PLASMIN INHIBITOR INTERACTIONS

The Effectiveness of α_2-Plasmin Inhibitor in the Presence of α_2-Macroglobulin*

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Plasmin is inhibited in vitro by several plasma protease inhibitors including α_2-macroglobulin (1-3), α_1-antitrypsin (1, 4, 5), $C\bar{1}$ inactivator (6, 7), and antithrombin-heparin cofactor (8). When these inhibitors are compared in purified systems, α_2-macroglobulin appears to be the dominant inhibitor.[1, 2] It is also an important inhibitor in vivo because when plasminogen activators are infused, plasmin-α_2-macroglobulin complexes have been identified (9). Nevertheless, neither the role of plasma protease inhibitors in modulating intravascular proteolytic events is established, nor is it clear whether the entire spectrum of possible inhibitors has been fully defined.

For example, a newly recognized plasmin inhibitor, termed α_2-plasmin inhibitor, has been shown to inhibit plasmin rapidly (10), thus adding a new aspect to considerations of the regulation of plasmin activity. In this communication, we present experiments which detail the interactions between α_2-plasmin inhibitor and α_2-macroglobulin in competing for plasmin.

Materials and Methods

Chemicals. All chemicals were reagent grade.

Plasma. Plasma for the purification of α_2-macroglobulin, α_2-plasmin inhibitor, and plasminogen was obtained from fresh whole blood collected in acid-citrate-dextrose anticoagulant.

Antisera. Rabbit antisera produced against the following human proteins were obtained from Behring Diagnostics, American Hoechst Corp., Somerville, N. J.: α_1-antitrypsin, $C\bar{1}$ inactivator, antithrombin-heparin cofactor, inter-α-trypsin inhibitor, and chymotrypsin inhibitor.

Plasminogen. Plasminogen from human plasma was purified by insoluble-lysine affinity chromatography as described by Deutsch and Mertz (11), followed by gel filtration chromatography (Bio-Gel A-0.5 m, Bio-Rad Laboratories, Richmond, Calif.). The final preparation possessed a specific activity of 20 CTA (Committee on Thrombolytic Agents) U/mg as assayed by a standard caseinolytic assay (12). Plasminogen was labeled with ^{125}I by the technique of McConahey and Dixon (13). Chloramine-T, 0.3 ml of a 100-μg/ml solution, was added to plasminogen, 1.3 mg in 0.5 ml 0.15 M phosphate buffer, pH 7.4, containing 1 mCi of carrier-free ^{125}I-sodium iodide. After a 5-min incubation at 0°C, 0.3 ml sodium metabisulfite (100 μg/ml) was added. The unbound sodium iodide was removed by gel filtration chromatography. Labeling occurred at 0.4 μCi/μg plasminogen.

Native or ^{125}I-labeled plasminogen was activated at 25°C by incubation in a 25% glycerol solution containing 0.04 M Tris-0.016 M lysine-0.08 M NaCl at pH 9.0 (14) with 500 Abbot U of

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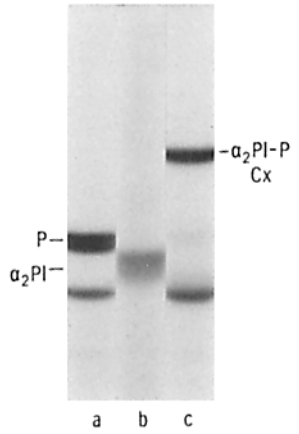

FIG. 1. Interaction of plasmin with α_2-plasmin inhibitor. Plasmin (P, 2.5 μg), gel a, and α_2-plasmin inhibitor (α_2-PI; 2.0 μg), gel b, were incubated together, gel c, for 2 min at 37°C. After incubation, these mixtures were analyzed in the absence of reduction, by electrophoresis on 5% SDS-acrylamide gels. The anode is toward the bottom of the figure. The location of the inhibitor-plasmin complex is indicated (α_2PI-P Cx).

urokinase/mg zymogen. Conversion to plasmin was documented by sodium dodecyl sulfate (SDS)[3] gel electrophoretic analysis of reduced samples of the plasminogen-urokinase incubation mixture which demonstrated complete conversion of the plasminogen band to the heavy and light chain of plasmin. Active site titration using *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride (*p*-NPGB) was performed as described by Chase and Shaw (15).

