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Positive Regulation of Activation of Plasminogen by Urokinase: Differences in K_m for (Glutamic acid)-plasminogen and Lysine-plasminogen and Effect of Certain α,ω-Amino Acids†

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ABSTRACT: The kinetics of activation of human Glu- and Lys-plasminogens by human urokinase and the effect of lysine on the kinetics of activation are analyzed in a simple assay with the active site titrant for plasmin 3′-(4-guanidinobenzoyloxy)-6′-hydroxyspiro[isobenzofuran-1(3*H*),9′-[9*H*]-xanthen]-3-one. The K_m for the activation of Glu-plasminogen is 200 μM and for Lys-plasminogen is 12 μM, while the k_{cat} values are 1.48 s^{-1} and 1.89 s^{-1}, respectively. In the presence of 0.1 M lysine, the K_m for the activation of Glu-plasminogen decreases 10-fold to 24 μM and for Lys-plasminogen is 7.5 μM, while the k_{cat} values are 1.78 s^{-1} and 1.62 s^{-1}, respectively. Lysine is a competitive inhibitor of urokinase with a K_i of 0.1 M. These data explain some of the well-documented effects of certain α,ω-amino acids on the activation of plasminogen and are relevant to their use in clinical therapy. Glu-plasminogen is not as activatable as Lys-plasminogen, because its K_m is 10-fold higher. Lysine renders Glu-plasminogen more activatable, because it lowers the K_m 10-fold to that of Lys-plasminogen. High concentrations of lysine inhibit the activation of both Glu- and Lys-plasminogens, because it is a competitive inhibitor of urokinase. Thus, at low concentrations certain α,ω-amino acids are fibrinolytic whereas at high concentrations they are antifibrinolytic. These experiments have led us to propose a new model for the positive regulation of fibrinolysis in vivo. In plasma the concentration of Glu-plasminogen is 100-fold lower than its K_m for activation by urokinase. Thus, even in the presence of urokinase little or no activation to plasmin occurs. Upon formation of a fibrin clot, Glu-plasminogen binds at its lysine binding sites to the clot [Wiman, B., & Wallén, P. (1977) *Thromb. Res. 10*, 213-222]. This lowers the K_m for activation 10-fold so that plasmin now begins to form. Once the clot is dissolved by plasmin, the lysine binding sites in Glu-plasminogen are no longer bound to fibrin, and thus the K_m for activation is increased 10-fold. Requiring for activation of plasminogen the simultaneous presence of both a plasminogen activator and a plasmin substrate to which plasminogen is bound ensures that the formation of plasmin occurs quickly and only where and when it is needed.

The fibrinolytic system is highly regulated (Kline & Reddy, 1980). 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. How, on the one hand, can plasminogen and plasminogen activators coexist 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 L-lysine. By implication, the data indicate how fibrin may regulate plasminogen activation.

Native plasminogen consists of a single polypeptide chain composed of 790 amino acids, including an NH_2-terminal glutamic acid (Wallén & Wiman, 1972). Plasmin is formed upon proteolytic cleavage of a single arginyl–valyl bond 560 amino acids from the NH_2-terminal end of Glu-plasminogen[1] (Robbins et al., 1967). The resulting two polypeptides, the 560 amino acid A chain and the 230 amino acid B chain, are held together by a disulfide bridge. The serine and histidine of the catalytic site are located in the B chain (Groskopf et al., 1969). Plasmin can catalytically cleave native Glu-plasminogen at the lysyl–lysyl bond at position 76–77 from the NH_2 terminal, yielding a small preactivation peptide and a 714 amino acid form of plasminogen called Lys-plasminogen

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[1] Abbreviations: FMGB, 3′-(4-guanidinobenzoyloxy)-6′-hydroxyspiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one; P_i/NaCl, phosphate-buffered saline; Glu-plasminogen, native plasminogen with an NH_2-terminal glutamic acid; Lys-plasminogen, plasminogen with an NH_2-terminal lysine that is produced by plasmin cleavage of the lysyl–lysyl bond at position 76–77 of Glu-plasminogen; A chain, the largest of the two polypeptide chains of plasmin; B chain, the smaller of the two polypeptide chains of plasmin; iPr_2P-F, diisopropyl fluorophosphate; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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(Wallén & Wiman, 1972; Robbins et al., 1967).

