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STUDIES ON THE MECHANISM OF THE ANTIFIBRINOLYTIC ACTION OF TRANEXAMIC ACID

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Summary

The effect of the antifibrinolytic agent tranexamic acid on the solubilization of ^{125}I-labeled fibrin by plasmin or by mixtures of plasminogen and plasminogen activator (tissue activator or urokinase) was studied. The time required to solubilize half of the radioactivity (S_{50}) decreased curvilinearly with the logarithm of the concentration of plasmin or plasminogen. Tranexamic acid caused a concentration-dependent retardation of fibrinolysis. When corresponding S_{50} values were converted to apparent concentrations of plasmin or plasminogen in the absence of tranexamic acid, sigmoidal relationships were obtained between the apparent plasmin(ogen) concentration and the logarithm of the concentration of tranexamic acid. When tissue activator (with a high affinity for fibrin) was used, the shape of these curves was compatible with single association reactions between plasminogen and tranexamic acid. A 50% decrease of the apparent plasminogen concentration was obtained at 1.2 μM tranexamic acid for native plasminogen (Glu-plasminogen) and 2.3 μM for proteolytically degraded plasminogen (Lys-plasminogen). The dissociation constants of the interaction between tranexamic acid and the high affinity lysine-binding site of Glu-plasminogen or Lys-plasminogen have previously been estimated at 1.1 and 2.2 μM, respectively. Direct measurements of the binding of ^{125}I-labeled plasminogen to fibrin clots revealed that tranexamic acid displaced plasminogen from the fibrin surface; a 50% displacement was obtained at 1.3 μM tranexamic acid for Glu-plasminogen and 5.0 μM for Lys-plasminogen. These observations are compatible with the interpretation that saturation of the high affinity lysine-binding site of plasminogen with tranexamic acid results in its displacement from the fibrin surface and abolishes its activation by fibrin-bound plasminogen activator.

When native or degraded plasminogen was activated with urokinase (with a low affinity for fibrin), tranexamic acid also caused a concentration-dependent retardation of fibrinolysis but of a much more complex nature. Saturation of the high affinity lysine-binding site in plasminogen with tranexamic acid had no significant influence on the solubilization rate of fibrin, indicating that binding of plasminogen to fibrin is of little importance for its activation by urokinase. At higher tranexamic acid concentrations an enhancement of Glu-plasminogen activation was observed, followed by interference of tranexamic acid with fibrinolysis by plasmin. These effects have already been described previously.

Tranexamic acid caused a concentration-dependent retardation of fibrinolysis by plasmin; a 50% reduction of the apparent plasmin concentration was obtained at 45 μM tranexamic acid.

Introduction

Human plasminogen contains structures, called lysine-binding sites, which are of importance not only for its interaction with antifibrinolytic amino acids such as tranexamic acid but also with the main physiological plasmin inhibitor, α_2-antiplasmin, and with fibrin. These lysine-binding sites appear to play a crucial role in the regulation of fibrinolysis [1,2].

Native human plasminogen (Glu-plasminogen) contains one lysine-binding site with a high affinity for tranexamic acid (K_d = 1.1 μM) and four or five with low affinity (K_d = 750 μM) [3]. Partially degraded plasminogen (Lys-plasminogen) has one site which binds tranexamic acid with K_d = 2.2 μM, one with K_d = 36 μM and about two or three sites with K_d = 1000 μM [3].

The rate of the reaction between plasmin and α_2-antiplasmin is strongly dependent on the availability of lysine-binding sites in the enzyme [4—6]. Saturation of the high affinity lysine-binding site of plasmin reduces the reaction rate about 50 times [5,6].

The binding of plasminogen to fibrin is also mediated through the lysine-binding sites and this interaction is virtually completely abolished in the presence of antifibrinolytic amino acids [7—9]. Displacement of plasminogen from the fibrin surface is probably responsible for the antifibrinolytic effect of these compounds. The relative role of the different lysine-binding sites in the interaction between plasminogen and fibrin is, however, not clearly established.

