Plasma Inhibitors of the Components of the Fibrinolytic Pathway in Man

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ABSTRACT The effect of highly purified inhibitor of the first component of complement ($C\bar{1}INH$), $\alpha 2$ macroglobulin ($\alpha 2M$), and $\alpha 1$ antitrypsin on the components of the fibrinolytic pathway in human plasma has been examined. $C\bar{1}INH$ was the only factor active upon the Hageman factor fragments functioning at the initial step of the fibrinolytic pathway. $\alpha 2M$ was the only factor active against the plasminogen activator and the most active inhibitor of plasmin. The inhibition of plasmin by $\alpha 2M$ appeared stoichiometric with one molecule of $\alpha 2M$ inhibiting two molecules of plasmin. All three plasma inhibitors were active against plasmin.

INTRODUCTION

The fibrinolytic pathway in human plasma is initiated by activation of Hageman factor (1, 2). The prealbumin fragments of active Hageman factor isolated from serum or derived experimentally from activated Hageman factor have been shown to activate a plasma protein, the plasminogen proactivator to the plasminogen activator, which in turn converts plasminogen to plasmin (3, 4).

The plasma protein inhibitors, $\alpha 1$ antitrypsin, $\alpha 2$ macroglobulin ($\alpha 2M$),[1] and the inhibitor of the first component of complement ($C\bar{1}INH$) have been examined for their effects on this pathway. Inhibition of Hageman factor fragments and plasminogen activator is accomplished only by $C\bar{1}INH$ and $\alpha 2M$, respectively, while all three control proteins are active against plasmin.

METHODS

Antisera to $\alpha 1$ antitrypsin, $\alpha 2M$, and albumin (Behring Diagnostics, Inc., Woodbury, N. Y.); hexadimethrine bromide (polybrene) (Aldrich Chemical Co., Inc., Milwaukee, Wis.); enzodiffusion fibrin plates, $\alpha 2M$ quantitative immunodiffusion plates and streptokinase (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.); $\alpha 1$ antitrypsin quantitative immunodiffusion plates (Miles Laboratories Inc., Kankakee, Ill.); benzoyl-d-l-arginine-p nitro analide HCl (BAPA) (Nutritional Biochemicals Corp., Cleveland, Ohio); crystalline trypsin (Worthington Biochemical Corp., Freehold, N. J.) were obtained as indicated. Hageman factor deficient and plasma thromboplastin antecedent- (PTA) deficient plasma were supplied by Sera-Tec Biologicals, New Brunswick, N. J. Concentration was performed by ultrafiltration using UM-10 membranes (Amicon Corp., Lexington, Mass.) in 500 ml, 50 ml, or 10 ml capacity Amicons as appropriate.

Plasma was prepared for the isolation of plasma proenzymes and inhibitors in ethylenediaminetetraacetate (EDTA) and polybrene as described (5). The conversion of pre-PTA[2] (5, 6), prekallikrein (5), and plasminogen proactivator (3, 4), by the Hageman factor fragments was measured by subsequent coagulation, bradykinin formation, and fibrinolysis, respectively. The $C\bar{1}INH$ was assessed functionally by its ability to inhibit the hemolytic activity of erythrocyte-bound $C\bar{1}$ ($EAC\bar{1}$) by either microtiter plate or tube titrations (7) and was quantitated by immunodiffusion (8, 9). The titer of 1613 U per microgram of $C\bar{1}INH$ obtained is comparable to the activity in normal human serum (7). $\alpha 1$ antitrypsin concentration was determined by quantitative immunodiffusion plates or by its inhibition of the esterase activity of trypsin upon BAPA as previously described (10). $\alpha 2M$ concentration was determined utilizing quantitative immunodiffusion plates.

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[1] *Abbreviations used in this paper:* $\alpha 2M$, $\alpha 2$ macroglobulin; BAPA, benzoyl-*d-l*-arginine-*p* nitro analide HCl; $C\bar{1}INH$, inhibitor of the activated first component of complement; $EAC\bar{1}$, erythrocyte-bound $C\bar{1}$; PTA, plasma thromboplastin antecedent; QAE, Quartenary aminoethyl; SE Sephadex, Sulphoethyl Sephadex; SP Sephadex, Sulphopropyl Sephadex.

[2] In order to avoid ambiguity and confusion as to the state of activation of the molecule we have referred to the precursor of PTA as pre-PTA throughout the text. Other workers may consider PTA as the precursor form and refer to the activated molecule as activated PTA.