Certain α,ω-amino acids have been shown to bind to both Glu- and Lys-plasminogens. There are two classes of binding sites on Glu-plasminogen for 6-aminohexanoic acid, one strong site with a dissociation constant, K_d, of 9 μM and five weaker sites with a K_d of 5 mM (Markus et al., 1978a). With Lys-plasminogen, there are also six binding sites, one with a K_d of 35 μM, one with a K_d of 0.26 mM, and four with a K_d of 10 mM (Markus et al., 1978b). L-Lysine also binds to these sites but with a much lower affinity. Its K_d for the strong ligand site on Glu-plasminogen is about 30-fold larger than that for 6-aminohexanoic acid (Markus et al., 1978a) and for the weaker sites about 150-fold larger (Brockway & Castellino, 1972). The binding of fragments of plasmin to lysine–Sepharose indicates the lysine binding sites are confined to the A chain of plasmin (Rickli & Otavsky, 1975). 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 6-aminohexanoic acid (Wiman & Wallén, 1977).

The binding of certain α,ω-amino acids alters some of the physical properties of Glu-plasminogen but not Lys-plasminogen. The presence of 6-aminohexanoic acid or lysine in a solution of Glu-plasminogen causes a reversible decrease in the sedimentation coefficient (Alkjaersig, 1964) from 5.75 to 4.85 S (Violand et al., 1978) and an increase of 7% in the relative fluorescence. The concentrations required to produce half of the change in sedimentation coefficient or in fluorescence intensity are about 2.7 mM for 6-aminohexanoic acid and 25.5 mM for lysine. Under similar conditions, no change in conformation can be detected with Lys-plasminogen. These and other experiments including gel filtration (Wallén & Wiman, 1972), circular dichroism (Sjöholm et al., 1973), and rotational diffusion measurements (Castellino et al., 1973) indicate that the binding of certain 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 is increased, and the circular dichroism pattern in the near-UV is changed (Wallén & Wiman, 1972; Violand et al., 1978; Sjöholm et al., 1973).

The presence of certain α,ω-amino acids also alters the kinetics of activation of plasminogen. In the absence of α,ω-amino acids, the rate of activation of Glu-plasminogen by urokinase is 10–20-fold less than that of Lys-plasminogen (Markus et al., 1978b; Claeys & Vermylen, 1974; Wallén & Wiman, 1975; Walther et al., 1975). In the presence of 6-aminohexanoic acid, the rate of activation of Glu-plasminogen by urokinase increases 10–20-fold whereas that of Lys-plasminogen is unaffected. At high concentrations of 6-aminohexanoic acid, the rates of activation of both Glu- and Lys-plasminogens by urokinase begin to decrease.

Recently, we developed a sensitive, quantitative assay for the activation of plasminogen to plasmin (Leytus et al., 1981). A sensitive, quantitative 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. The assay consists of two steps. 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}–10^{-12} M. With this assay, we have begun to characterize the activation of plasminogen and the factors that regulate it.

Materials and Methods

FMGB, 3′-(4-guanidinobenzoyloxy)-6′-hydroxyspiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one, is similar structurally to the active site titrant 3′,6′-bis(4-guanidinobenzoyloxy)-5-[*N*′-(4-carboxyphenyl)thioureido]spiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one, which was recently synthesized and characterized (Leytus et al., 1981; Livingston et al., 1981). The synthesis, purification, and characterization of FMGB are described elsewhere (Melhado et al., 1982). Aprotinin was purchased as Trasylol from Mobay Chemical Corp. Human urokinase was purchased from Leo Pharmaceuticals. Phosphate-buffered saline (P_i/NaCl) contained 0.137 M NaCl, 2.68 mM KCl, 8.00 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.91 mM $CaCl_2$, and 0.49 mM $MgCl_2$ at pH 7.2. Lysine–Sepharose 4B and cyanogen bromide activated Sepharose 4B were purchased from Pharmacia. Diisopropyl fluorophosphate (iPr_2P-F) was purchased from Aldrich. 6-Aminohexanoic acid was purchased from Calbiochem. Cheng Chin polyamide layer sheets were purchased from Pierce. Dansyl chloride, dansylated amino acid standards, and soybean trypsin inhibitor were purchased from Sigma. All electrophoresis reagents were purchased from Bio-Rad. The discontinuous pH, $NaDodSO_4$/polyacrylamide gel system described by Laemmli (1972) was used.