In the present paper evidence is presented that primarily the high affinity lysine-binding site of plasminogen is involved in its binding to fibrin, that saturation of this binding site with tranexamic acid displaces plasminogen from the fibrin surface and that this results in a retardation of fibrinolysis if tissue plasminogen activator is used for activation.

Materials and Methods

Plasminogen

Native plasminogen (NH_2-terminal glutamic acid, Glu-plasminogen) was prepared from human plasma by affinity chromatography on lysine-Sepharose

[10] and gel filtration on Ultrogel AcA 44. The two types of plasminogen with different affinities towards lysine-Sepharose (plasminogen I and plasminogen II) were separated using a linear gradient of 6-aminohexanoic acid, as described by Brockway and Castellino [11].

Partially degraded plasminogen (Lys-plasminogen) was generated by limited proteolysis of Glu-plasminogen (6 mg/ml in 0.1 M NH_4HCO_3) by plasmin (molar ratio, 1 : 100) for 30 min at 37°C [12]. The solution was then passed over a 0.9 × 15 cm column of aprotinin-Sepharose (0.6 mg protein per ml gel). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified protein showed complete conversion to Lys-plasminogen, and end group analysis by Edman degradation showed only lysine and valine as NH_2-terminal amino acids.

Low molecular weight plasminogen (triple loop number 5 of the plasmin A chain and the intact plasmin B chain, M_r 39 000) was obtained by elastase digestion of plasminogen, followed by gel filtration on Sephadex G-75 and affinity chromatography on lysine-Sepharose essentially as described by Sottrup-Jensen et al. [13]. The concentration of the different types of plasminogen was measured spectrophotometrically using $A^{1\%}_{1cm} = 16.1$ at 280 nm [14]. Plasminogen was labeled with ^{125}I ($Na^{125}I$, IRE, Fleurus, Belgium) with a substitution level of 0.07 atoms of iodine per molecule, according to the method of McFarlane [15].

Fibrinogen

Fibrinogen was prepared from freshly frozen blood bank plasma according to the method of Blombäck and Blombäck [16]. The concentration was determined spectrophotometrically using $A^{1\%}_{1cm} = 15.1$ at 280 nm [16]. ^{125}I-labeled fibrinogen was obtained by labelling with $Na^{125}I$, using a reduced volume version of the chloramine-T method [17].

Thrombin

Human thrombin was purified essentially as described by Fenton et al. [18]. The concentration was determined spectrophotometrically using $A^{1\%}_{1cm} = 18.0$ at 280 nm [18].

Tissue plasminogen activator

Tissue plasminogen activator was prepared from human uterine tissue as described previously [19]. It was a kind gift from Dr. D.C. Rijken (Center for Thrombosis and Vascular Research, Leuven). The activity was expressed in urokinase equivalent units by comparison of their fibrinolytic activity on plasminogen-enriched fibrin films [20].

Other reagents

Urokinase was a kind gift from Abbott Laboratories, North Chicago, IL, U.S.A. (courtesy of Dr. Sewell). Plasmin, the plasmin substrate D-Val-Leu-Lys-Nan (S-2251, Coatest), streptokinase (Kabikinase) and tranexamic acid were gifts from Kabi AB, Stockholm, Sweden. Elastase was purchased from Sigma, St. Louis, MO, U.S.A.; *p*-nitrophenyl-*p'*-guanidinobenzoate, Tween 80 and gela-

tin from Merck, Darmstadt, F.R.G.; Sephadex products from Pharmacia, Uppsala, Sweden; and Ultrogel AcA 44 from LKB, Stockholm, Sweden.