Isolation of components of the fibrinolytic pathway

Hageman factor fragments. Hageman factor prealbumin fragments were prepared by Quarternary aminoethyl (QAE) Sephadex chromatography followed by rechromatography on QAE Sephadex and were then fractionated by Sephadex G-100 gel filtration, as described in the accompanying paper (11). The Hageman factor prealbumin fragments eluted on Sephadex G-100 at a mol wt of 32,500, were concentrated to approximately 20–25 μg/ml, divided into aliquots, and stored at −70°C for routine use. Disc gel electrophoresis (12) revealed prealbumin bands with only trace contamination with albumin.

The intermediate sized Hageman factor fragment was isolated from human plasma as outlined in the accompanying paper (11) by QAE Sephadex chromatography followed by rechromatography on QAE Sephadex and was then fractionated by Sephadex G-100 gel filtration. The intermediate sized Hageman factor fragment eluted from Sephadex G-100 at a mol wt of 80,000 and was aliquoted and stored at −70°C for further use. Assessment of the preparation by disc gel electrophoresis revealed two bands in the β-globulin region, one corresponding to the Hageman factor fragment and the other to a transferrin contaminant.

Plasminogen proactivator. The plasminogen proactivator was isolated by QAE Sephadex, Sulphoethyl (SE) Sephadex, and Sephadex G-150 chromatography as described (4, 11). The plasminogen proactivator eluted from Sephadex G-150 at a mol wt of approximately 100,000 and was pooled, concentrated to 10 ml, and stored at −70°C. The preparation contained trace prekallikrein and IgG contamination, when assessed by bioassay and Ouchterlony or alkaline disc gel electrophoresis, respectively.

Plasminogen. Plasminogen was prepared by affinity chromatography and Sephadex G-100 gel filtration as described (4, 13). Disc gel electrophoresis revealed a single broad band identified as plasminogen by functional analysis of an unstained sliced replicate disc gel. There were no contaminating proteins detected. Plasmin was prepared by activating 500 μl of plasminogen (100 μg/ml) with 50 μl (1,500 U) of streptokinase for 30 min at 30°C and assayed on human fibrin plates (4).

Isolation of inhibitors of the fibrinolytic pathway

α1 antitrypsin. 100 ml of plasma dialyzed against 0.0035 M phosphate buffer pH 7.8 were applied to a 5 × 100 cm column of QAE Sephadex equilibrated with the same buffer. The column was washed with 600 ml of equilibrating buffer and eluted with a linear salt gradient of 2,500 ml of equilibrating buffer and 2,500 ml of 0.0035 M phosphate buffer pH 7.8 containing 0.3 M sodium chloride. The column was run at 50 ml/h, and 10 ml fractions were collected. α1 antitrypsin was assayed by counterelectroimmunodiffusion (14), utilizing undiluted anti-α1 antitrypsin antisera in all chromatographic procedures. The precipitin arcs observed were quantitated on a 0–4+ scale by inspection; α1 antitrypsin eluted between 0.07 and 0.15 M sodium chloride as shown in Fig. 1. Tubes 220–245 were pooled so as to exclude much of the C1INH and α2M; the pool was concentrated to 20 ml by ultrafiltration, dialyzed for 5 h in 0.05 M sodium acetate buffer pH 5.0, and applied to a Sulphopropyl (SP) Sephadex column (3.5 × 30 cm) equilibrated with the same buffer. α1 antitrypsin was obtained in the effluent by washing the column with 200 ml of equilibrating buffer at 50 ml/h. The effluent fractions were pooled, adjusted to pH 7.45

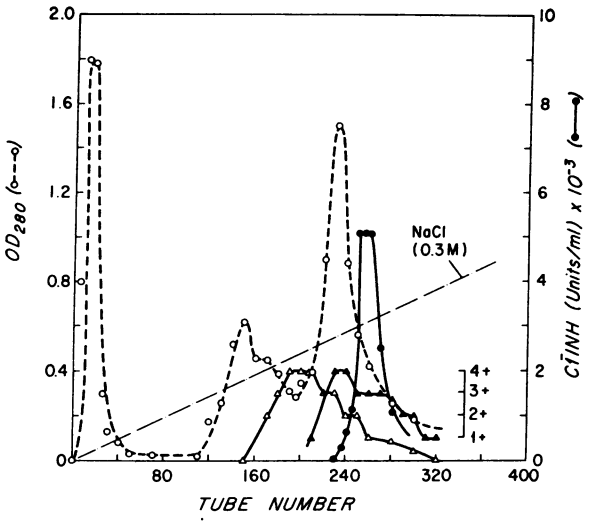

FIGURE 1 Isolation of C1INH (closed circles), α1 antitrypsin (closed triangles), and α2 macroglobulin (open triangles) by chromatography of human plasma on QAE Sephadex.

