A CHARACTERIZATION OF
PLASMINOGEN AND THE EFFECTS
OF LYSINE ON ITS ACTIVATION

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INTRODUCTION

The zymogen plasminogen has come under considerable study in recent years. Plasmin, the active form of plasminogen, is largely responsible for the dissolution of fibrin blood clots in all mammals, but has also been noted for other activities. Some of these activities are: accessory in ovulation (1), tissue remodeling, trophoblast implantation, and embryogenesis (2), inflammation (3), mammary gland involution (4), cell migration (5), and proinsulin cleavage (6). Plasmin itself is a very potent, non-specific protease and yet, is still involved in all these functions. Being a non-specific protease, plasmin must therefore be highly regulated at all times. Our major concern, however, is the interactions of plasminogen in the fibrinolytic system which also is highly regulated (7). The basic components are plasminogen, plasminogen activators, and plasmin. Under normal physiological conditions the basic components of the fibrinolytic system are present in the blood and most extracellular fluids. Plasminogen at a level of about 0.5% of the total serum protein (8), and plasminogen activators in trace amounts, co-exist together with plasminogen being activated specifically and locally only at a fibrin clot. How, on one hand, can plasminogen and plasminogen activators co-exist without all the plasminogen being converted to plasmin and yet, on the other hand, how can plasminogen be converted to plasmin at a fibrin clot? Here we address these questions by analyzing the kinetics of activation of two forms of plasminogen by urokinase in the absence and presence of lysine. By implication, the data shows how fibrin may regulate plasminogen activation.

Native plasminogen prepared by affinity chromatography from fresh plasma has glutamic acid at its NH₂-terminal (9) and asparagine at its COOH-terminal
The plasminogen molecule is composed of 790 amino acids and is stabilized by 24 disulfide bridges. Plasmin is formed by proteolytic cleavage of a single arginyI-valine bond (10,11) at a position 560 amino acids from the NH₂-terminal glutamic acid. The larger chain termed the A-chain, or heavy chain (mol. wt. about 60,000), contains the lysine binding sites and is derived from the NH₂-terminal portion of the molecule. The smaller chain termed the B-chain, or light chain (mol. wt. about 25,000), contains the serine and histidine of the active site (10). Plasmin can catalytically cleave native Glu-plasminogen at a lysyl-lysine bond at position 76-77 from the NH₂-terminal yielding a small peptide and a form of plasminogen termed Lys-plasminogen (12).

Certain α-,ω-amino acids have been shown to bind to both Glu- and Lys-plasminogen. There are two classes of binding sites on Glu-plasminogen for ε-amino-N-caproic acid, one strong site with a dissociation constant, K₀, of 9 μM and five weaker sites with a K₀ of 5 mM (13). With Lys-plasminogen, there are also six binding sites, one with a K₀ of 35 μM, one with a K₀ of 0.26 mM, and four with a K₀ of 10 mM (14). L-lysine also binds to these sites but with a much lower affinity. Lysines K₀ for the first ligand site on Glu-plasminogen is about 30-fold larger for ε-amino-N-caproic acid and about 150-fold larger for the secondary sites (15). The binding of fragments of plasmin to lysine-Sepharose indicates that the lysine binding sites are confined to the A-chain of plasmin (16). Furthermore, these lysine binding sites are the sites at which plasminogen binds to fibrin, because binding of plasminogen to fibrin can be abolished by the presence of ε-amino-N-caproic acid (17).

The binding of α-,ω-amino acids to the lysine binding sites alters some of the physical properties of Glu-plasminogen but not Lys-plasminogen. The
presence of ε-amino-N-caproic acid or lysine in a solution of Glu-plasminogen causes a reversible decrease in the sedimentation coefficient (18) from 5.75 to 4.85 (19) and an increase of 7% in the realitive fluorescence. The concentrations required to produce one-half of the change in the sedimentation coefficient or in fluorescence intensity are about 2.7 μM for ε-amino-N-caproic acid and 25.5 μM for lysine. Under similar conditions, no change in conformation can be detected with Lys-plasminogen. Those and other experiments including gel filtration (20), circular dichroism (21), and rotational diffusion measurements (22) indicate that the binding of α-,ω-amino acids to Glu-plasminogen results in an "opening" of the structure. This conformational change is similar to that observed upon conversion of Glu-plasminogen to Lys-plasminogen or to plasmin. In both cases, the sedimentation coefficient is decreased, the Stokes’ radius increased, and the circular dichroism pattern in the near UV is changed (20,19,21).

