PROTEASE INHIBITORS AND HUMAN PLASMIN: INTERACTION IN A WHOLE PLASMA SYSTEM*

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Summary: *Using trace amounts of [^{125}I]-plasminogen and conventional biochemical techniques, the distribution of the labeled zymogen amongst the various protease inhibitors was studied in whole plasma before and after activation with urokinase and streptokinase. A small percentage of the labeled enzyme was bound to α_2-macroglobulin while a majority was complexed to a component in plasma immunologically distinct from the well known human antiplasmins. The inhibitor was identified as α_2-antiplasmin and confirmed the existence of this antiprotease recently described by others. These data also suggest that the other antiplasmins may play a minor, yet important role in the regulation of plasmin activity under different physiological conditions.*

The generation of plasmin from its inactive zymogen, plasminogen, represents the central event in the fibrinolytic mechanism. Once evolved in plasma, this endopeptidase may catalyze the hydrolysis of circulating fibrinogen as well as intravascular deposits of fibrin. However, other non-hemostatic plasma proteins of equal biological importance are also susceptible to the action of this enzyme. Fortunately, normal human plasma is capable of localizing and restricting intravascular proteolysis in a variety of ways. This protective mechanism is supported by an abundance of plasma protease inhibitors. Those which have been shown to possess significant antiplasmin activity in vitro are: α_2-macroglobulin (1), α_1-antitrypsin (2), antithrombin-heparin cofactor (3), $C\bar{1}$-esterase inhibitor (4) and inter-α-trypsin inhibitor (5). Recently, several authors have reported on the existence of yet another antiprotease which rapidly inhibits and irreversibly interacts with the fibrinolytic

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Abbreviations used: CTA, Committee on Thrombolytic Agents; SDS, sodium dodecyl sulfate; UK, urokinase; SK, streptokinase; LSAC, lysine-sepharose affinity chromatography.

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enzyme. This inhibitor, termed α_2-antiplasmin, has a molecular weight of approximately 60-80,000 and post-β_1 mobility upon immunoelectrophoresis in agarose gels. In addition, these authors have demonstrated that α_2-antiplasmin is the fastest reacting antiplasmin in whole plasma (6,7,8,9).

Our laboratory is currently investigating the quantitative significance of each of these antiproteases in regulating the activity of human plasmin in a whole plasma system. These studies present evidence to substantiate the existence of the newly described inhibitor; in addition, data is presented on the interaction of human plasmin with the protease inhibitors in plasma under various experimental conditions of activation of the fibrinolytic system.

MATERIALS AND METHODS

Chemicals and Reagents. Fresh, non-frozen human plasma was kindly provided by the Hoxworth Blood Center, Cincinnati, OH. [^{125}I]-Iodine, as NaI (carrier-free), was the product of New England Nuclear, Boston, Mass. UK (Sterling-Winthrop, Rensselear, N.Y.) was authorized by Dr. J.C. Fratantoni, Division of Blood Diseases and Resources, NIH. SK was the gift of Hoechst-Roussel Pharmaceuticals, Somerville, N.J. Monospecific antisera to the human protease inhibitors and to plasminogen were purchased from Behring Diagnostics, Somerville, N.J. Rabbit, anti-human α_2-antiplasmin antiserum was the kind gift of Dr. D. Collen, Leuven, Belgium. All other commercial material was of reagent grade or better.

Plasminogen Purification and Iodination. Plasminogen was purified from fresh human plasma by the affinity chromatographic technique of Deutsch and Mertz (10) and Sephadex G-100 gel filtration. The preparation exhibited an average specific activity of 21.5 CTA units per mg in the standard caseinolytic assay (11) and was homogeneous as judged by immunoelectrophoresis (12) and SDS-gel electrophoresis (13). The purified zymogen was iodinated by our modification (14) of the Chloramine T method of Hunter and Greenwood (15). The [^{125}I]-plasminogen had no detectable loss of activity upon activation with UK when measured by the standard assay (11) or on heated fibrin plates (16) and co-electrophoresed with purified unlabeled zymogen.