with 4 N sodium hydroxide, concentrated by ultrafiltration to 4 ml, and applied to a 5 × 100 cm column of Sephadex G-100 equilibrated in 0.0035 M phosphate buffer pH 7.8 containing 0.15 M sodium chloride. The column was run at 30 ml/h and 10 ml fractions collected. The α1 antitrypsin peak obtained at 53% bed volume was pooled, concentrated to 20 ml, and utilized for all subsequent studies. This preparation contained no functional C1INH as assayed in a hemolytic system, no α2M as assessed by counterelectroimmunodiffusion, no inter-alpha trypsin inhibitor as measured by the Ouchterlony technique, and no detectable plasmin or plasminogen activator activity. Alkaline disc gel electrophoresis (Fig. 2) revealed two bands, representing α1 antitrypsin and albumin, as shown by elution of unstained replicate gels and analysis by counterelectroimmunodiffusion with undiluted anti-α1 antitrypsin antisera and antialbumin antisera, as well as two unidentified bands. The isoelectric point of α1 antitrypsin determined by elution from sliced polyacrylamide gels after isoelectric focusing (15) was 5.2, as shown in Fig. 3 A.

α2 macroglobulin. 100 ml of plasma dialyzed against 0.0035 M phosphate buffer pH 7.8 were applied to a QAE Sephadex (5 × 100 cm) column equilibrated with the same buffer. The column was washed with 600 ml of equilibrating buffer and eluted with a linear salt gradient of 2,500 ml of equilibrating buffer and 2,500 ml of 0.0035 M phosphate buffer pH 7.8 containing 0.3 M sodium chloride. The column was run at 50 ml/h, and 10 ml fractions were collected. α2M was assayed by counterelectroimmunodiffusion using undiluted anti-α2M antisera in all chromatographic procedures. The precipitin arcs observed were quantitated on a 0–4+ scale by inspection; α2M eluted between 0.05 and 0.10 M NaCl as shown in Fig. 1. A pool containing α2M (tubes 170–212) was concentrated to approximately 4 ml and fractionated by upward flow at 10 ml/h utilizing a 5 × 100 cm column of Sepharose 6 B equilibrated with 0.0035 M phosphate buffer pH 7.8 containing 0.15 M NaCl. 10 ml fractions were collected per hour, and α2M eluted at 61% bed volume. The α2M peak was pooled, concentrated to 4.0 ml,

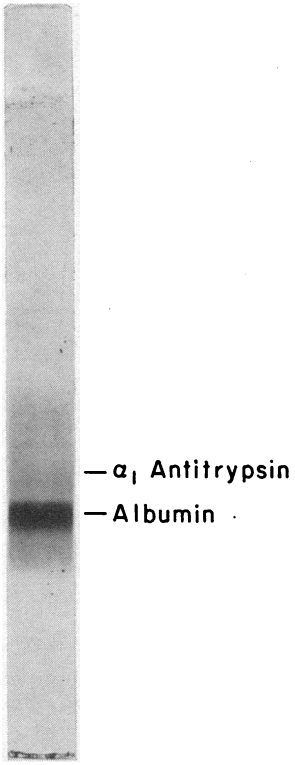

FIGURE 2 Disc gel electrophoresis of α1 antitrypsin obtained by QAE Sephadex (Fig. 1), SP Sephadex, and Sephadex G-100 chromatography.

and fractionated by upward flow at 10 ml/h on a 5 × 100 cm column of Sephadex G-200 equilibrated with 0.0035 M phosphate buffer pH 7.8 containing 0.15 M NaCl. Fractionation was performed at 10 ml/h and 10 ml fractions were collected; α2M eluted at 30% bed volume. The α2M peak was pooled, concentrated to 30 ml, and stored at −4°C for further use. This preparation contained no detectable C1INH, α1 antitrypsin, or inter-alpha trypsin inhibitor as assayed by counterelectroimmunodiffusion or Ouchterlony analysis and no detectable plasmin or plasminogen activator activity. Alkaline disc gel electrophoresis (Fig. 4) revealed one major band representing α2M as shown by elution of unstained replicate gels and analysis by counterelectroimmunodiffusion with undiluted anti-α2M antisera, as well as three minor bands. The isoelectric point of α2M determined by elution from sliced gels after isoelectric focusing was 6.4 as shown in Fig. 3 B.