The commercially available plasminogen activator urokinase, which is extracted from urine, is the most widely used activator for the study of plasminogen. Urokinase activates plasminogen to plasmin by cleavage of a single arginyl-valine bond whereas, plasmin cleaves the NH₂-terminal peptide of Glu-plasminogen (25). It was once thought that urokinase cleaved the NH₂-terminal peptide of plasminogen, but further studies show that this cleavage was indeed autoproteolysis by plasmin. The general scheme for the activation of native Glu-plasminogen to Lys-plasmin in vitro and in vivo is as follows: (25)

\[ \text{Glu-plasminogen} \rightarrow_{\text{Pmg-Act.}} \text{Glu-plasmin} \quad \text{slow} \]
\[ \text{Glu-plasminogen} \rightarrow_{\text{Glu-Pan}} \text{Lys-plasminogen} \quad \text{fast} \]
\[ \text{Lys-plasminogen} \rightarrow_{\text{Pmg-Act.}} \text{Lys-plasmin} \quad \text{fast} \]

When Glu-plasminogen is activated by urokinase (or plasminogen activators), the plasmin formed will autocatalytically cleave other Glu-plasminogen
molecules to Lys-plasminogen which in turn will be readily activated to plasmin. Glu-plasminogen will not be activated except in high concentrations of plasminogen and urokinase. In the presence of α-,ω-amino acids, however, the kinetics of the activation of plasminogen are altered. In the absence of α-,ω-amino acids, the rate of activation of Glu-plasminogen by urokinase is 10- to 20-fold less than that of Lys-plasminogen (14, 27, 28, 29). In the presence of ε-amino-N-caproic acid, the rate of activation of Glu-plasminogen by urokinase increases 10- to 20-fold whereas that of Lys-plasminogen is unaffected. At high concentrations of ε-amino-N-caproic acid, the rates of activation of both Glu- and Lys-plasminogen by urokinase begin to decrease.

Recently, we developed a sensitive, quantitative assay for the activation of plasminogen to plasmin (30). This type of assay was needed because when high concentrations of plasmin are allowed to build up in an assay before determining the amount of plasmin formed, that plasmin will begin to degrade itself, plasminogen, and the plasminogen activator. Our assay consists of two steps. First plasminogen is incubated with a plasminogen activator, and then the amount of plasmin formed is measured by an active site titration with a new, fluorogenic substrate. The assay is sensitive enough to be able to detect, quantitatively, plasmin at concentrations as low as $10^{-9}$ to $10^{-12}$ M. With this assay, we have begun to characterize the activation of plasminogen and the factors that regulate it.
MATERIALS AND METHODS

Materials used for plasminogen purification including DEAE (Diethylaminoethyl) sephadex A-50, DEAE Sephacel, Sephadex G-25 and Lysine-Sepharose were purchased from Pharmacia; $\epsilon$-amino-$N$-caproic acid ($\epsilon$-ACA) was purchased from Calbiochem, and Diiisopropylfluorophosphate (DFP) and Benzamidine HCl were obtained from Aldrich. For the purpose of activations and NH$_2$-terminal analysis, ur-\textsuperscript{V}ename (UK) was purchased from Leo Pharmaceuticals as a lyophyized powder at 2240 Plough units per vial; the aprotinin Trasylol\textsuperscript{a} was obtained from Mobay Chemical Corp.; Urea and Trichloroacetic acid (TCA) were purchased from Mallinckrodt; and Cheng Chin poylamide layer sheets were obtained from Pierce. Tris (hydroxymethyl) HCl under the name of Trizma HCl, was purchased from Sigma as well as Dansyl-Chloride\textsuperscript{b} (DNS-Cl), all other Dansyl-amino acid standards and L-lysine. All reagents used for electrophoresis were obtained from Bio-Rad. The Dimethylformamide (DMF), purchased from Fisher, was redistilled fresh before use. Phosphate buffered saline (PBS) used in all plasminogen dilutions contained:

\[
0.137 \text{ M NaCl, 2.68 mM KCl, 8.00 mM Na}_2\text{HPO}_4
\]

\[
1.47 \text{ mM KH}_2\text{PO}_4, 0.91 \text{ mM CaCl}_2 \text{ and 0.49 mM MgCl}_2
\]

with pH adjusted to 7.4

\textsuperscript{a}Dansyl and DNS are the abbreviations for 5-dimethylaminonapthalene-1-sulfonyl

Purification - Both Dog plasminogen and Human plasminogen were used in the following experiments. Dog plasminogen was obtained from freshly drawn blood, while Human plasminogen was purified from outdated plasma fractions.
The purification of both types of plasminogen was performed by the method of Deutsch and Mertz (31). The procedure is as follows: The whole blood or plasma was spun twice at 27,000 x g in 250 ml plastic centrifuge bottles in a refrigerated centrifuge at 4°C. The plasma, with all particulate matter removed, was then pumped into a Lysine-Sepharose column (2.6 x 90 cm) at a flow rate of 24 ml/hr, washed with PBS buffer, minus magnesium and calcium, and then with 0.3 M KPO₄, 3 mM EDTA buffer (pH 7.4) to remove non-specifically bound proteins. Human plasminogen was removed from the column with a gradient of 0 to 0.16 M ε-ACA in 0.1 M KPO₄ buffer while Dog plasminogen was removed with a gradient of 0 to 0.3 M ε-ACA in 0.1 M KPO₄. The gradient separates the plasminogen into two fractions (Figure 1) according to differences in carbohydrate content (32) and are designated FI and FII respectively. Each was pooled individually and the serine protease inhibitor, DFP, was added to a final concentration of 4 mM to inhibit any plasminogen activator and plasmin that may be present. Solid ammonium sulfate [(NH₄)₂SO₄] at 0.31 grams per ml of plasminogen, was added to precipitate the plasminogen and to remove the ε-ACA. After centrifugation, the plasminogen was redissolved in 2-3 ml PBS and placed on a Sephadex G-25 column to remove the (NH₄)₂SO₄ salts. After collecting the plasminogen fractions, the plasminogen was concentrated to about 10 mg/ml in an Amicon ultrafiltration stirred cell with a Diaflo PM10 membrane.