Assays. Plasmin, as well as plasminogen after activation with UK, were assayed by the [^{125}I]-α-casein method as originally described by Highsmith and Rosenberg (3) with some modifications (17). The qualitative presence of the protease inhibitors was determined by immunodiffusion (18) using monospecific antisera prepared against each of the inhibitor antigens.

Experimental Protocol. Fresh, citrated human plasma was drawn from normal donors and mixed with tracer amounts (<2μg) of [^{125}I]-plasminogen. The plasminogen activators, UK or SK, were then added to the mixture at either 22° or 37°. The activation conditions were as follows: at 37°-2,500 I.U. of SK/ml plasma for 15 min. or 500 CTA units of UK/ml plasma for 45 min.; at 22°-2,500 I.U. of SK/ml plasma for 3 hrs. or 500 CTA units of UK/ml plasma for 6 hrs. The resulting mixtures were then applied to a Sephadex G-200 column (2.6 x 100

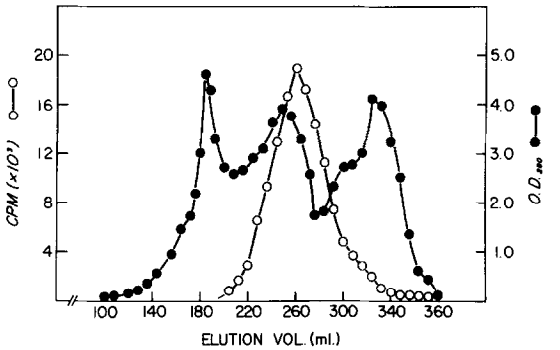

Figure 1. Sephadex G-200 gel filtration of [^{125}I]-plasminogen added to 10 ml of fresh human plasma in the absence of plasminogen activators. Closed circles (●-●) represent the absorbance at 280 nm while open circles (o-o) depict radioactivity.

cm) equilibrated with 0.15M NaCl in 0.01M Tris-HCl, pH 7.5 at 4°. The resulting peak radioactive fractions, containing either unactivated plasminogen, free plasmin or plasmin-inhibitor complexes were then subjected to LSAC (10) and eluted with epsilon-amino caproic acid in order to isolate these fibrinolytic components. Equal size aliquots of the fractions from this eluate were pooled, dialyzed against the appropriate buffer and analyzed for: 1) completeness of plasminogen activation and for the presence of free plasmin by the [^{125}I]-α-caseinolytic assay, and 2) the qualitative presence of the aforementioned inhibitors by immunodiffusion. In addition, the composition and molecular weights of the proteins in the pooled aliquot were physically monitored by SDS-gel electrophoresis in the absence of reducing agents. The amount of plasmin bound to the various inhibitors was estimated by sectioning the gels and counting the bands for radioactivity. Duplicate gels were stained and utilized in the figures below.

RESULTS

Fig. 1 depicts the Sephadex G-200 elution profile of [^{125}I]-plasminogen added to whole plasma in the absence of UK. The labeled zymogen is seen to elute as a single peak at approximately 260 ml. LSAC and subsequent SDS-gel electrophoresis of this fraction of plasma resulted in a homogeneous mixture of plasminogen and [^{125}I]-plasminogen. However, when UK is first added to the plasma containing labeled plasminogen, the elution profile is altered (Fig. 2). The resultant shift of the peak radioactive fractions reflects the formation of higher molecular weight complexes between the activated labeled enzyme and the plasma inhibitors. The two peaks, A and B, were then processed and ana-