Human α_2-Macroglobulin. Human α_2-macroglobulin was prepared as previously described (16). The final product migrated as a single protein band after reduction as analyzed by SDS-acrylamide gel electrophoresis. Concentrations of α_2-macroglobulin were established by radial immunodiffusion (Immuno Plate, Hyland Div. Travenol Laboratories, Inc., Costa Mesa, Calif.).

Human α_2-Plasmin Inhibitor. Human α_2-plasmin inhibitor was isolated from outdated blood bank plasma as described by Moroi and Aoki (10). Upon cellulose acetate electrophoresis, the final preparation demonstrated one band with α_2-mobility. Upon SDS-gel electrophoresis, the material displayed a major band with an apparent M_r = 67,000, consistent with that obtained by Moroi. A minor contaminant, M_r = 64,000, probably similar to that reported by Moroi, was also apparent (Fig. 1, gel b). Using specific antibodies and double-diffusion analysis, no detectable α_2-macroglobulin, $C\bar{1}$ inactivator, α_1-antitrypsin, antithrombin-heparin cofactor, inter-α-trypsin inhibitor, or chymotrypsin inhibitor were identified in the α_2-plasmin inhibitor preparation.

After incubation of α_2-plasmin inhibitor with plasmin, SDS-gel electrophoretic analysis of the unreduced mixture demonstrated a loss of the protein bands in the α_2-plasmin inhibitor and plasmin positions, and a new higher molecular weight band was identified (Fig. 1, gel c). The apparent M_r of this band was 142,000, consistent with the formation of a complex between plasmin (M_r = 75,000) and α_2-plasmin inhibitor. The lower molecular weight band in Fig. 1, gels a and c, was a component of the urokinase preparation added to activate plasminogen.

The concentration of functionally active plasmin inhibitor in the final α_2-plasmin inhibitor preparation was obtained by titration of a known concentration of plasmin with the inhibitor similar to the method described by Müllertz and Clemmensen (17). The inhibition of plasmin was found to be linearly related to the volume of inhibitor added to the incubation mixture containing plasmin and the substrate H-D-valyl-leucyl-lysine-*p*-nitroanilide. The concentration of active inhibitor was 101 μg/ml.

SDS-Polyacrylamide Gel Electrophoresis (5% gels). This was performed according to the method of Weber and Osborn (18). Samples for analysis were added to an equal volume of a solution containing 10 M urea and 2% SDS, and boiled for 5 min.

[3] *Abbreviations used in this paper*: M_r, molecular weight; *p*-NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride; SDS, sodium dodecyl sulfate.

Studies of the Binding of ^{125}I-Plasmin to α_2-Macroglobulin and α_2-Plasmin Inhibitor. ^{125}I-Plasmin was prepared by the activation of ^{125}I-plasminogen with urokinase as described above, and the activity was established by active site titration with *p*-NPGB. The experimental conditions in these binding studies used physiologic concentrations of α_2-macroglobulin (3.5 μM [19]) since it has been found that this inhibitor is less effective in binding plasmin in more dilute systems (unpublished observations). Since the α_2-plasmin inhibitor reacts rapidly with plasmin even at 0°C (10), all incubation mixtures containing the inhibitors were equilibrated at 37°C before the addition of plasmin. Plasmin was added to α_2-macroglobulin or to mixtures of α_2-macroglobulin and increasing concentrations of α_2-plasmin inhibitor. The concentrations of α_2-plasmin inhibitor used in these studies (0.06–0.75 μM) were less than those found for normal plasma (1.1 μM [10]). The reaction was terminated by the addition of the SDS-urea solution and boiling. After SDS-gel electrophoresis, the gels were sliced into 2-mm segments and counted in a Searle 1185 gamma counter (Searle Analytic Inc., Des Plaines, Ill.).

Amidolytic Assay. Standard plasmin curves were established using the substrate H-D-valyl-leucyl-lysine-*p*-nitroanilide (AB Bofors Nobel Division, Molndal, Sweden) using minor modifications of the methods detailed by the manufacturers. A constant concentration of plasmin was added to a constant concentration of α_2-macroglobulin or to a mixture of α_2-macroglobulin and increasing concentrations of α_2-plasmin inhibitor in the concentrations indicated in Table II. The final incubation volume was 1.2 ml and contained 330 μg substrate. After a 15-min incubation at 37°C, 30% acetic acid (0.3 ml), was added to stop the reaction, and the absorbance was recorded at 410 nm. Residual plasmin activity was established from the linear plasmin standard curves. α_2-Macroglobulin was found not to inhibit the amidolytic activity of plasmin since the residual activity of a mixture of α_2-macroglobulin and plasmin was similar to the activity expressed by plasmin alone.