α2-macroglobulin and α1-antitrypsin were not necessarily prepared from the same samples.

C1INH. The C1INH was purified by QAE Sephadex, SP Sephadex, Sephadex G-200, and Sephadex G-150 chromatography as described (11). This preparation had no detectable α1 antitrypsin or α2M as assessed by Ouchterlony analysis, no detectable plasmin or plasminogen activator as assayed on human fibrin plates, and gave a double band on alkaline disc gel electrophoresis in a region from which unstained gels yielded active C1INH upon elution (11).

RESULTS

Inhibition of plasmin

C1INH. 20 μl of plasmin (150 μg/ml) were incubated with 20 μl of decreasing concentrations of purified C1INH (45,000 U/ml) plus 20 μl of phosphate buffered saline pH 7.8 or with 40 μl of buffer alone for 30 min at 37°C and the residual fibrinolytic activity determined. The preparation of plasmin with buffer alone generated 47 μg/ml of plasmin. As indicated in Fig. 5 A, when the concentration of C1INH is increased, the effective plasmin is progressively inhibited.

α1 antitrypsin. 25 μl of plasmin (50 μg/ml) were incubated for 30 min at 37°C with an equal volume of decreasing concentrations of purified α1 antitrypsin (2.1 mg/ml), having the same functional activity as an equal

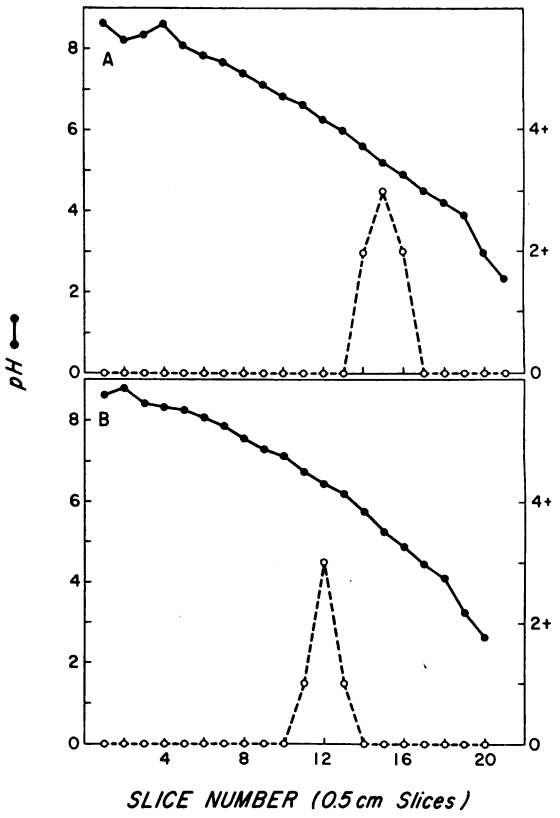

FIGURE 3 Isoelectric focusing of α1 antitrypsin obtained by QAE Sephadex, SP Sephadex, and Sephadex G-100 chromatography (A) and of α2 macroglobulin obtained by QAE Sephadex, Sepharose 6B, and Sephadex G-200 chromatography (B). Identification of each inhibitor was estimated on a 0 to 4+ scale by inspection of precipitin arcs obtained by counterelectroimmunodiffusion employing specific antisera (14).

volume of plasma when measured by trypsin inhibition (10), and the residual fibrinolytic activity determined. The preparation of plasmin with buffer alone yielded 24 μg/ml of plasmin. As shown in Fig. 5 B, increasing concentrations of α1 antitrypsin resulted in a diminution of plasmin activity. The inhibitory activity of α1 antitrypsin upon plasmin, however, appeared relatively weak yielding only 36% inhibition at concentrations of α1 antitrypsin comparable to that of whole plasma in the presence of a plasmin concentration approximately 10% of the plasminogen concentration of whole plasma (16).

α2M. 50 μl of plasmin (100 μg/ml) were incubated with decreasing concentrations of α2M (247 μg/ml) or buffer at 37°C for 30 min, and the residual fibrinolytic activity determined. The control preparation of plasmin with buffer generated 52 μg/ml of plasmin. As indicated in Fig. 6, as the concentration of α2M increased, the residual fibrinolytic activity decreased proportionately. 45% inhibition was reached at an α2M concentration of 124 μg/ml.