Separation of the Human Glu and Lys forms of plasminogen was originally carried out by the method of Wiman and Wallen who used DEAE-Sephadex A-50 with a gradient of 0.04 M Tris HCl pH 8.8, 0.06 M NaCl to 0.04 M Tris HCl pH 7.8, 0.44 M NaCl (20). This procedure was later modified by using DEAE-Sephacel with a gradient of 0.04 M Tris HCl pH 8.5, 0.04 M NaCl to 0.04 M Tris HCl pH 7.5, 0.22 M NaCl. Fractions were collected dropwise and an elution profile by optical density was obtained (Figure 2). The Glu- and Lys-plasminogen forms
were pooled separately, precipitated with \((\text{NH}_4)_2\text{SO}_4\), as before, to concentrate the plasminogen and finally was dialized against PBS to remove the \((\text{NH}_4)_2\text{SO}_4\) salts. The samples were then diluted to 10-15 mg/ml, aliquoted into 0.1 ml volumes, quick frozen and stored at -20°C until use.

**Conversion of Glu-plasminogen to Lys-plasminogen** - Some preparations of plasminogen contained a mixture of Glu-plasminogen and Lys-plasminogen. These preparations were completely converted to Lys-plasminogen by incubation with plasmin in a ratio of 200:1-Pmg:Pmn on a molar basis, for 24 hours at 4°C. After the incubation, 3.5 ml of Trasylol-Sepharose beads was added to remove the plasmin and after 12 hours, removed with a sintered glass filter. Plasmin was generated by incubating plasminogen with Urokinase-Sepharose beads in the presence of 25% glycerol and 0.1 M lysine for 45 minutes. The concentrations of plasminogen were determined by using an extinction coefficient, \(E_{280}^{1%}\) of 16.9. Purity of the Glu- and Lys-plasminogen was determined by NH₂-terminal analysis and by SDS acrylamide gel electrophoresis. Urokinase and aprotinin were coupled to sepharose by the CNBr method and stored refrigerated in buffer until use.

**NH₂-terminal analysis** - Determination of the NH₂-terminal amino acid of proteins was performed using the method of Gros and Labouesse (33). Into a 1 ml glass ampule was placed 5-10 nmole of protein in 0.1 ml buffer. Added to this was 84 mg Urea (CN⁻ and NH₄⁺ free); 0.05 ml 0.4 M PO₄ buffer, pH 8.6; 0.084 ml of DMF; and 0.1 ml of a 10 mg/ml solution of Dansyl-Cl in acetonitrile. This mixture was allowed to react for 20-30 minutes at 37°C. The dansylated protein was then precipitated by the addition of two volumes of cold 20% TCA and spun at 30,000 x g and 4°C for 5 minutes. The pellet was
washed with 1 N HCl and dissolved in 0.4 ml of 6 N HCl. The peptide bonds were then hydrolyzed in the sealed ampules for 18 hours at 105°C. After hydrolysis, the ampule was opened and the sample dried under an air stream. The dried residue was then redissolved with 0.01 ml of 50% aqueous pyridine (v/v). Two dimensional chromatography was performed at room temperature on 5 x 5 cm polyamide layer sheets using the procedure of Woods and Wang (34). In the lower left-hand corner of the plate, approximately 0.5 µl of the redissolved protein was placed. Directly behind this spot was placed the previously dissolved dansyl-amino acid standards of o-DNS-Tyrosine, DNS-Arginine, ε-DNS-Lysine, DNS-Glutamic acid, di-DNS-Lysine and DNS-NH₂. The first dimension of H₂O:formic acid, 200:3 (v/v) was run left to right, while the second dimension of benzine:pyridine: acetic acid, 9:1:1 (v/v/v) was run bottom to top. The volume was varied depending on the original concentration of the protein. Comparison of the standards on the back of the plate will match up to the unknown spot on the front yielding an accurate determination of the NH₂-terminus. DNS-Arginine, O-DNS-Tyrosine, and ε-DNS-Lysine will appear for all proteins due to their residue groups, found internally on proteins, becoming dansylated, as will DNS-OH and DNS-NH₃ due to the dansylation of water and any free NH₄⁺ (Figure 3 & 4).