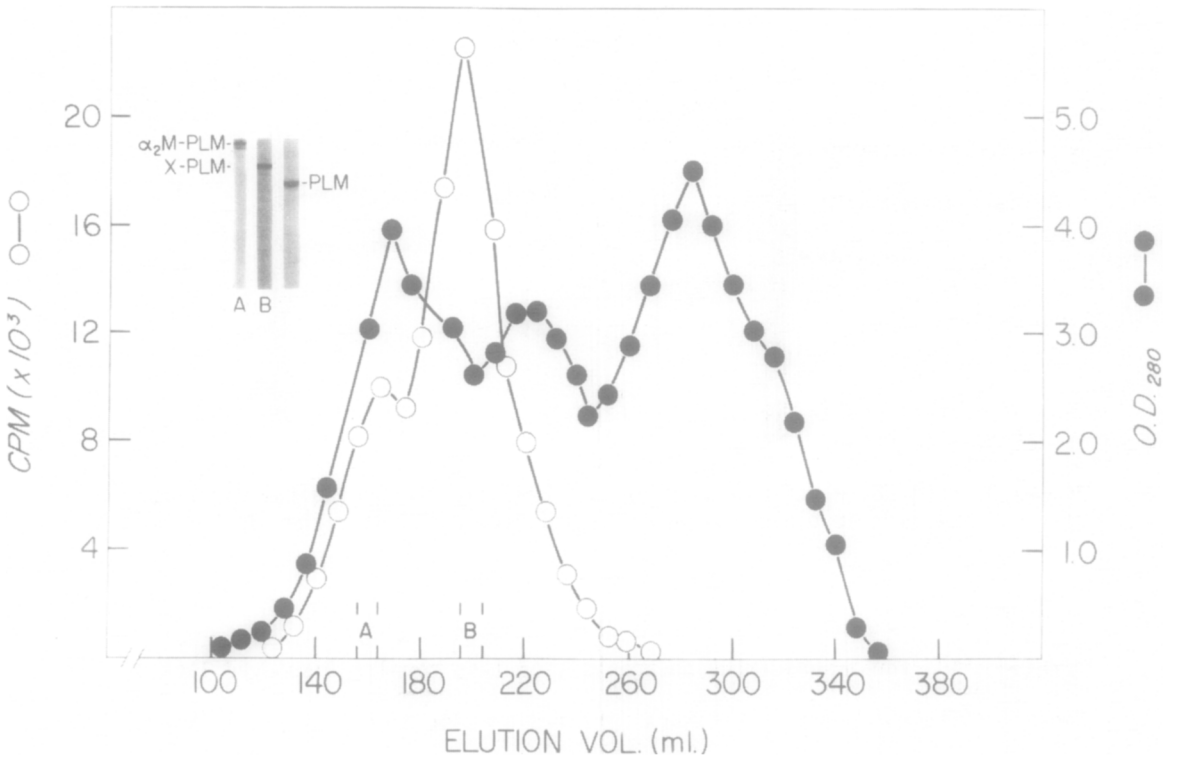

Figure 2. Sephadex G-200 elution profile of [^{125}I]-plasminogen added to 10 ml of fresh human plasma in the presence of UK.

lyzed as mentioned above. The inset indicates the SDS-gel analysis following LSAC of the peak radioactive fractions. The components of the mixture were identified by immunodiffusion and from their estimated molecular weights relative to standard marker proteins. Peak A contains a lesser, but significant amount of [^{125}I]-plasmin bound to α_2-macroglobulin. Peak B, containing the major amount of radioactive enzyme, consists of plasmin complexed to another substance in plasma indicated by an X. It is indeed a complex of plasmin with inhibitor as evidenced by: 1) the presence of radioactivity solely within the band; 2) the lack of any measurable proteolytic activity in this fraction; and 3) the increased molecular weight as compared to the control gel of purified plasmin alone also shown in the inset for comparison. The inhibitor is des-