Activation of plasminogen by streptokinase

Streptokinase (2000 I.U./mg plasminogen) was used to measure maximal activatability of the different plasminogen types, which were dissolved to a final concentration of 8 mg per ml at 25°C in 0.1 M phosphate buffer, pH 7.30, containing 25% glycerol. After incubation for 16 h at 4°C the concentration of generated plasmin was determined by active site titration with *p*-nitrophenyl-*p'*-guanidinobenzoate [21]. The extent of activation obtained with the different preparations used in the present study was over 95% (low molecular weight plasminogen could be activated to 85%). Plasmin (Kabi) and low molecular weight plasmin (upon activation with streptokinase) were titrated with *p*-nitrophenyl-*p'*-guanidinobenzoate before use.

Solubilization of ^{125}I-labeled fibrin by mixtures of plasminogen and tissue plasminogen activator

Fibrinolysis was measured essentially as described by Unkeless et al. [22]. ^{125}I-labeled fibrinogen was diluted with unlabeled fibrinogen in distilled water to a final concentration of 150—180 μg/ml and $4 \cdot 10^6$ cpm/ml. Of this solution 100 μl was introduced in each well of a tissue culture plate (Falcon, Becton and Dickinson, CA, U.S.A.). After drying at 37°C for 15 h, 100 μl of a 0.04 μg/ml solution of thrombin was added to the fibrinogen layer. After incubation for 15 h at 37°C the liquid was decanted and each well washed three times with 100 μl phosphate-buffered saline, pH 7.4, containing 0.25% gelatin and 0.006% Tween 80. In this way more than 70% of the ratioactivity applied to the wells was incorporated in the fibrin film. The plates were stored at room temperature and used for up to one week. Before use each well was washed twice again to remove small amount of soluble radioactive material.

Different concentrations of plasminogen and human tissue plasminogen activator or urokinase (final concentration 0.25 I.U. per ml) were incubated in the wells in a final volume of 200 μl and the tissue culture plates were rotated at 37°C. At fixed time intervals 10-μl samples were removed, and released ^{125}I was counted in a Berthold scintillation counter BF 5300 (Benelux Analytical Instruments, Vilvoorde, Belgium). The released cumulative radioactivity was plotted against time and the time required to solubilize half of the radioactive fibrin (S_{50}) was determined. Alternatively, 200 μl of different concentrations of plasmin or low molecular weight plasmin were incubated in the wells and the samples were treated in the same way.

Influence of tranexamic acid on the solubilization of ^{125}I-labeled fibrin

The different forms of plasminogen were preincubated with tranexamic acid for 30 min. These mixtures were then added to wells containing ^{125}I-labeled fibrin and 0.05 I.U. of tissue plasminogen activator or urokinase to a final plasminogen concentration of 50, 100 or 200 nM and tranexamic acid concentrations of 0—15 μM or 0—1000 μM in a final volume of 200 μl. The released ^{125}I was measured as described above and the time required to solubilize half of the radioactive fibrin (S_{50}) was determined.

Alternatively, plasmin was preincubated with tranexamic acid to final plasmin concentrations of 0.4 or 0.8 nM and 0—400 μM tranexamic acid, before transfer of 200 μl of these solutions to the ^{125}I-labeled fibrin wells and further treatment as described.

^{125}I-labeled plasminogen binding to fibrin clots in the presence of tranexamic acid

^{125}I-labeled Glu-plasminogen or Lys-plasminogen were diluted with unlabeled plasminogen to final concentrations of 50 nM and approximately 8000 cpm per ml. To these solutions tranexamic acid (0—2.3 mM final concentration) and fibrinogen (1.2 mg/ml final concentration) were added in a total volume of 2 ml. Clots formed at room temperature within 15 min after addition of 0.2 μg human thrombin were rotated onto a glass rod. After incubation for 1 h at 37°C the clot was dried by blotting on filter paper and the radioactivity associated with fibrin was measured.