Protein. Protein was measured by the Lowry procedure (20) with bovine serum albumin (Pentex Biochemical, Kankakee, Ill.) as the reference standard.

Results

The Interaction of ^{125}I-Plasmin with α_2-Macroglobulin and α_2-Plasmin Inhibitor as Analyzed by SDS-Acrylamide Gel Electrophoresis. To define the relative importance of purified α_2-macroglobulin and α_2-plasmin inhibitor as plasmin inhibitors, the amount of ^{125}I-plasmin bound to each in incubation mixtures of the two was determined. This proved feasible as preliminary studies established that complexes between radiolabeled plasmin and either inhibitor did not dissociate after electrophoresis in SDS gels. Further, the electrophoretic mobility of the plasmin, α_2-macroglobulin complex near the top of the gel[1] was sufficiently different from that of the plasmin, α_2-plasmin inhibitor complex (Fig. 1) to make it possible to separate these complexes in mixtures of inhibitors and plasmin. Thus, analysis of incubation mixtures of ^{125}I-plasmin, α_2-macroglobulin, and varying concentrations of α_2-plasmin inhibitor by SDS-acrylamide gel electrophoresis demonstrated the quantitative contribution of each in binding plasmin (Table I).

In the present studies, concentrations of plasmin were selected which were well below the previously established 1 to 1 M binding capacity of α_2-macroglobulin.[1] Under these conditions, comparison of the binding of ^{125}I-plasmin to mixtures of the inhibitors at 10 and 120 s of incubation demonstrated that the reactions were complete by 10 s since no shift of radioactivity between inhibitor-enzyme complexes was detected. As indicated in Table I, when a constant amount of plasmin (0.28 μM) was added to α_2-macroglobulin (3.5 μM) and increasing concentrations of α_2-plasmin inhibitor (0.06–0.75 μM), the α_2-plasmin inhibitor always bound nearly as much plasmin as it did in the absence of

TABLE I

The Binding of ^{125}I-Plasmin by α_2-Plasmin Inhibitor in the Presence or Absence of α_2-Macroglobulin: Effect of Varying the Concentration of α_2-Plasmin Inhibitor

Concentration α_2-PI	Incubation mixtures of ^{125}I-plasmin plus:		
	α_2M + α_2-PI*		α_2-PI‡
	^{125}I-plasmin bound to§		^{125}I-plasmin bound to
	α_2M	α_2-PI	α_2-PI
μM	%	%	%
0	46.6	–	–
0.06	41.3	5.9	0
0.13	36.9	11.7	11.0
0.38	12.7	29.9	32.9
0.75	5.4	38.0	40.6

* 5 μl ^{125}I-plasmin (0.28 μM final concentration) was added to a mixture of 10 μl α_2-macroglobulin (α_2M; 3.5 μM final concentration) and 5 μl buffer (0 μM α_2-PI) at 37°C, or to a mixture containing α_2M and 5 μl of the concentrations of α_2-plasmin inhibitor (α_2-PI) indicated in the table. For these concentrations of α_2-PI, the molar ratios relative to the concentration of α_2M (3.5 μM) are as follows: 0.06 μM = 0.02; 0.13 μM = 0.4; 0.38 μM = 0.11; 0.75 μM = 0.21.
After a 10-s incubation, a solution of SDS and urea was added to terminate the reaction. The mixtures were then analyzed by SDS-acrylamide gel electrophoresis, and 2-mm gel segments were counted in a gamma counter.

‡ 5 μl ^{125}I-plasmin (0.28 μM) was incubated with buffer (0 μM α_2-PI) or with varying concentrations of α_2-PI as indicated in the table and under the conditions described above. No α_2-macroglobulin was included in these mixtures.

§ Percent plasmin bound was calculated as the counts per minute associated with the ^{125}I-plasmin-α_2-macroglobulin complex or with the ^{125}I-plasmin-α_2-plasmin inhibitor complex divided by the sum of the cpm of these complexes plus the radioactivity of the unbound plasmin. All values are the mean of duplicate determinations.