Utilizing 900,000 as the mol wt of α2M (16) and 80,000 as the mol wt of plasmin (16), the number of moles of plasmin inhibited relative to the moles of α2M input was calculated. As shown in Fig. 7 A, a straight line relating moles of plasmin inhibited to moles of α2M was obtained. By plotting the molar ratio of plasmin per α2M against moles of α2M input, a molar ratio approximating 2.0 was obtained for these reactants as indicated in Fig. 7 B.

Inhibition of plasminogen activator

1 ml of the concentrated QAE Sephadex effluent obtained from 40 ml of plasma was treated with 50 μl of Hageman factor fragments (25 μg/ml) to convert the prekallikrein and plasminogen proactivator to kallikrein and plasminogen activator, respectively. Duplicate 20 μl samples of this QAE Sephadex effluent were incubated

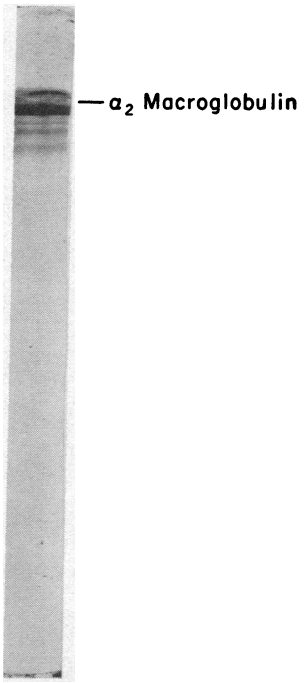

FIGURE 4 Disc gel electrophoresis of α2 macroglobulin obtained by QAE Sephadex (Fig. 1), Sepharose 6B, and Sephadex G-200 chromatography.

with an equal volume of C1INH (50,000 U/ml), α2M (1.1 mg/ml) or phosphate-buffered saline pH 7.8 for 45 min at 37°C. 20 μl of plasminogen (200 μg/ml) were added to one of each duplicate mixture, incubated for 45 min at 37°C, and the plasmin generated determined, while the other duplicate mixture was assayed for kallikrein. The mixtures containing plasminogen activator alone or plasminogen activator and C1INH generated 65 and 62 μg/ml of plasmin, indicating no inhibition of the plasminogen activator by C1INH. In contrast, the mixture

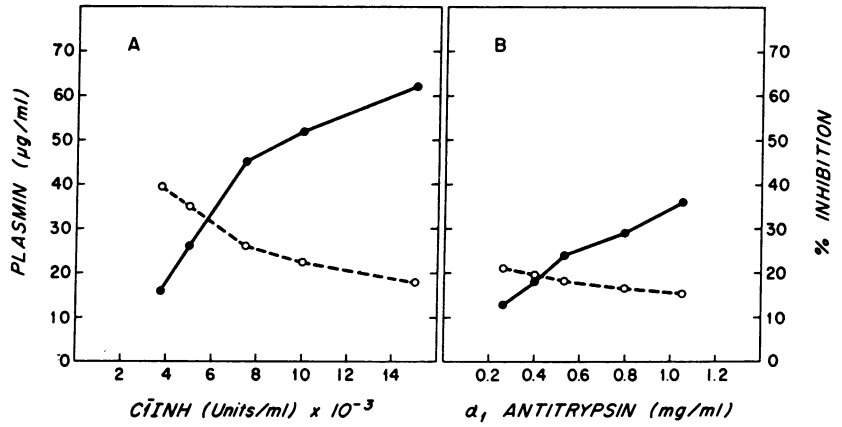

FIGURE 5 Inhibition by C1INH (A) and by α1 antitrypsin (B) of plasmin fibrinolytic activity (open circles). Per cent inhibition is plotted as closed circles.

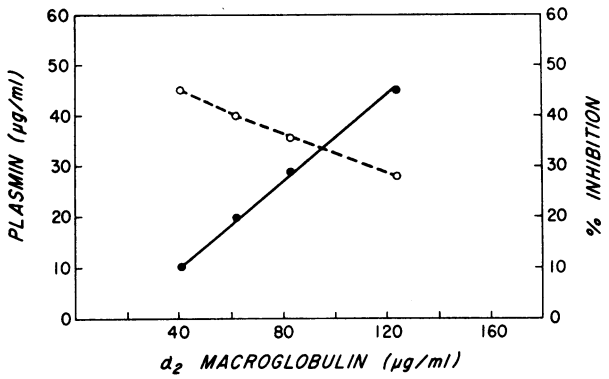

FIGURE 6 Inhibition by α2 macroglobulin of plasmin fibrinolytic activity (open circles). Per cent inhibition is plotted as closed circles.

containing plasminogen activator and α2M generated no detectable plasmin activity from plasminogen. The CĪINH and α2M inhibited the kallikrein activity 70% and 75%, respectively.