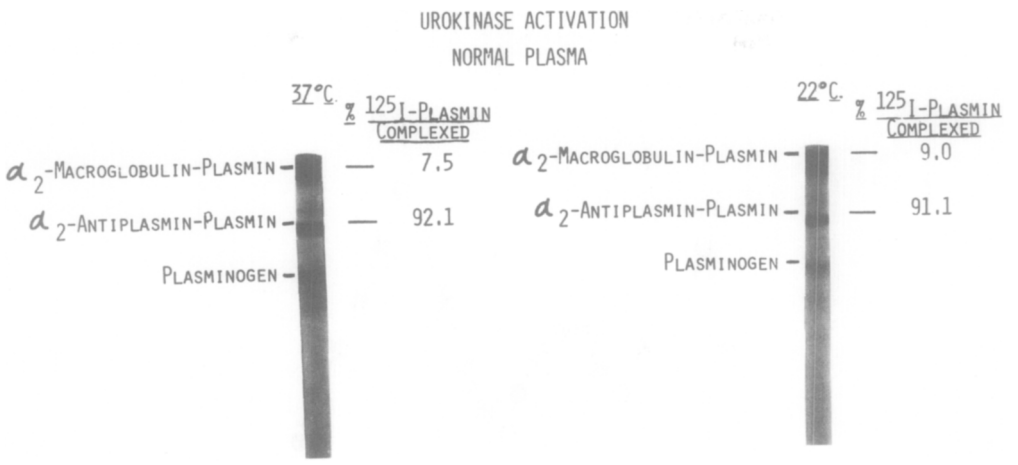

Figure 3. SDS-gel electrophoresis of the LSAC eluate showing the distribution of [^{125}I]-plasmin in plasma to which UK had been added at either 37° or 22°.

ignated "X", for immunidiffusion analysis of this complex displayed lack of identity with monospecific antisera directed against each of the well known antiplasmins. However, a reaction of full identity occurred when this complex was analyzed using α_2-antiplasmin antiserum kindly provided by Dr. D. Collen.

The next series of experiments was designed to evaluate the distribution of plasmin amongst the various plasma protease inhibitors in plasma under differing conditions of plasminogen activation. The concentration of activator, whether SK or UK and the length of incubation were predetermined to be optimal for complete plasminogen activation. The experimental protocol was the same as described earlier except that prior gel filtration on Sephadex G-200 was omitted in order to insure that the lysine-sepharose eluate would contain all of the protease inhibitor-plasmin complexes which had formed.

Fig. 3 dipicts the SDS-gel analysis of the distribution of [^{125}I]-plasmin in normal plasma to which UK had been added at either 37° or 22°. In both conditions, the presence of three major bands are seen representing: 1) a very small amount of unactivated plasminogen determined by assays; and 2) the higher molecular weight complexes of plasmin with the protease inhibitors. Clearly,

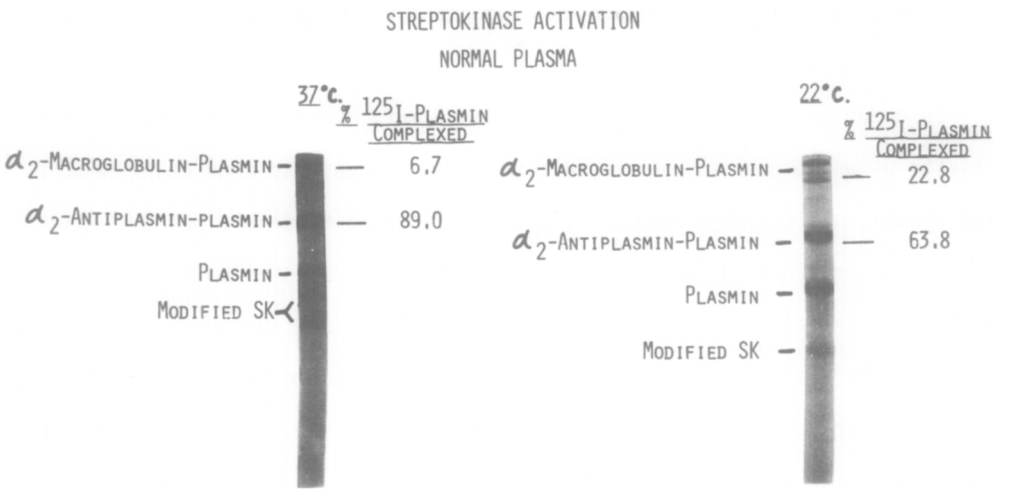

Figure 4. SDS-gel electrophoresis of the LSAC eluate showing the distribution of [^{125}I]-plasmin in plasma to which SK had been added at either 37° or 22°.

the majority of the plasmin (∿ 90%) is bound to α_2-antiplasmin. A small, but significant percentage is bound to α_2-macroglobulin (∿ 8%). No significant difference in this profile was noted when the rate of plasminogen activation was altered by activating plasma at 22°.