Activations of plasminogen - The activation of plasminogen to plasmin by urokinase was assayed using the active site titrant FMGB which has been previously described (30,35). FMGB was stored in redistilled DMF at a concentration of 3.88 x 10⁻² M and when needed, diluted to 1 x 10⁻³ M in DMF. Appropriate dilutions of plasminogen in PBS buffer were made from which 90 µl was added to 10 µl of 2240 Plough units/ml urokinase and allowed to react at room temperature. At varying times, 10 µl of the reaction mixture was added to
1 ml of 1 x 10^{-6} M FMGB. The resulting fluorescence was then read on a fluorometer and was quantitated to the amount of plasmin formed. A fluorescence baseline was set by adding 10 \mu l of unactivated plasminogen and an appropriate dilution of urokinase, as in the reaction, to 1 ml 1 x 10^{-6} M FMGB. This eliminated the background fluorescence seen by spontaneous hydrolysis of the FMGB. For activations in the presence of lysine, 10 \mu l of a 1 M lysine solution was added to 80 \mu l of plasminogen before the addition of 10 \mu l urokinase, to yield a solution of 0.1 M lysine. The same is true for activations in the presence of benzamidine to acquire the appropriate concentration of benzamidine. For the activations in the presence of glycerol, the plasminogen dilutions were made by diluting 1:1 with 50% glycerol in PBS buffer to yield a 25% glycerol solution. Activations in the presence of aprotinin can not be monitored by FMGB assays due to the fact that aprotinin irreversibly inhibits plasminogen at the active site but, were used instead to assay the activation on SDS slab gels. For this, 1000KIU aprotinin will inhibit 1 mg of active plasmin. KIU is the international abbreviation for Kallikrein Inactivator Units.

**Acrylamide Gel Electrophoresis** - Acrylamide gel electrophoresis was carried out on a Hoefer acrylamide gel system and was performed by the method described by Laemmli (36). Discontinuous pH gel slabs (10%) were prepared in 2 M Tris HCl pH 6.8 (stacking) and pH 8.8 (running) containing 0.1% dodecyl sulfate (SDS): N,N,N-tetramethylethlenediamine and ammonium persulfate were used as catalysts. The running buffer was 0.25 M Tris and 1.9 M glycine with 0.05% SDS at pH 8.3. Samples between 10 to 20 \mu g of protein in 0.1% \beta-mercaptoethanol and 6% SDS were placed in the slots. Electrophoresis was carried out at a constant 125 volts for 10 hours at 4\degree C. The slab gels were
stained for one hour with 0.8% coomassie blue in 25% isopropanol-10% glacial acetic acid, and destained in 25% methanol-75% glacial acetic acid overnight.

\[ \frac{K_M}{k_{cat}} \] Determinations - To obtain the kinetic parameters of \( K_M \) and \( k_{cat} \), an activation assay using FMGB similar to that described above was performed. Six to eight plasminogen concentrations were prepared and the concentrations determined by absorbance at 280 nm. The plasminogen samples ranged from 13 mg/ml to 0.5 mg/ml and were activated with 11.2 PU/ml urokinase. Each dilution was stopped at the exact same time point, between six and eight minutes, by adding 10 \( \mu \)l of the reaction to 1 ml of \( 1 \times 10^{-6} \) M FMGB and the resulting fluorescence was then read on the fluorometer. Graphs and computations were obtained from preprogrammed tapes on a Hewlett Packard model 9825B desk computer/calculator.
RESULTS

Separation of the Glu and Lys forms of plasminogen. After the initial isolation of plasminogen by affinity chromatography, the NH$_2$-terminus Glu and Lys forms of plasminogen were separated on the ion exchanger DEAE-Sephadex A-50. The initial procedure used a pH gradient from 8.8 to 7.8 in 0.04 M Tris and a salt gradient of 0.06 M NaCl to 0.44 M NaCl. This procedure worked but not without problems. One inherent property of DEAE-Sephadex is that in low ionic strength solutions (I=0.05) the beads swell to about 10 ml per dry gram but, in high ionic strength solutions (I=0.4) the beads will only swell to about 7 ml per dry gram. This produces the first problem, the entire column bed shrinking by as much as one-fifth the original height. As the column shrinks, pressure builds up causing stress to be applied to the connecting tubes. These problems were alleviated by using DEAE-Sephacel. This ion exchanger contains the same functional group as the sephadex type but the beads are made from cellulose derivatives instead of dextrose units. This gives the beads a more rigid form, swelling less than 5% for ionic strength changes from I=0.05 to I=0.5. Also, the total capacity of DEAE-Sephadex is less, therefore, substances are eluted at lower ionic strengths. This elution problem was solved by lowering the gradient to 0.04 M NaCl. The elution profile of plasminogen off of DEAE-Sephacel is shown in Figure 2.

NH$_2$-terminal analysis of the different forms of plasminogen. Outdated Human plasma usually contains varying amounts of Lys-plasminogen. Figure 3 shows two TLC plates, one of pure Glutamic acid NH$_2$-terminus plasminogen and the other of pure Lysine NH$_2$-terminus plasminogen. Some preparations of Lys-
plasminogen also contain traces of Valine-plasminogen and sometimes even Methionine-plasminogen. Figure 4c shows a preparation with contaminating traces of Val-plasminogen. Val-plasminogen has the same properties as Lys-plasminogen, the only difference being that plasmin has cleaved the NH₂-terminal Lysine to yield Valine at position 78. In preparing Lys-plasminogen from Glu-plasminogen, the 76 amino acid peptide must first be removed, otherwise the results of dansylation will show a mixture of Glu and Lys forms of plasminogen. The removal of the peptide was accomplished by passing the plasminogen through a small Sephadex G-75 column before dansylation.