α_2-macroglobulin. This effect was apparent in α_2-plasmin inhibitor to α_2-macroglobulin molar ratios as small as 0.02.

The amount of the radiolabeled plasmin which was identified in complexes with the inhibitors (46%) was in agreement with the results of active site titration of the plasmin preparation used, which demonstrated a 50% activity. For reasons which are poorly understood, in the mixture of enzyme and inhibitor with the lowest concentration of α_2-plasmin inhibitor (0.06 μM), no inhibitor-plasmin complex was identified after SDS-gel electrophoresis (three experiments), although binding to this concentration of inhibitor was detectable in the presence of α_2-macroglobulin.

In other experiments not shown in Table I, preincubation of α_2-macroglobulin (3.5 μM) with ^{125}I-plasmin for 2 min at 37°C, followed by the addition of α_2-plasmin inhibitor (0.75 μM) for a similar incubation period, demonstrated that all the active plasmin remained bound to α_2-macroglobulin and none became associated with the α_2-plasmin inhibitor. In an experiment reversing the addition sequence in which ^{125}I-plasmin was added to α_2-plasmin inhibitor first followed by addition of α_2-macroglobulin, plasmin was identified in the α_2-plasmin inhibitor, plasmin complex, and none became associated with α_2-

macroglobulin. Thus, we conclude that after initial binding, there is no transfer of enzyme from one inhibitor to the other.

The Binding of Plasmin to α_2-Macroglobulin in the Presence of α_2-Plasmin Inhibitor as Determined by an Amidolytic Assay. To test for the binding of unlabeled, native plasmin to α_2-macroglobulin in the presence of varying concentrations of α_2-plasmin inhibitor, a second technique was utilized. This method of analysis was made possible by the observation that the hydrolytic activity of plasmin against low molecular weight substrates was preserved after complex formation with α_2-macroglobulin (3, 21). We first established that the hydrolysis of the chromogenic synthetic peptide H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide by plasmin was inhibited completely by α_2-plasmin inhibitor, but was not inhibited when plasmin was bound to α_2-macroglobulin. Furthermore, we established that α_2-plasmin inhibitor caused negligible inhibition of the amidolytic activity of the α_2-macroglobulin–plasmin complex. Therefore, when plasmin is added to mixtures of both inhibitors, the amount of enzyme bound to α_2-macroglobulin can be quantitated by the preservation of amidolytic activity.

Assays using this substrate confirmed the effectiveness of α_2-plasmin inhibitor in competing with α_2-macroglobulin for plasmin (Table II). Increasing concentrations of α_2-plasmin inhibitor in a mixture of a constant concentration of plasmin and α_2-macroglobulin caused a reduction in the amidolytic activity of the incubation mixture which was proportional to the final concentration of α_2-plasmin inhibitor added. Comparison of the plasmin activity of the incubation mixtures containing α_2-plasmin inhibitor and plasmin with those mixtures which also included α_2-macroglobulin demonstrated no significant differences. Thus, the α_2-plasmin inhibitor competed with α_2-macroglobulin as effectively for native plasmin as it did for the radiolabeled enzyme.

Discussion

Previous studies from our laboratory have examined the interactions between plasmin and mixtures of purified plasma protease inhibitors in the concentrations found in plasma. In mixtures containing α_2-macroglobulin, α_1-antitrypsin, and $C\bar{1}$ inactivator, the α_2-macroglobulin was found to bind most of the plasmin which was added.[1] In a similar analysis, α_2-macroglobulin competed effectively for plasmin in the presence of antithrombin-heparin cofactor.[2] The addition of heparin, however, enabled the antithrombin-heparin cofactor to bind slightly more plasmin that did the α_2-macroglobulin. Thus, in the competition among plasma inhibitors for plasmin in the absence of heparin, α_2-macroglobulin binds plasmin more rapidly than does α_1-antitrypsin, $C\bar{1}$ inactivator, and antithrombin-heparin cofactor.