When SK was used as the source of activator, the lysine-sepharose eluate is seen to exist of the same three major bands as seen with UK, plus some additional minor ones representing degradation products of SK* (Fig. 4). The quantitative distribution of the labeled enzyme activated at 37° appears similar to that achieved by UK in that ∿ 90% is bound to α_2-antiplasmin and ∿ 7% bound to α_2-macroglobulin. However, at 22° the pattern is altered in that the amount bound to α_2-macroglobulin increases to ∿ 23% with a concommittant decrease in the amount bound to α_2-antiplasmin (∿ 65%). Also of interest is the significant amount of uninhibited plasmin consistently seen with SK activation. Although this band was faintly visible with UK activation, subsequent assays

*This was confirmed in experiments using [^{125}I]-streptokinase and the same experimental protocol in which the gels were sectioned and counted for radioactivity.

confirmed that it represented unactivated plasminogen in that case and not "free" plasmin as seen with SK.

In order to estimate the contribution of antithrombin to the antiplasmin activity of whole plasma, additional experiments have been done using heparinized plasma. In previous studies (3) using purified material, we have shown that in the presence of heparin, antithrombin inactivates plasmin in a very rapid, irreversible fashion. However, in a whole plasma system, a significant amount of the activated fibrinolytic enzyme was complexed to antithrombin, but only in the presence of this anticoagulant (data not shown). Precise quantitation of the amount bound to this inhibitor is not possible using this experimental protocol, for the resulting complex co-electrophoreses with that of a plasmin-α_2-antiplasmin complex.

DISCUSSION

Limited proteolysis is a central feature of many enzyme systems including the coagulation, kinin and fibrinolytic pathways. Some of these processes involve the transition of the inactive zymogen into a biologically active protein in a single step; other pathways may involve multiple steps in which amplification of small stimuli to major physiological responses occurs (19). A critically important feature in determining whether an enzymatic response to an appropriate stimuli is physiological or pathological is the ability of human plasma to localize and restrict intravascular proteolysis. This is accomplished in part by the presence in plasma of numerous protease inhibitors. These studies deal with the interaction of the fibrinolytic enzyme, plasmin, with the inhibitors in a whole plasma system. The data clearly reveal that α_2-antiplasmin is the primary antiplasmin in human plasma and are in agreement with observations made by others using different methodologies (7,8). In addition, our findings illustrate the importance of this inhibitor under varied experimental conditions in that the precise amount of activated enzyme bound to this inhibitor was found to be dependent on both the activator used as well as the rate of plasminogen activation.

Other investigators (7,8) have reported that plasmin binds to α_2-macroglobulin only after α_2-antiplasmin is saturated with enzyme. However, our present results and those reported elsewhere (9,20) indicate that a small, yet consistent percentage of the fibrinolytic enzyme is bound to α_2-macroglobulin under all conditions tested. Since complexes of plasmin with this high molecular weight inhibitor are unique in that they are proteolytically active (1), these data may help to explain why therapeutic fibrinolysis can be achieved in spite of the large molar excess of protease inhibitors in plasma.

Also of interest in this report is the consistent finding of "free", uninhibited plasmin in plasmas activated with SK, but not with UK. It is well established (21,22,23) that SK can form a stoichiometric complex with human plasmin that is readily dissociable in SDS (24). Therefore, our results may be indicative of this dissociation process occurring in the experimental protocol rather than the existence of "free" plasmin in these plasmas. In support of this interpretation is the fact that modified SK was apparent in the eluates of the lysine-sepharose affinity column. Furthermore, in order for SK to bind to the affinity ligand, it must be complexed to either plasminogen and/or plasmin. Therefore, it appears that proteolytically active SK-plasmin complexes are formed following the addition of this plasminogen activator to whole plasma. Since SK-plasmin complexes possess fibrinolytic activity (25), these results may have relevance to the speculation that SK, despite problems with antigenicity and toxicity, may be clinically more effective than UK in promoting intravascular thrombolysis. Further studies are being undertaken to investigate the interaction of this activator-enzyme complex with the plasma protease inhibitors.

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