Results

Fig. 1 illustrates the solubilization of ^{125}I-labeled fibrin by varying concentrations of Glu-plasminogen following activation with tissue plasminogen activator (0.25 I.U./ml). Similar curves were obtained for plasminogen I, plasminogen II and Lys-plasminogen. At concentrations between 5 and 200 nM of plasminogen the rate of solubilization increased with increasing plasminogen concentration. Solubilization curves obtained with higher or lower concentrations of tissue plasminogen activator had a similar appearance but the solubilization rates were, respectively, faster or slower.

When low molecular weight plasminogen (10—5000 nM) was activated with tissue plasminogen activator, a slow and nearly linear increase of the cumulative released radioactivity was observed, and S_{50} values could not be determined accurately. When Glu-plasminogen or Lys-plasminogen were activated with 0.25 I.U./ml of urokinase, solubilization curves were obtained similar to those shown in Fig. 1.

The solubilization of ^{125}I-labeled fibrin by plasmin or by low molecular weight plasmin also yielded sigmoidal curves with similar appearance to those shown in Fig. 1.

Fig. 2a illustrates the relationship between the time required to solubilize 50% of the fibrin (S_{50}) and the plasminogen concentration for Glu-plasminogen, plasminogen I, plasminogen II and Lys-plasminogen following activation with tissue plasminogen activator. It appears that plasminogen II dissolves fibrin about two to four times faster than plasminogen I since about 2—4-fold higher concentrations of plasminogen I are required to yield the same S_{50} values as obtained with plasminogen II. It also appears that Lys-plasminogen is 3—5-fold more efficient than Glu-plasminogen. Using different concentrations of tissue plasminogen activator, the same relationships were found.

The relationship between S_{50} and the concentration of Glu- or Lys-plasminogen following activation with urokinase is shown in Fig. 2b and that between S_{50} and the concentration of plasmin in Fig. 2c. In both cases the rate of fibrinolysis was found to be more strongly dependent on the concentration of plas-

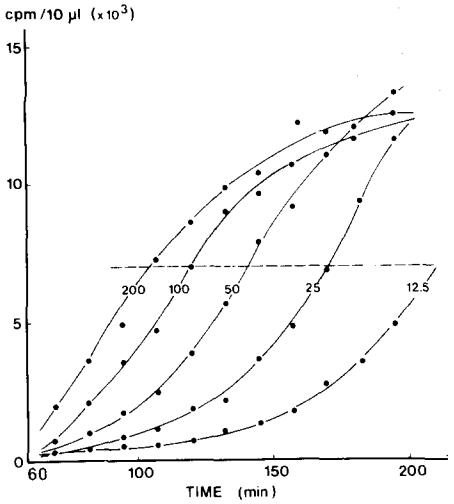

Fig. 1. Cumulative release of ^{125}I from a ^{125}I-labeled fibrin film by mixtures of human Glu-plasminogen (12.5—200 nm) and tissue plasminogen activator (0.25 I.U./ml). The dotted line indicates solubilization of 50% of the ^{125}I-labeled fibrin film.

minogen or plasmin than observed with tissue plasminogen activator.

Tranexamic acid induces a concentration-dependent retardation of fibrinolysis by Glu-plasminogen, plasminogen I, plasminogen II and Lys-plasminogen when activated with tissue plasminogen activator. This influence on the solubilization of ^{125}I-labeled fibrin is illustrated for Glu-plasminogen in Fig. 3. For each concentration of tranexamic acid the S_{50} value was determined (dashed line). Using conversion curves, such as shown in Fig. 2a, obtained in simultaneous experiments, the corresponding apparent plasminogen concentrations were determined.