In order to establish that the apparent inhibition of plasminogen activator by α2M was not attributable to an effect on the assay for plasmin generated, this inhibitor as well as CĪINH and α1 antitrypsin were chromatographically separated from the plasminogen activator after interaction of inhibitor with plasminogen activator. One-half milliliter of the concentrated QAE Sephadex effluent was incubated with 0.5 ml of buffer plus either 0.5 ml of CĪINH (31 μg/ml), α2M (1.1 mg/ml), α1 antitrypsin (2.1 mg/ml), or buffer for 30 min at 37°C. Each mixture was then dialyzed for 4.5 h with 0.0035 M phosphate buffer pH 7.8 and a 10 μl sample removed from each dialysate. Each mixture was then applied to a 2 × 10 cm column of QAE Sephadex, a 25 ml effluent obtained utilizing starting buffer, and the latter concentrated to 1.0 ml for assay of residual kallikrein and plasminogen activator activity. The results are presented in Table I. CĪINH completely inactivated the kallikrein present in the original QAE Sephadex effluent, but had no effect upon the plasminogen activator. α2M inhibited both kallikrein and the plasminogen activator, whereas α1 antitrypsin revealed no inhibition of either enzyme. The final QAE Sephadex effluent contained no detectable CĪINH, α2M, α1 antitrypsin, or other plasmin inhibitors which might interefere with the assay for plasminogen activator or kallikrein. The loss of plasminogen activator and kallikrein as a result of dialysis and chromatography alone was less than 33%.

To examine the dose response effect of α2M, 0.5 ml of a QAE Sephadex effluent containing kallikrein and plasminogen activator was incubated with 0.5 ml of twofold falling dilutions of α2M (1.85 mg/ml), α1 antitrypsin (3 mg/ml), or buffer alone. The mixtures were dialyzed against 2,000 ml of 0.0035 M phosphate buffer pH 7.8 for 4.5 h and applied to 2 × 10 cm QAE Sephadex column equilibrated with the dialysis buffer. The columns were washed with 25 ml of equilibrating buffer, and each effluent concentrated to 0.5 ml for assay of residual kallikrein and plasminogen activator. The plasminogen activator in buffer or exposed to α1 antitrypsin generated 25 μg of plasmin from plasminogen (100 μg/ml). The inhibition of plasminogen activator obtained with α2M ranged from 17% to 100% as shown in Fig. 8. The inhibition of kallikrein by α2M was similar. The effluents tested contained no detectable plasmin or plasminogen. The effluent exposed to the highest concentration of α2M (1.85 mg/ml) yielded 10% inhibition of streptokinase activated plasminogen, suggesting that the peak inhibitory value of 100% at an α2M concentration of 1.85 mg/ml may be an overestimate.

Inhibition of Hageman factor fragments

In the accompanying paper (11) the ability of the CĪINH to inhibit the action of the Hageman factor fragments upon its three substrates, prekallikrein, pre-PTA and plasminogen proactivator is demonstrated. To evaluate the effect of α2M (1.1 mg/ml) and α1 antitrypsin (2.1 mg/ml) on the ability of the Hageman factor frag-

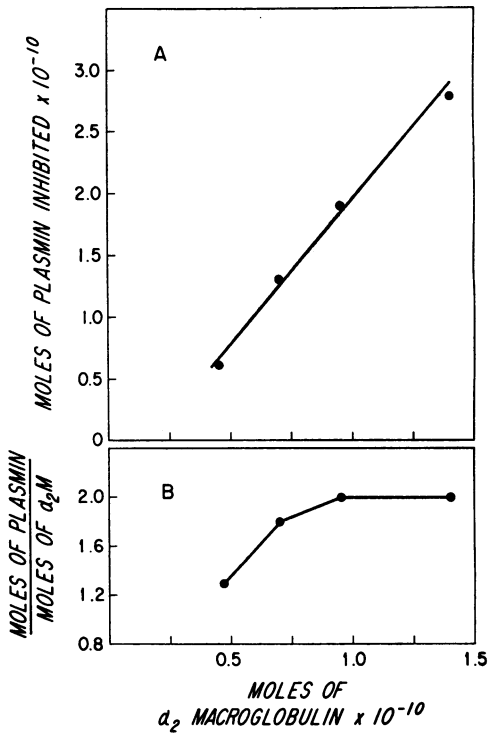

FIGURE 7 Molar inhibition by α2 macroglobulin of plasmin (A) and molar ratio of plasmin inhibited to α2 macroglobulin input (B).