The NH₂-terminus of Dog plasminogen is reported to be blocked (38) with a pyroglutamic acid. Pyroglutamic acid is a glutamic acid which has its γ-carboxy group bound to its α-amino group and thus is cyclized upon itself. Since the amino group is blocked, the dansyl-chloride is unable to react and no spot will be visible on the resulting TLC. Our results confirm this, yielding a very faint DNS-Glutamic acid spot that is almost too faint to be photographed. Figure 4b shows Dog plasminogen represented on a TLC plate. Here the faint DNS-Glu spot represents a small fraction of Glu-plasminogen that is not cyclized. Lys-plasminogen is also found in Dog preparations.

The activation of Glu- and Lys-plasminogen by urokinase and the effect of lysine. The activation of Glu- and Lys-plasminogen by urokinase in the presence and absence of lysine is shown in Figure 5. Solutions of PBS containing 13.7 μM Glu-plasminogen or 7.5 μM Lys-plasminogen, 224 Plough units of urokinase, and either 0.1 M lysine or no lysine were incubated at room temperature. After the indicated time intervals, aliquots were withdrawn and the amount of plasmin present was immediately determined by an active site titration with FMB. The results indicate that this concentration of lysine
dramatically affects both the rate and extent of activation of Glu-plasminogen whereas it has little effect on the activation of Lys-plasminogen.

Assays of the activation of plasminogen to plasmin have to be judged carefully. The results may be influenced by the fact that it is very difficult to achieve complete activation without losses due to plasmin autodigestion (7,37). Figure 6a shows an example of this autodigestion. The asterisk (*) shows Lys-plasminogen activated in the presence of aprotinin whereas the cross (X) shows the same sample of Lys-plasminogen activated in the absence of aprotinin. The plasminogen activated in the presence of aprotinin, (*), was placed in SDS-glycerol-β-mercaptoethanol to stop the reaction at the indicated time points and was run out on a 10% acrylamide gel. The gel was then scanned with a densitometer and quantitated to yield the percent activated. This can be done since the rate of disappearance of plasminogen or the rate of appearance of the A- and B-chains of plasmin parallels the kinetics of plasmin formation. This analysis also indicates that greater than 95% of the plasminogen is activatable. The plasminogen activated in the absence of aprotinin, (X), was determined by an active site titration with FMGB. From Figure 6a one can see that the plasminogen activated without inhibitor is being degraded and plateaus before reaching 100% activated. At longer time periods the slope becomes negative as more plasminogen is destroyed by plasmin. The aprotinin, Trasylol binds very tightly to the active site of plasmin (26) and for this reason cannot be used for FMGB assays. If however, a competitive inhibitor such as benzanidime, which can move easily in and out of the active site, is used, one may be able to inhibit the autodegradation by plasmin. Figure 7a shows the activation of Glu-plasminogen in the presence of 10^{-3} M benzanidime. At a concentration of 10^{-3} M the benzanidime is 10-fold higher than the reported K_{i} value of 10^{-4} M. One can assume then that at any given time, ap-
The activation of Glu- and Lys-plasminogen by urokinase on 10% acrylamide gel. Figure 6a shows the activation of Glu-plasminogen from 0 to 30 minutes (A) in the absence of lysine and the activation of Lys-plasminogen from 30 to 0 minutes (D) respectively, also in the absence of lysine. Both gels indicate that plasmin is indeed digesting itself.

One last experiment that was performed to prove the autodigestion by plasmin formed in the absence of lysine, is activated very slowly indicating the plasmin formed is not converting the remaining Glu-plasminogen to Lys-plasminogen therefore is still available to cleave the remaining Glu-plasminogen.

Cleavage, it seems, is a stabilizing effect on plasmin which inhibits the autodigestion of plasminogen. Figure 7b shows the activation of Glu-plasminogen by urokinase in the presence of 25% glycerol. The sample activated in the absence of lysine (*) is activated very slowly indicating the plasmin formed is not converting the remaining Glu-plasminogen to Lys-plasminogen.
plasminogen were activated in the presence of the aprotinin. The gel clearly shows the difference in molecular weight between Glu- and Lys-plasminogen and the plasmin A-chains, both due to the 76 amino acid NH₂-terminal peptide. Even at double the concentration of urokinase and three times the activation time period, Glu-plasminogen was only activated to a small percent whereas the Lys-plasminogen was totally activated. Figure 8b shows the same Glu-plasminogen activated in the presence of 0.1 M lysine for 30 to 10 minutes (f-h) respectively. Here we see that in the presence of lysine, the activation of Glu-plasminogen nearly parallels that of Lys-plasminogen. The remainder of the gel space, Figure 8b (b-e), was devoted to prove the autodigestion of plasminogen by plasmin. Slots b and c are Glu-plasminogen without and with aprotinin respectively and, slots d and e are Lys-plasminogen without and with aprotinin also respectively. Slot b clearly shows the plasminogen band and the plasmin A-chain of Glu-plasminogen running equal with Lys-plasminogen and Lys-plasmin A-chains indicating that the plasmin formed degrades the remaining plasminogen and plasmin. However, in the presence of aprotinin (slot c) the plasminogen and plasmin A-chain still run parallel with those of Glu-plasminogen.