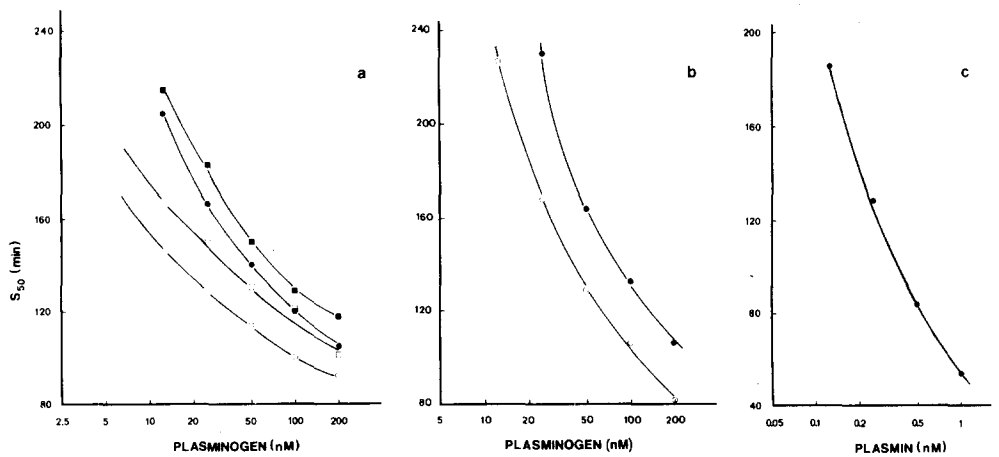

Fig. 2. Influence of plasmin(ogen) concentration on the time required to solubilize 50% of the ^{125}I-labeled fibrin. a, activation with 0.25 I.U./ml tissue plasminogen activator; b, activation with 0.25 I.U./ml urokinase; c, following addition of 0.1—1 nM plasmin. ●, Glu-plasminogen (plasmin in c); ○, Lys-plasminogen; ■, plasminogen I; □, plasminogen II.

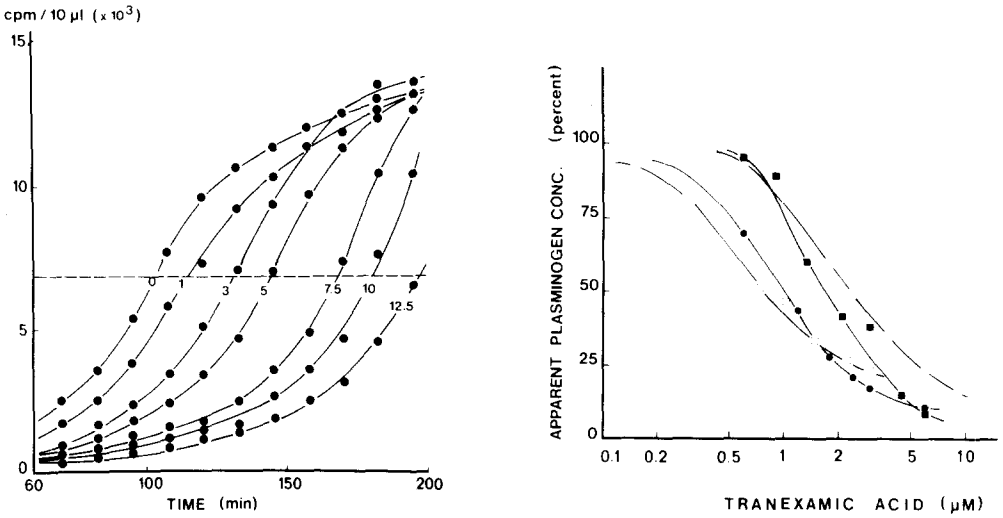

Fig. 3. Influence of tranexamic acid (0—12.5 µM) on the cumulative release of ^{125}I from a ^{125}I-labeled fibrin film by mixtures of human Glu-plasminogen (200 nM) and tissue plasminogen activator (0.25 I.U. per ml). The dashed line indicates solubilization of 50% of the ^{125}I-labeled fibrin film.

Fig. 4. Plots of the logarithm of the concentration of tranexamic acid (0—10 µM) versus the apparent plasminogen concentration (in percent of 50 nM added to the mixture) for Glu-plasminogen (●), Lys-plasminogen (○), plasminogen I (■) and plasminogen II (□).