TABLE I

Inhibition of Plasminogen Activator and Kallikrein by CĪINH, α2M, and α1 Antitrypsin

	Plasmin generated from plasminogen	Inhibition of plasminogen activator	Bradykinin generated from kininogen	Inhibition of kallikrein
	μg/ml	%	*ng/ml*	%
Kallikrein + plasminogen activator* + buffer	33	—	3,000	—
Kallikrein + plasminogen activator + CĪINH (31 μg/ml)	32	0	0	100
Kallikrein + plasminogen activator + α1 antitrypsin (2.1 mg/ml)	45	0	5,000	0
Kallikrein + plasminogen activator + α2M (1.1 mg/ml)	12	64	0	100

* A mixture of kallikrein and plasminogen activator obtained by activation of a concentrated QAE Sephadex effluent with the Hageman factor fragments was incubated with buffer or the inhibitors indicated, dialyzed, and the active enzymes recovered by a second passage over QAE Sephadex.

ments to activate prekallikrein in fresh plasma, 10 μl of each inhibitor or buffer was incubated with 10 μl of Hageman factor prealbumin fragments (25 μg/ml) at 37°C for 30 min and the bradykinin generated from 100 μl of fresh plasma in 2 min at 37°C measured. The preparation of Hageman factor fragments and buffer generated 2,500 ng of bradykinin/milliliter of fresh plasma; there was no inhibition of prekallikrein activation by α1 antitrypsin or α2M. To examine inhibition of Hageman factor fragment pre-PTA activation by α1 antitrypsin (2.1 mg/ml) and α2M (1.1 mg/ml), 25 μl of each inhibitor or buffer was incubated at 37°C for 15 min with 25 μl of intermediate sized Hageman factor fragment (mol wt 80,000), and the correction of the partial thromboplastin time of Hageman factor-deficient plasma determined (11). There was no inhibition of the coagulant activity of the Hageman factor preparation preincubated with α2M or α1 antitrypsin. A control preparation of CĪINH (31 μg/ml), however, inhibited 80% of the coagulant activity of the Hageman factor fragment. In order to assess the effect of α2M and α1 antitrypsin upon the ability of Hageman factor fragments to activate plasminogen protactivator, 10 μl of the Hageman factor fragments (25 μg/ml) were incubated with 10 μl of α2M (1.1 mg/ml), 10 μl of α1 antitrypsin (2.1 mg/ml) or buffer for 30 min at 37°C. 5 μl of each mixture were interacted with 50 μl of highly purified plasminogen proactivator for 48 h at 4°C, and a 20 μl sample of each mixture then removed and further incubated with 20 μl of plasminogen (200 μg/ml) for 1 h at 37°C to generate plasmin. 45 μg of plasmin/milliliter were generated in each reaction mixture.

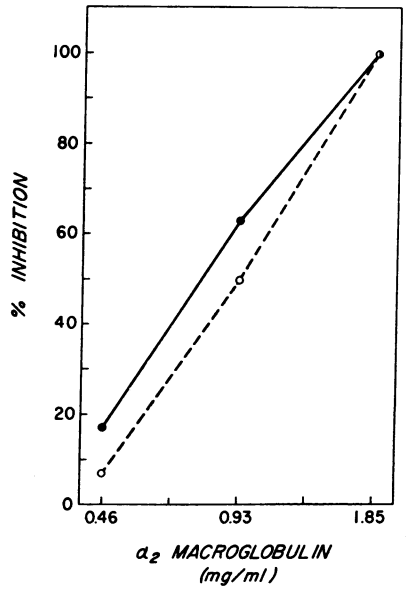

FIGURE 8 Inhibition by α2 macroglobulin of the ability of the plasminogen activator to generate plasmin from plasminogen (solid line) and of kallikrein to generate bradykinin in heat-inactivated plasma as a kininogen source (5) (dotted line).

DISCUSSION

The activation of Hageman factor in plasma is known to result in the development of fibrinolytic activity which is not attributable to the direct action of activated Hageman factor or its fragments (1, 2) upon plasminogen. The conversion of plasminogen proactivator to plasminogen activator by activated Hageman factor or its fragments is now recognized as an essential intermediate step in the fibrinolytic sequence (3, 4). Regulation of the fibrinolytic sequence previously recognized only in terms of the action of C$\bar{1}$INH, α1 antitrypsin, and α2M on plasmin (17–19) is now extended to include the inhibition of the plasminogen activator by α2M and inhibition of the Hageman factor fragments by C$\bar{1}$INH (11).