Purification of Human Glu- and Lys-plasminogens. Human Glu-plasminogen was purified from fresh human plasma. Blood was drawn into anticoagulant citrate phosphate dextrose adenine solution Blood-Packs (Fenwal Laboratories) that were placed on ice. Once flow had started, 2.5 mL of 10000 kallikrein inactivator units/mL aprotinin and 2.5 mL of 5 mg/mL soybean trypsin inhibitor were injected into each bag. After collection, the blood was centrifuged at 4 °C for 30 min at 27000*g* to remove particulate matter. The supernatant, 1000 mL, was applied at 4 °C to a 2.6 by 70 cm column of lysine–Sepharose at a flow rate of 24 mL/h (Deutsch & Mertz, 1970). The column was washed with P_i/NaCl minus calcium and magnesium until the A_{280} of the effluent was less than 0.05. Bound contaminating plasma proteins were eluted by washing the column with a solution containing 0.3 M phosphate–0.003 M EDTA, pH 7.4, until the A_{280} of the effluent was less than 0.05. The plasminogen was then eluted by 0.015 M 6-aminohexanoic acid in 0.1 M phosphate–0.003 M EDTA, pH 7.4, at a flow rate of 24 mL/h. The peak fractions were pooled and then incubated at 4 °C for 30 min with 0.004 M iPr_2P-F to ensure that any contaminating plasmin or plasminogen activator was inactivated. The plasminogen was precipitated to remove iPr_2P-F and 6-aminohexanoic acid by adding 0.31 g of $(NH_4)_2SO_4$/mL of solution, stirring at 4 °C for 30 min, and then centrifuging at 4 °C for 30 min at 30000*g*. The precipitate was dissolved in less than 4 mL of P_i/NaCl, and that solution was desalted by chromatography on a 3 by 30 cm column of Sephadex G-25. The peak fractions were pooled and concentrated by filtration under nitrogen pressure with an Amicon ultrafiltration membrane. The plasminogen, at concentrations greater than 8 mg/mL, was stored in aliquots at −70 °C. The yield from 1000 mL of plasma was about 150 mg.

Human Lys-plasminogen was obtained by converting Glu-plasminogen to Lys-plasminogen with plasmin. For example, 30 mg of Glu-plasminogen at a concentration of 10 mg/mL in P_i/NaCl was incubated with plasmin at a molar ratio of Glu-plasminogen to plasmin of 200:1. After 24 h at 4 °C, 3.5 mL of aprotinin–Sepharose beads was added to the reaction

mixture to remove the plasmin. After 12 h at 4 °C with continuous inversion, the beads were removed by filtration on a sintered glass filter. The concentration of human Glu- or Lys-plasminogen was determined by using an extinction coefficient, $E^{1\%}_{280}$, of 16.9.

Human plasmin was obtained by converting Glu-plasminogen to plasmin with urokinase. For example, 5 mg of Glu-plasminogen at a concentration of 10 mg/mL in P_i/NaCl containing 0.1 M lysine was incubated with 0.5 mL of urokinase–Sepharose beads. After 45-min of continuous mixing by inversion at room temperature, the beads were removed by filtration on a sintered glass filter. Glycerol was added to 25%, and the plasmin was stored at −70 °C.

The coupling of urokinase and aprotinin to Sepharose beads was accomplished by dissolving 6600 Plough units of human urokinase in 1 mL of coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) or 100 000 kallikrein inactivator units of aprotinin in 10 mL of coupling buffer and adding the solutions to 1 or 3.5 mL, respectively, of cyanogen bromide activated Sepharose 4B in coupling buffer. The suspensions were continuously mixed by inversion for 2 h at room temperature. The beads were then washed with several volumes of coupling buffer, followed by treatment with 1 M ethanolamine, pH 8.0, to block any remaining active groups. Adsorbed protein was removed by a series of four cycles of high pH (coupling buffer) and low pH (0.1 M sodium acetate, 0.5 M NaCl, pH 4) washes. The beads were then suspended in coupling buffer and stored in 50% glycerol at 4 °C.

NH_2-Terminal Amino Acid Determination. The NH_2-terminal amino acid of plasminogen was determined by using the procedure of Gros & Labouesse (1969) with a few slight modifications. Briefly, the procedure involved mixing in a 1-mL glass ampule 5–10 nmol of plasminogen in 0.4 M sodium phosphate, pH 8.6, 0.084 mL of dimethylformamide, 0.1 mL of a 10 mg/mL solution of dansyl chloride in acetonitrile, and solid urea such that its final concentration was 4 M. Dansylation of the NH_2-terminal amino acid was allowed to proceed at 37 °C for 30 min. The dansylplasminogen was then precipitated by adding 2 volumes of cold 20% trichloroacetic acid and incubating at 4 °C for 30 min. The precipitate was collected by centrifugation at 30000*g* for 5 min at 4 °C, was washed once with 1 N HCl, and was then dissolved in 0.4 mL of 6 N HCl. The peptide bonds were then hydrolyzed in sealed ampules for 18 h at 105 °C. Two-dimensional chromatography was performed at room temperature on 5 by 5 cm polyamide sheets by using, with slight modification, the procedure of Woods & Wang (1967). Chromatography in the first dimension was performed in water/formic acid, 200/3, and in the second dimension in benzene/pyridine/acetic acid, 9/1/1. On the opposite side of the sheet dansylated amino acid standards were chromatographed. An example of an NH_2-terminal amino acid analysis of Glu- and Lys-plasminogen is shown in Figure 1.