Fig. 4 shows the relationship between the apparent plasminogen concentration and the concentration of tranexamic acid for Glu-plasminogen, plasminogen I, plasminogen II and Lys-plasminogen. In two separate experiments a decrease of the apparent plasminogen concentration to 50% was obtained at concentrations of tranexamic acid of 1.0 and 1.3 µM for Glu-plasminogen, 1.75 and 1.45 µM for plasminogen I, 0.75 and 0.45 µM for plasminogen II and 2.25 and 2.35 µM for Lys-plasminogen.

Tranexamic acid caused a concentration-dependent retardation of fibrinolysis by plasmin yielding curves with a similar appearance to those shown in Fig. 3 but requiring higher concentrations of tranexamic acid. When S_{50} values were converted to apparent plasmin concentrations (as described for plasminogen) a sigmoidal relationship was obtained between the apparent plasmin concentration and the concentration of tranexamic acid with a 50% decrease at 45 µM of tranexamic acid (Fig. 5). Similar results were obtained for low molecular weight plasmin.

When Glu- or Lys-plasminogen were preincubated with tranexamic acid and activated by urokinase, a concentration-dependent retardation of fibrinolysis was observed. However, when S_{50} values were converted to apparent plasminogen concentrations, much more complex relationships were observed between the apparent plasminogen concentrations and the concentration of tranexamic acid (Fig. 6). For Glu-plasminogen an initial slight decrease in the apparent plasminogen concentration is followed by an increase and finally by a strong decrease at very high concentrations of tranexamic acid. For Lys-plasminogen a biphasic relationship is obtained between the apparent plasminogen

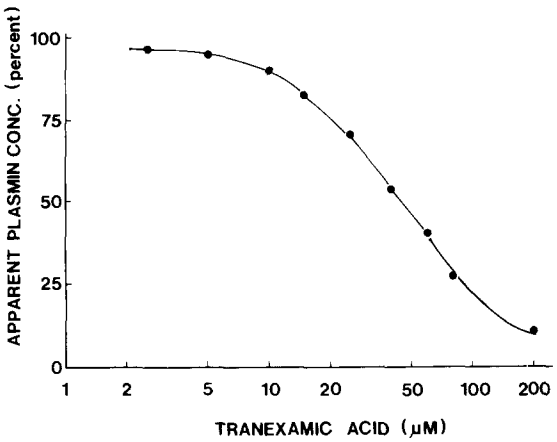

Fig. 5. Plots of the logarithm of the concentration of tranexamic acid (2.5—200 μM) versus the apparent plasmin concentration (in percent of 0.4 nM added to the wells).

concentration and tranexamic acid, with a slow decrease at low concentrations and a fast decrease at higher concentrations.

Fig. 7 illustrates the influence of tranexamic acid on the binding of ^{125}I-labeled Glu-plasminogen and Lys-plasminogen to fibrin. In the absence of tranexamic acid about 6.2% of ^{125}I-labeled Glu-plasminogen and about 22% of ^{125}I-labeled Lys-plasminogen remained associated with fibrin. With increasing concentrations of tranexamic acid these values decreased to a level of 1.25% of Glu-plasminogen and 1.9% of Lys-plasminogen at concentrations above 1 mM tranexamic acid. We assumed that these limit values are due to mechanical entrapment of labeled plasminogen into the fibrin network and subtracted these backgrounds from the values obtained at lower concentrations of tranexamic acid. The binding of plasminogen to fibrin in the presence of tranexamic acid was then expressed in percentage of the values obtained in its absence. The binding of Glu-plasminogen to fibrin was reduced to 50% at 1.3