The chromatographic isolation of each inhibitor was such that the final product was free of detectable contamination by the other two. On disc gel electrophoresis, the C$\bar{1}$INH was free of other detectable proteins (11), the α1 antitrypsin contained albumin and two trace bands (Fig. 2), and the α2M revealed three undefined bands (Fig. 4). The isoelectric point of α1 antitrypsin was 5.2 (Fig. 3 A) and of α2M was 6.4 (Fig. 3 B). Functional analysis of the C$\bar{1}$INH revealed that the number of effective molecules/microgram of protein was consistent with that observed in whole serum (7). The concentration of α1 antitrypsin measured by immunodiffusion analysis of the purified preparation required to inhibit the esterolytic activity of trypsin was approximately twice an identical antigenic amount of α1 antitrypsin in normal plasma. The finding that 1 mol of α2M inhibited 2 mol of plasmin (Fig. 7) attested to the functional integrity of the purified protein.

The capacity of the C$\bar{1}$INH to prevent the action of the Hageman factor fragments in converting the plasminogen proactivator to plasminogen activator is noted in the companion study and extends to inhibition of prekallikrein and pre-PTA activation as well (11). The concentration of C$\bar{1}$INH effective in this regard did not interfere with the action of the immediate conversion products and subsequent steps involved in completion of each biologic reaction sequence. In comparable experiments neither α1 antitrypsin nor α2M inhibited the ability of the Hageman factor fragments to convert plasminogen proactivator, prekallikrein, or pre-PTA to their respective active enzymes.

In order to study the inhibition of the plasminogen activator it was necessary to minimize the concentration of the inhibitors during the subsequent assay of the residual plasminogen activator activity. Thus, the reactants were subjected to QAE Sephadex chromatography and the active enzymes sought in the fractions in which they were identified after exposure to buffer alone. The recovery of plasminogen activator or kallikrein in buffer alone was 67% and the value was similar upon interaction with α1 antitrypsin. Thus the lack of recovery with α2M and C$\bar{1}$INH pretreatment was attributed to inhibition of the active enzyme. Carry over of inhibitory material was excluded by both functional and Ouchterlony analysis. With this experimental design it was established that α2M inhibited the plasminogen activator in a dose response fashion (Fig. 8), the effect being quite comparable to the inhibition of kallikrein. Although the protein concentration of the two enzymes in the experiment is not known, it is noteworthy that they were both derived from their precursors by activation with Hageman factor fragments of a plasma fraction known to contain all of each proenzyme which can be derived from plasma. Further, the concentration of α2M yielding 100% inhibition is in good agreement with its concentration in normal plasma. When the same source of proenzymes was activated and the inhibitory effect of α2M compared with C$\bar{1}$INH and α1 antitrypsin, only α2M inhibited the plasminogen activator (Table I). Both α2M and C$\bar{1}$INH were active against kallikrein, while α1 antitrypsin failed to inhibit either enzyme. Inhibition of kallikrein by α1 antitrypsin observed by others involved longer incubation times (20) or less pure preparations of α1 antitrypsin (21). The failure of C$\bar{1}$INH to prevent the action of plasminogen activator is not only in contradistinction to its effect on kallikrein, but also to its ability to inhibit PTA (22).

Inhibition of the fibrinolytic activity of plasmin by C$\bar{1}$INH (17) and inhibition of the caseinolytic activity of human plasmin by α1 antitrypsin (18) and by α2M (19) have been previously observed. Utilizing highly purified preparations of each inhibitor, a dose response type inhibition (Figs. 5 A, 5 B, and 6) was noted upon the fibrinolytic activity of plasmin. The concentration of α1 antitrypsin and C$\bar{1}$INH required for these experiments represented falling dilutions of a concentration approaching that in plasma, whereas α2M had a similar activity beginning at one-tenth its plasma concentration. The inhibition appeared to be stoichiometric with one molecule of α2M inhibiting two molecules of plasmin (Fig. 7), a result similar to that obtained by Ganrot (19) in examining trypsin inhibition by α2M. Using casein as a substrate for plasmin, Ganrot (19) determined an equimolar binding ratio between α2M and plasmin.

To summarize, C$\bar{1}$INH is the only factor active upon the Hageman factor fragments, functioning at the initial step of the fibrinolytic pathway. α2M appears to be the other critical inhibitor of the fibrinolytic pathway, being the only factor active against the plasminogen activator and the most active inhibitor of plasmin.

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