc gel electrophoresis of α_2-plasmin inhibitor, iPr_2P-trypsin and their mixture. The inhibitor (15.2 µg) and iPr_2P-trypsin (6.8 µg to 27.2 µg) were mixed in 0.05 M Tris · HCl/0.15 M NaCl/5 mM $CaCl_2$, pH 7.4, and subjected to gel electrophoresis at pH 8.9. The inhibitor (15.2 µg) and native trypsin (3.5 µg) were treated by the same way as a control. b,c; Gel electrophoresis of the inhibitor, carboxyamidomethyl trypsin and their mixture in the absence (b) and presence (c) of sodium dodecyl sulfate. The inhibitor (19.8 µg) was mixed with carboxyamidomethyl trypsin (9.6 µg) in 0.05 M Tris · HCl/0.15 M NaCl/5 mM $CaCl_2$, pH 7.4, and subjected to electrophoresis. The inhibitor (19.8 µg) and native trypsin (3.5 µg), were treated by the same way as a control. Inhib.: the inhibitor, Tryp.: native trypsin, DIP.T: iPr_2P-trypsin, CAM.T: carboxyamidomethyl trypsin.

sin was also studied. The reaction was analyzed similarly to the reaction with anhydrotrypsin by polyacrylamide gel electrophoresis at pH 8.9 and sodium dodecyl sulfate polyacrylamide gel electrophoresis. The complex of the inhibitor and carboxyamidomethyltrypsin was detected on gels without sodium dodecyl sulfate (Fig. 4b) but not on gels containing sodium dodecyl sulfate (Fig. 4c). These indicate the formation of the dissociable complex of the inhibitor and carboxyamidomethyl trypsin similarly to that of the inhibitor and anhydrotrypsin.

Discussion

α_2-plasmin inhibitor is known to form an undissociable complex with plasmin, trypsin or urokinase [1]. Such complex formation through a covalent cross-linkage was also found to occur in the reaction of other plasma inhibitors. such as α_1-antitrypsin [6], antithrombin III [7] and Cl-esterase inhibitor [20] with proteases. It was found that the crosslinking was formed between α_1-antitrypsin and active site-containing B chain of α-trypsin and the cross-linking could be cleaved by treatment with hydrazine [6]. Similar results were also obtained in the studies on antithrombin III [7,8]. Moreover, α_1-antitrypsin did not form a complex with trypsin when Ser-183 of trypsin had been modified [13]. Consequently, it was suggested that an acyl bond was formed between the carbonyl carbon of the inhibitor and the γ-oxygen of Ser-183 of the enzyme.

In the present study the complex of α_2-plasmin inhibitor and plasmin or trypsin was dissociated by the treatment with hydrazine to a protein of a molecular weight 55 000 and the constituent enzyme moiety. These results suggest that the covalent bond formed between the inhibitor and plasmin or trypsin in the complex formation is an ester, and a similar mechanism to the reaction of α_1-antitrypsin and trypsin [6,13] could be proposed for the reaction between α_2-plasmin inhibitor and the enzymes: at the complex formation an acyl bond is formed between the active serine of the enzyme and the carbonyl carbon of a residue at the reactive site of the inhibitor.

On the other hand, carboxyamidomethyl trypsin and anhydrotrypsin which are catalytically inactive were reported to retain the ability to bind to substrate analogues [15]. Ako et al. [10,11] also showed that anhydrotrypsin bound strongly to various protein trypsin inhibitors of non-plasma origin. However, anhydrotrypsin was found not to form a complex with α_1-antitrypsin. This indicates that the active serine of trypsin plays an essential role in the complex formation with α_1-antitrypsin [13]. Unlike in the reaction of anhydrotrypsin and α_1-antitrypsin, α_2-plasmin inhibitor was found to form a dissociable complex with anhydrotrypsin and with carboxyamidomethyl trypsin. But α_2-plasmin inhibitor did not form a complex with iPr_2P-trypsin, which may be attributed to the steric hindrance caused by the bulky isopropylphosphoryl group. Since anhydrotrypsin and carboxyamidomethyl trypsin can be considered to be structurally similar to the native enzyme, the results in the present study suggest that the binding of α_2-plasmin inhibitor to trypsin is partly due to a sum of weak forces in a complementary fit between the inhibitor and the enzyme in addition to a covalent linkage at the active serine of the enzyme.

As for the inhibition reaction of plasma inhibitors, such as α_1-antitrypsin [6,13], antithrombin III [7,8], Cl-esterase inhibitor [20] and α_2-plasmin inhibitor, the following mechanism could be deduced from the present and published data:

$$I + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} IE \overset{k_2}{\rightarrow} C$$

where I, E, IE and C represent the inhibitors, proteolytic enzymes, the complex not covalently crosslinked, and the complex crosslinked by a covalent (acyl) bond, respectively. In this hypothetical mechanism, the dissociable Michaelis complex IE is considered to be a complex which is enzymatically inactive if not dissociated, since the active site of an enzyme is enveloped by the inhibitor in the complex. However, this dissociable complex is unstable. Therefore, the most efficient step of the inhibition reaction by plasma inhibitors is the step forming a covalently cross-linked complex C. In the reaction of α_1-antitrypsin and trypsin $K_s = k_{-1}/k_1$ and k_2 must be relatively large, since α_1-antitrypsin inhibits trypsin rapidly by forming a complex but the inhibitor did not form a complex with anhydrotrypsin [6,13]. In the reaction of α_2-plasmin inhibitor and trypsin or, presumably, plasmin, K_s must be small and k_2 large, since the enzymes were instantaneously inhibited and the inhibitor formed a complex with anhydrotrypsin or carboxyamidomethyl trypsin. In the slow inhibition, such as those of plasmin by α_1-antitrypsin [21], thrombin by antithrombin III [7], and urokinase by α_2-plasmin inhibitor, K_s must be relatively large and k_2 small, since the complex C is slowly formed. The low K_s value in the reaction of α_2-plasmin inhibitor and the enzymes implies that the inhibitor could inhibit the enzymes also in a competitive manner in the presence of the substrates. This would be the reason why α_2-plasmin inhibitor inhibits instantaneously enzymes even in the presence of substrate, which is the case of the inhibition of activator-induced clot lysis.

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