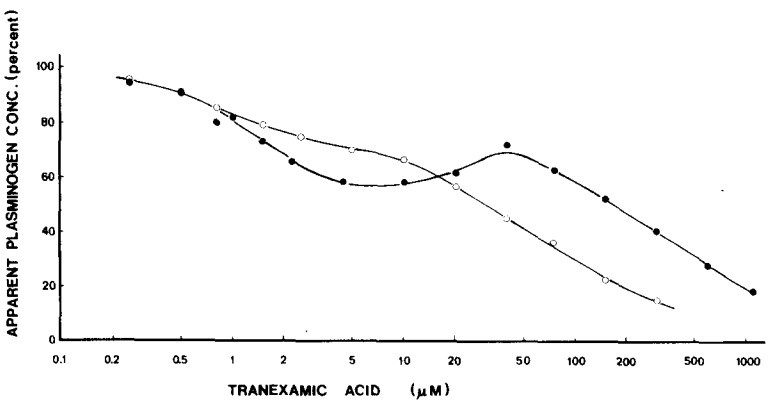

Fig. 6. Plots of the logarithm of the concentration of tranexamic acid (0.25—1100 μM) versus the apparent plasminogen concentration (in percent of 50 nM added to the mixture) for Glu-plasminogen (●) and Lys-plasminogen (○).

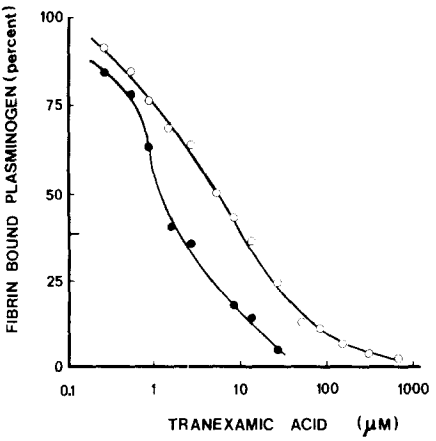

Fig. 7. Influence of tranexamic acid on the binding of ^{125}I-labeled Glu-plasminogen (●) or Lys-plasminogen (○) to fibrin clots. The data are corrected for aspecific binding and/or mechanical entrapment at high concentration of tranexamic acid (1 mM) and expressed as percent of the values obtained in the absence of tranexamic acid.

μM tranexamic acid and that of Lys-plasminogen at 5.0 μM, as determined in four separate experiments.

Discussion

The present study deals with the role of the lysine-binding sites of plasminogen in the solubilization of a fibrin film by mixtures of plasminogen and tissue plasminogen activator. In agreement with earlier findings we observed that the rate of solubilization of a fibrin film depends on the concentrations of fibrin, plasminogen and plasminogen activator and that the curve representing the cumulative released radioactivity in time followed a sigmoidal pattern [22, 23], composed of a lag phase, on exponential increase of the solubilization and termination of the reaction due to exhaustion of substrate (Fig. 1). Since the solubilization of fibrin is a sigmoidal process the time required to solubilize half of the fibrin (S_{50}) appeared to be a more accurate measure of the reaction rate than initial rates of solubilization, which were very low. Fibrin films of 15 μg/well were used because under those conditions reproducible clots and reasonably short solubilization rates were obtained with the use of small amounts of tissue plasminogen activator. S_{50} values decreased curvilinearly when plotted against the logarithm of plasminogen concentration in the concentration range between 5 and 200 nM. At higher plasminogen concentrations the influence of the plasminogen concentration on the S_{50} value decreases gradually. This is probably due to saturation of fibrin-bound plasminogen activator with its substrate. Wallén and Rånby [24] have indeed recently estimated the K_m of this reaction to be 150 nM.

It appeared that the type of plasminogen influenced the rate of fibrinolysis. Thus Lys-plasminogen was about three to five times more efficient than Glu-plasminogen. This might have been anticipated since Lys-plasminogen is more easily activated (at least by urokinase) than Glu-plasminogen [25—27] and

binds more strongly to fibrin [8,9]. The two forms of Glu-plasminogen which can be separated by chromatography on lysine-Sepharose also induce a slightly different rate of fibrinolysis, plasminogen II being more efficient than plasminogen I. In purified systems, however, plasminogen I is more rapidly activated than plasminogen II [28], and this was confirmed in the present study. Thus, the activatability by urokinase in solution appears to bear no direct relationship to activation rates on fibrin clots by fibrin-bound tissue plasminogen activator. A low molecular weight form of plasminogen lacking the lysine-binding sites reacted very poorly on a fibrin film. This observation is in line with the hypothesis that the lysine-binding sites in plasminogen play a key role in the regulation of fibrinolysis [1,2].