**Determination of the mode of inhibition and the inhibitor constant for the inhibition by lysine of the activation of Lys-plasminogen by urokinase.**

The effect of lysine on the initial velocity for the activation of Lys-plasminogen by urokinase appears to be one of simple inhibition. If so, the mode of inhibition and the inhibitor constant, $K_i$, can be obtained. To do this, we incubated three different concentrations of Lys-plasminogen with urokinase and varying concentrations of lysine. After six minutes at room temperature, the amount of plasmin formed was measured by an active site titration with FMBG. The data are presented in Figure 9 in the form of a Dixon
plot. Because the three lines intersect above the abscissa, the mode of inhibition by lysine is competitive. The point of intersection of the three lines indicates that the $K_i$ for lysine is 0.1 M. Thus, the inhibition of the activation of both Glu- and Lys-plasminogen by lysine occurs because, both plasminogen and lysine compete for binding to the active site of urokinase.

The effect of lysine on the initial rates of activation of Glu- and Lys-plasminogen by urokinase. The effects of lysine on the initial rates of activation of Glu- and Lys-plasminogen by urokinase are shown in Figure 10. These experiments were performed by incubating the plasminogens with urokinase and the indicated concentrations of lysine at room temperature for seven minutes and then immediately measuring the amount of plasmin formed by an active site titration with FMGB. Only a small amount of plasmin was allowed to form during the assay, thereby preventing the conversion of Glu-plasminogen to Lys-plasminogen and any self-proteolysis. The results indicate that increasing concentrations of lysine progressively increase the initial velocity of the activation of Glu-plasminogen by urokinase. A plateau was reached at 0.05 M lysine. Above 0.1 M lysine, the initial velocity progressively decreases as the lysine concentration is progressively increased. A different pattern is observed with Lys-plasminogen. The initial velocity progressively decreases as the lysine concentration is increased. Above a lysine concentration of 0.1 M, the decrease in initial velocity parallels the decrease in the initial velocity for Glu-plasminogen.

Determination of the $K_m$ and $k_{cat}$ for the activation of Glu- and Lys-plasminogen by urokinase and the effect of lysine on these parameters. Clearly the initial rates of activation of Glu- and Lys-plasminogen by urokinase are different and clearly lysine stimulates the initial rate of activation of
Glu-plasminogen. Are these differences simply reflective of differences in $K_M$ or $k_{cat}$? To measure these macroscopic kinetic constants, we incubated at room temperature solutions of PBS containing various concentrations of Glu- and Lys-plasminogen, urokinase, and either 0.1 M lysine or no lysine. After five minutes, the amount of plasmin formed was determined by an active site titration with FMGB. The urokinase concentration was made low and the time for activation was kept short so that high concentrations of plasmin could not build up and convert some of the Glu-plasminogen to Lys-plasminogen. The data are represented in Figure 11 in the form of a Hanes-Woolf linear transformation of the Michaelis-Menten rate equation.

The data indicates that the activation of Glu- and Lys-plasminogen by urokinase in the presence and absence of lysine follows Michaelis-Menten kinetics. The $K_M$ for the activation of Glu-plasminogen is 116 μM and the $k_{cat}$ is 1.46 sec$^{-1}$, while the $K_M$ for the activation of Lys-plasminogen is 12 μM and the $k_{cat}$ is 1.59 sec$^{-1}$. In the presence of 0.1 M lysine, the $K_M$ for the activation of Glu-plasminogen decreases to 14.5 μM and the $k_{cat}$ is 1.78 sec$^{-1}$, while the $K_M$ for the activation of Lys-plasminogen is 7.5 μM and the $k_{cat}$ is 1.62 sec$^{-1}$. The $K_M$ values in the presence of 0.1 M lysine were calculated by dividing the apparent $K_M$ values by $(1 + 1/K_i)$, where $I$ is the lysine concentration, to compensate for the competitive inhibition by lysine. $I=1$
DISCUSSION

The combined procedures of Deutsch and Mertz and Wiman and Wal len provide a simple method for the purification of the native and modified forms of plasminogen. The combination of these two procedures allows for the study of the micro-heterogeneous forms of both Glu- and Lys-plasminogen. Unfortunately, time limits did not permit us to study the effects of different carbohydrate moieties on the activation of plasminogen but, preliminary studies such as this, help open the door for future studies.

Activations of plasminogen should be performed with care. If the resulting plasmin is not inhibited during the course of a long activation, the plasmin will destroy the plasminogen activator and if Glu-plasminogen is used, the plasmin will convert the Glu-plasminogen to Lys-plasminogen. This conversion of Glu- to Lys-plasminogen, in effect, causes a change in the substrate for urokinase thereby causing a change in the activation kinetics. Our results show that this change in activation and combined conversion does in fact occur. Activations of plasminogen should be done in the presence of an inhibitor such as the aprotinin, Trasylol. If the activations are monitored with an active site substrate such as FMGB, the activations are best done in the presence of 25% glycerol.