Measurement of Kinetics of Activation of Plasminogen by Urokinase. A two-step procedure was used to monitor the kinetics of activation of plasminogen to plasmin by urokinase. Activation reactions in P_i/NaCl contained plasminogen, urokinase, and, in some cases, lysine. After specified time intervals at room temperature (25 ± 2 °C), aliquots were withdrawn and the amount of plasmin formed was determined by an active site titration with FMGB. This was accomplished by adding the aliquots to 1.0-mL solutions of P_i/NaCl containing 2 μM FMGB, incubating for 2 min at room temperature, and measuring the increase in fluorescence. The difference between this increase in fluorescence and that of an identical solution but lacking urokinase is directly proportional to the concentration of plasmin. K_m and k_{cat} values were obtained by using the iterative method described by Cleland (1967) and a computer program to fit the data points to the Michaelis–Menten rate equation.

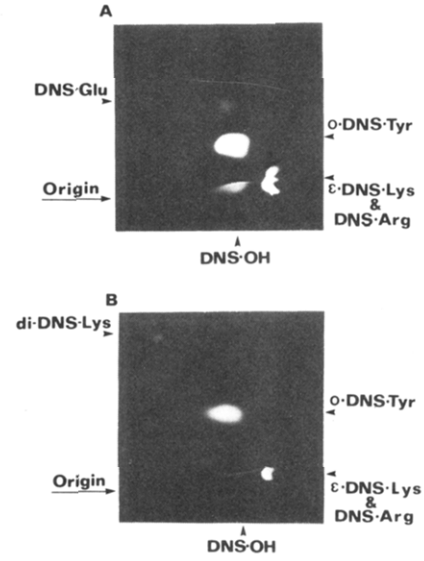

FIGURE 1: Two-dimensional thin-layer chromatography of the NH_2-terminal amino acids of (A) Glu-plasminogen and (B) Lys-plasminogen. The origin is in the lower left corner. Chromatography in the first dimension (left to right) was performed in water/formic acid (200/3) and in the second dimension (bottom 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 the polyamide sheets.

The concentration of plasmin was determined by using the active site titrant FMGB for the esterase activity of plasmin (Melhado et al., 1982). The rate of acylation is extremely fast ($k_2 = 0.94\ s^{-1}$), and the rate of deacylation is extremely slow ($k_3 = 6.27 \times 10^{-6}\ s^{-1}$). The K_s is 7.26×10^{-6} M and the $K_{m(app)}$ is 4.86×10^{-11} M. FMGB exhibits a slight intrinsic fluorescence. Upon acylation of plasmin, the highly fluorescent product fluorescein is released. Because one plasmin molecule reacts with one FMGB molecule to release one fluorescein molecule, the concentration of plasmin can be determined by using a standard curve relating fluorescence to the molar concentration of fluorescein.

Fluorescence was measured in a Perkin-Elmer MPF-44A fluorescence spectrofluorometer with a Univeral digital readout. The excitation and emission wavelengths were 491 and 514 nm, respectively, both set with a bandwidth of 4 nm. The fluorometer was standardized with rhodamine B embedded in a polymethacrylate block so that the relative fluorescence in different experiments was comparable.

Results

Activation of Glu- and Lys-plasminogens by Urokinase and Effect of Lysine. The activation of Glu- and Lys-plasminogens by urokinase in the presence and absence of lysine is shown in Figure 2. Solutions of P_i/NaCl containing 11.9 μM Glu-plasminogen or 6.75 μM Lys-plasminogen, 2240 Plough units/mL 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 FMGB. The results indicate that this concentration of lysine dramatically affects both the rate and extent of activation of Glu-plasminogen whereas it has little affect on the activation of Lys-plasminogen.