Preincubation of plasminogen and tranexamic acid resulted in a concentration-dependent delay of fibrinolysis as evidenced by a prolongation of the S_{50} values with increasing concentration of tranexamic acid. When the S_{50} values obtained in the presence of tranexamic acid were converted to their corresponding apparent plasminogen concentrations in the absence of tranexamic acid, a sigmoidal relationship was obtained between the apparent plasminogen concentration and the logarithm of the tranexamic acid concentration. The appearance of this sigmoidal curve appeared to be compatible with a decrease of the apparent plasminogen concentration as a result of a single association reaction between plasminogen and tranexamic acid when tissue activator was used for activation. A 50% reduction of the apparent plasminogen concentration was obtained at a tranexamic acid concentration of 1.2 μM for Glu-plasminogen and 2.3 μM for Lys-plasminogen. These values are nearly identical to those obtained for the dissociation constant between tranexamic acid and the high affinity lysine-binding site in Glu-plasminogen or in Lys-plasminogen [3]. Tranexamic acid indeed reduced the extent of binding of ^{125}I-labeled plasminogen to fibrin; a 50% decrease was obtained at 1.3 μM tranexamic acid for Glu-plasminogen and of 5.0 μM for Lys-plasminogen. These data are in reasonably good agreement with those obtained for the dissociation constants between tranexamic acid and the high affinity lysine-binding sites in Glu-plasminogen and Lys-plasminogen.

Preincubation of plasmin with tranexamic acid showed a sigmoidal decrease of the apparent plasmin concentration compatible with a single association reaction between plasmin and tranexamic acid with a dissociation constant of 45 μM. At concentrations below 10 μM of tranexamic acid no interference with the digestion of fibrin was observed. Similar results were obtained with low molecular weight plasmin lacking lysine-binding sites, indicating that availability of these sites is no longer of critical importance for the fibrinolytic activity of the enzyme. Comparison of the conversion curves of Fig. 2a and Fig. 2c indicate that only very small amounts of plasmin are generated throughout the experiments reported in Figs. 1 and 3, indicating that the equilibrium plasminogen-tranexamic acid is not shifted by generation of plasmin and that the fibrinolytic activity of plasmin is not modified by tranexamic acid.

Since tranexamic acid (0—10 μM) has no influence on the activation of plasminogen by tissue plasminogen activators in the absence of fibrin, it is concluded that its inhibitory effect in the presence of fibrin is due to dissociation of plasminogen from the fibrin surface as a result of saturation of its high affinity lysine-binding site.

The influence of tranexamic acid on the solubilization of labeled fibrin by Glu-plasminogen and Lys-plasminogen following activation by urokinase is more complex. Saturation of the high affinity lysine-binding site of either form of plasminogen has little influence on the lysis rate, indicating that activation of fibrin-bound plasminogen is not of prime importance for fibrinolysis. At high concentrations of tranexamic acid the fibrinolytic process is retarded, most likely as a result of interference with formed plasmin. For Glu-plasminogen, however, the apparent plasminogen concentration appears to increase at concentrations of tranexamic acid between 10 and 100 μM and the final decrease seems to require higher concentrations of tranexamic acid than in the case of Lys-plasminogen. This effect can be explained by earlier observations that tranexamic acid at concentrations above 10 μM induces a conformational change in the Glu-plasminogen molecule leading to enhanced activation by urokinase in solution [3,29]. From these observations it is concluded that tranexamic acid has a much weaker and less specific effect on fibrinolysis following activation of plasminogen with urokinase.

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