The major conclusions from these experiments are summarized in Table 1. The results show that the conversion of plasminogen to plasmin is controlled by its Michaelis-Menten constant, $K_m$, and not by its catalytic constant $k_{cat}$. In the past, all enzyme systems were found to be controlled by the catalytic
constant. In this, the fibrinolytic system, we have found a new mode by which the system is controlled. The data presented in this paper also explains some of the paradoxical observations made on the activation of plasminogen. Glu-plasminogen does not appear to be as easily activated by urokinase as is Lys-plasminogen, Figure 5, because its $K_M$ is 10-fold higher. At low concentrations of lysine, the initial rate of activation of Glu-plasminogen by urokinase is stimulated, because the $K_M$ is decreased. This stimulation of the initial activation rate does not occur with Lys-plasminogen because its $K_M$ for urokinase is not altered by lysine. At higher concentrations of lysine, the activation of both Glu- and Lys-plasminogen is inhibited because lysine at those concentrations becomes a competitive inhibitor of urokinase.

Our data have implications for the use of $\alpha$, $\omega$-amino acids as clinical therapeutic agents. At low concentrations these amino acids are both fibrinolytic and antifibrinolytic. They are fibrinolytic in that they induce a general activation of plasminogen because they lower the $K_M$. This results in a general increase in the concentration of plasmin and thus a decrease in the concentration of plasminogen and plasmin inhibitors in the circulation. These amino acids are antifibrinolytic in that occupation of the lysine binding sites prevents plasminogen from binding to fibrin. Therefore, the concentration of plasmin at a fibrin clot may not be high enough to dissolve it. At high concentrations, these amino acids are solely antifibrinolytic. Not only is plasminogen prevented from binding to fibrin because the lysine binding sites are occupied but also, plasminogen cannot be activated because lysine is a competitive inhibitor of plasminogen activators. In this case the concentration of plasminogen and plasmin inhibitors in the plasma is not decreased.

Our data have led us to propose a model for the regulation of fibrino-
lysis in vivo which we are currently testing. In the absence of a fibrin clot, the Glu-plasminogen concentration in plasma, which is 1-2 μM (8,39), is much lower than the $K_M$ of 116 μM for the activation of Glu-plasminogen to plasmin. Thus, in the steady state, plasminogen is allowed to circulate without being activated. Once a fibrin clot forms, however, Glu-plasminogen binds to the clot at its lysine binding sites. This not only increases the Glu-plasminogen concentration at the site of the clot but also lowers the $K_M$ for activation of Glu-plasminogen so that it is now activatable. This plasmin formed begins to degrade the fibrin, and also converts Glu-plasminogen to Lys-plasminogen. This results in a lowering of the $K_M$ and accelerates the production of plasmin. Once the clot is dissolved, the $K_M$ for the activation of Glu-plasminogen is increased so that it is no longer activatable. The plasmin that had formed is inactivated by inhibitors. And the system returns to the steady state, having altered the system only at the site of the clot for the short period of time required to dissolve it.
### Table 1

**Kinetic Constants**

A. Kinetic constants for the activation of plasminogen by urokinase.

<table>
<thead>
<tr>
<th>Plasminogen Type</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu-plasminogen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>minus lysine</td>
<td>116</td>
<td>1.48</td>
</tr>
<tr>
<td>plus 0.1 M lysine</td>
<td>14.5</td>
<td>1.78</td>
</tr>
<tr>
<td>Lys-plasminogen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>minus lysine</td>
<td>12</td>
<td>1.89</td>
</tr>
<tr>
<td>plus 0.1 M lysine</td>
<td>7.5</td>
<td>1.62</td>
</tr>
</tbody>
</table>

B. Kinetic constant for inhibition of urokinase by lysine.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urokinase</td>
<td>100</td>
</tr>
</tbody>
</table>
BIBLIOGRAPHY


FIGURE LEGENDS

Figure 1 Elution profile of Human plasminogen, separated as to carbohydrate content, on a Lysine-Sepharose column. Gradient of 0 to 0.166 M ε-ACA in 0.1 M KPO$_4$, 3 mM EDTA buffer at pH 7.4. First peak is designated FI and second peak FII.

Figure 2 Elution profile by absorbance at A$_{280}$ of Human plasminogen on a DEAE-Sephacel column. The first peak is Glutamic acid and the second is Lysine NH$_2$-terminus plasminogen. Gradient of 0.06 M NaCl, 0.04 M Tris pH 8.5 to 0.22 M NaCl, 0.04 M Tris pH 7.5.

Figure 3 Two dimensional thin layer chromatography of the NH$_2$-terminal amino acids of (A) Glu-plasminogen and (B) Lys-plasminogen. The origin is in the lower left corner. Chromatography in the first dimension, left to right, was performed in water:formic acid, 200/3 and the second dimension, bottom to top, in benzene:pyridine:acetic acid, 9/1/1. The spots were identified from the dansylated amino acid standards chromatographed on the back of polyamide sheets.