Moroi and Aoki (10) have recently isolated a plasma inhibitor of plasmin, termed the α_2-plasmin inhibitor, which rapidly formed a complex with plasmin and which completely inhibited the proteolytic and esterolytic activity of the enzyme. This inhibitor appears to be identical to the partially purified, rapidly acting plasmin inhibitor described by Müllertz and Clemmensen (17). Both Müllertz (22) and Collen (23) have documented that in vitro activation of small amounts of plasminogen in plasma results in the appearance of the α_2-plasmin inhibitor–plasmin complex, which precedes the formation of the plasmin–α_2-

TABLE II

Effect of Varying the Concentration of α_2-Plasmin Inhibitor on the Binding of Plasmin to α_2-Macroglobulin as Assayed Using a Tripeptide Substrate for Plasmin

Concentration α_2-PI	Incubation mixtures of plasmin plus:	
	Plasmin activity	
	α_2M + α_2-PI*	α_2-PI‡
μM	μM	μM
0	0.39	0.39
0.06	0.37	0.35
0.13	0.29	0.31
0.25	0.14	0.12
0.5	0.03	0

* 5 μl plasmin (0.4 μM final concentration of plasmin as determined by active site titration with *p*-NPGB) was added to a mixture of 5 μl α_2-macroglobulin (4.6 μM final concentration) and 5 μl buffer (0 μM α_2-PI) at 37°C, or to a mixture containing α_2M and 5 μl of the concentrations of α_2-plasmin inhibitor indicated in the table. For these concentrations of α_2-PI, the molar ratios relative to the concentration of α_2M (4.6 μM) are as follows: 0.06 μM = 0.01; 0.13 μM = 0.03; 0.25 μM = 0.05; 0.5 μM = 0.11.

After a 2-min incubation, 330 μg of the substrate (H-D-valyl-leucyl-lysine-*p*-nitroanilide) in a 1.2-ml volume was added and the mixture incubated an additional 15 min at 37°C. The reaction was stopped by the addition of 30% acetic acid (0.3 ml), and the absorbance at 410 nm recorded. The readings were converted to plasmin activity using a standard plasmin curve. All values are the mean of duplicate determinations.

‡ 5 μl plasmin (0.4 μM) was incubated with buffer (0 μM α_2-PI) or with varying concentrations of α_2-PI as indicated in the table and under the conditions described above. No α_2-macroglobulin was included in these mixtures.

macroglobulin complex. These observations suggest that the α_2-macroglobulin may function as a plasmin inhibitor after the saturation of the α_2-plasmin inhibitor.

In the present study, we have examined directly the ability of α_2-plasmin inhibitor to bind plasmin in the presence of α_2-macroglobulin. The results of two different methods designed to assess complex formation between plasmin and these inhibitors were entirely comparable and indicated that ^{125}I-plasmin bound as readily to these inhibitors as did the native enzyme. Comparison of the distribution of radiolabeled plasmin between the two inhibitors at 10 and 120 s of incubation showed an identical binding pattern, indicating that the reactions were rapid, being completed by 10 s. Preincubation of plasmin with one inhibitor followed by the addition of the other established that transfer of the enzyme between the inhibitors did not occur.

The reported plasma concentration of α_2-plasmin inhibitor (1.1 μM [10]), and of α_2-macroglobulin (3.5 μM [16]), represents a molar ratio of 0.3. In our binding studies, α_2-plasmin inhibitor to α_2-macroglobulin molar ratios from 0.01 to 0.21

have been examined. Over this range of α_2-plasmin inhibitor concentrations, approximately as much plasmin was bound to the α_2-plasmin inhibitor in mixtures which contained α_2-macroglobulin as in those which did not. This indicates that when both inhibitors simultaneously compete for plasmin, the α_2-plasmin inhibitor binds the enzyme much more rapidly than does the α_2-macroglobulin. These data strengthen the concept that the α_2-plasmin inhibitor may represent the primary plasmin inhibitor in the circulating blood.

Summary

α_2-Plasmin inhibitor and α_2-macroglobulin were allowed to compete for the protease plasmin. The binding of the enzyme to these inhibitors was assessed by two different but comparable methods. The interactions were completed in 10 s of incubation, and transfer of plasmin from one inhibitor to the other did not occur. Almost as much plasmin was bound to α_2-plasmin inhibitor in mixtures containing a large molar excess of α_2-macroglobulin relative to plasmin or α_2-plasmin inhibitor, as was bound in mixtures not containing α_2-macroglobulin. These studies demonstrate directly the effectiveness of α_2-plasmin inhibitor in binding and inhibiting plasmin in the presence of α_2-macroglobulin, and suggest that the α_2-plasmin inhibitor may be the major circulating plasmin inhibitor.

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