Figure 4 Two dimensional thin layer chromatography of (A) the standard dansyl-amino acids, (B) Dog plasminogen, (C) Human Lys-plasminogen, and (D) Human Glu-plasminogen. Chromatography is identical to Figure 3.

Figure 5 The activation of (A) Glu-plasminogen and (B) Lys-plasminogen by urokinase and the effect of lysine. Activation reactions of 0.1 ml were prepared in PBS containing 224 Plough units of urokinase, either 13.2 μM Glu-plasminogen or 7.5 μM Lys-plasminogen and either 0.01 M lysine (○) or no lysine (●). After the indicated time intervals, 0.01 ml aliquots were withdrawn and the plasmin formed immediately determined by an active site titration with FMGB.

Figure 6a The activation of Lys-plasminogen by urokinase in the presence of aprotinin (●) and absence of aprotinin (X). The activation in the presence of aprotinin (●) was run on a 10% acrylamide SDS gel and scanned using an Ortec densitometer to quantitate the percent activated. The activation in the absence of aprotinin (X) was monitored by active site titration with FMGB.

Figure 6b Densitometer tracings of the activation of Lys-plasminogen by urokinase in the absence of aprotinin. The change in the molecular weight indicated by the shift of the plasmin A-chain (arrow), sup-
ports the fact that plasmin degrades itself as high concentrations of plasmin accumulate.

Figure 7a The activation of Glu-plasminogen by urokinase in the presence of 10⁻³ M benzamidine and 0.1 M lysine (X) or no lysine (*). The activation was monitored as in Figure 5.

Figure 7b The activation of Glu-plasminogen by urokinase in the presence of 25% glycerol and 0.1 M lysine (X) or no lysine (*). The activation was monitored as in Figure 5.

Figure 8 Activation of plasminogen by urokinase on SDS 10% acrylamide gels. All activations were performed at room temperature with 0.1 M lysine and 1,000 Kallikrein inactivator units aprotinin per mg of plasminogen unless otherwise indicated. Each lane contains 10 μg of protein. Gel A: The activation of Glu-plasminogen by 8960 Plough units/ml of urokinase in the absence of lysine for 0 minutes (a & j), 30 minutes (b), 60 minutes (c), 90 minutes (d), and 120 minutes (e), and the activation of Lys-plasminogen by 4480 Plough units/ml urokinase in the absence of lysine for 30 minutes (f), 20 minutes (g), 10 minutes (h), and 0 minutes (i). Gel B: The activation of Glu-plasminogen for 20 minutes by 4480 Plough units/ml urokinase in the absence of aprotinin (b) or in the presence of aprotinin (c), the activation of Lys-plasminogen for 20 minutes by 4480 Plough units/ml urokinase in the absence of aprotinin (d) or in the presence of aprotinin (e), the activation of Glu-plasminogen by 4480 Plough units/ml urokinase for 30 minutes (f), 20 minutes (g), 10 minutes (h), and 0 minutes (a & j), and the activation of Lys-plasminogen for 10 minutes in the absence of lysine (i). Pmg denotes plasminogen; Pan-A denotes the plasmin A-chain; and Pan-B denotes the plasmin B-chain.

Figure 9 Determination of the mode of inhibition and the inhibitor constant for the inhibition by lysine of the activation of Lys-plasminogen by urokinase. Activation reactions of 0.03 ml in PBS were prepared containing 22 Plough units of urokinase, the indicated concentrations of lysine and either 11 μM (α), 7.33 μM (λ), or 4.38 μM (γ) Lys-plasminogen. After incubation at room temperature for six minutes, 0.02 ml aliquots were removed and the amount of plasmin was determined with FMGB. The data are presented in the form of a Dixon plot.

Figure 10 The effect of lysine on the initial rates of activation of Glu- and Lys-plasminogen by urokinase. Activation reactions were performed at room temperature in 0.03 ml of PBS containing 10 μM Glu-plasminogen (α) or 10 μM Lys-plasminogen (ε), 22 Plough units of urokinase and the indicated concentrations of lysine. After seven minutes, 0.02 ml aliquots were removed and the amount of plasmin present titrated with FMGB.
Figure 11 Determination of the $K_M$ and $k_{cat}$ for the activation of (A) Glu-plasminogen and (B) Lys-plasminogen by urokinase and the effect of lysine on these parameters. Activation reactions were performed at room temperature in 0.05 ml of PBS containing the indicated concentrations of plasminogen, 11.2 Plough units of urokinase, and either 0.1 M lysine (∇) or no lysine (●). After five minutes, the reactions were diluted to 0.1 ml with PBS containing 2 μM FMGB to titrate the amount of plasmin formed. The data are presented in the form of a Hanes-Woolf linear transformation.
FIG. 3

A

DNS-Gly

Origin

DNS-OH

o-DNS-Tyr

c-DNS-Lys &
DNS-Arg

B

dl-DNS-Lys

Origin

DNS-OH

c-DNS-Tyr &
DNS-Lys &
DNS-Arg
FIG. 10

Velocity, fmol plasmin/min

Lysine, M

Velocity, fmol plasmin/min

0 0.05 0.10 0.15 0.20 0

2100 1800 1500 1200 900 600 300 0

6000 4500 3000 1500 0