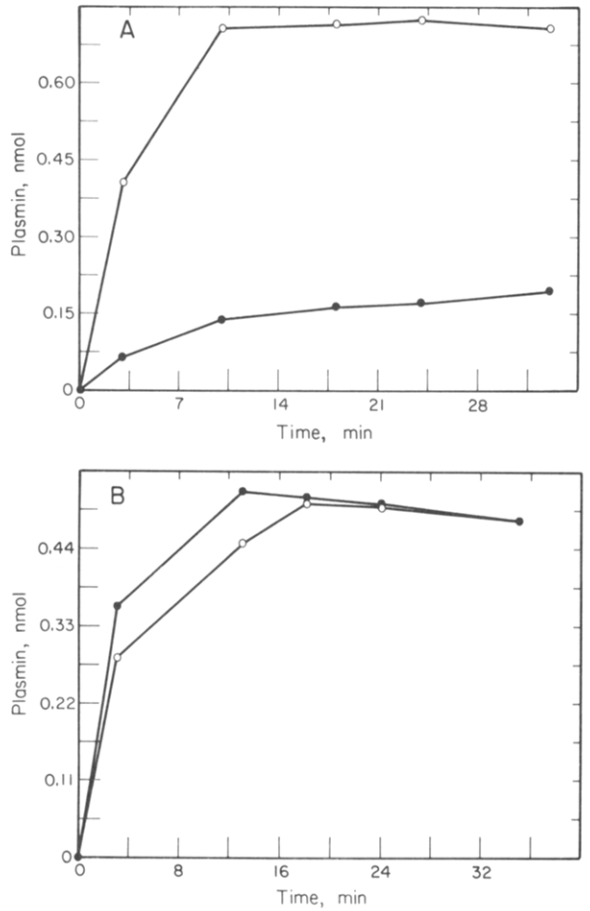

FIGURE 2: Activation of (A) Glu-plasminogen and (B) Lys-plasminogen by urokinase and effect of lysine. Activation reactions of 0.1 mL were prepared in P_i/NaCl containing 224 Plough units of urokinase, either 11.9 μM Glu-plasminogen or 6.75 μM Lys-plasminogen, and either 0.1 M lysine (O) or no lysine (●). After the indicated time intervals, 0.01-mL aliquots were withdrawn and the plasmin formed was immediately determined by an active site titration with FMGB.

The activation of Glu- and Lys-plasminogens by urokinase in the presence and absence of lysine was also analyzed by electrophoresis in $NaDodSO_4$/polyacrylamide gels (Figure 3). The reaction conditions were similar to those in Figure 2 except that aprotinin, a plasmin inhibitor, was present in some reactions. 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 in Figure 2. This analysis by gels also indicates that greater than 95% of the Glu- and Lys-plasminogens is activatable. The reason three of the curves in Figure 2 reached a plateau before all the plasminogen appeared to be activated was that at this point the concentration of plasmin had become high enough so that the plasmin had begun to cleave itself, while the rest of the plasminogen was being activated. If the kinetics in Figure 2 had been monitored for longer time intervals, the rate of plasmin formation would appear to be negative. This analysis by gels is also consistent with the NH_2-terminal amino acid analysis of the Glu- and Lys-plasminogens. The plasmin A chain from the activation of Glu-plasminogen in the absence of aprotinin comigrates with the plasmin A chain from Lys-plasminogen activated in the presence of aprotinin, whereas the plasmin A chain from Glu-plasminogen activated in the presence of aprotinin migrates more slowly, implying the activation polypeptide on the plasmin A chain is still present. Also, the migration of Lys-plasminogen is slightly faster than that of Glu-plasminogen.

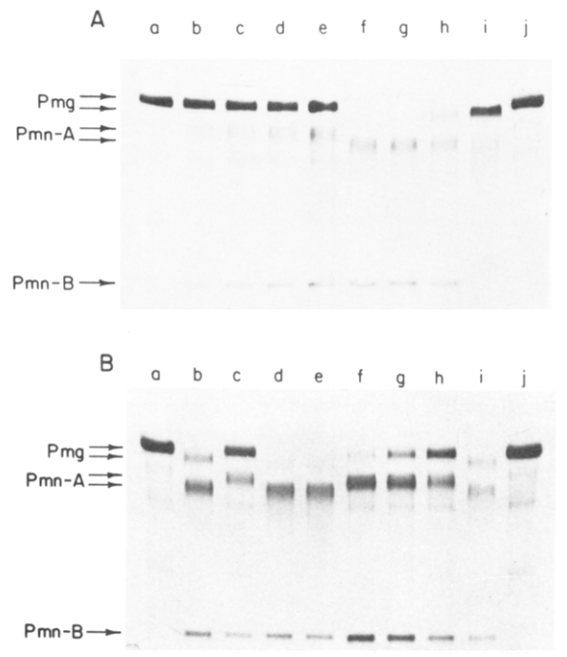

FIGURE 3: Analysis of activation of Glu- and Lys-plasminogens by urokinase in the presence and absence of lysine and aprotinin by $NaDodSO_4$/polyacrylamide gel electrophoresis. All activation reactions were performed at room temperature in 0.05 mL of P_i/NaCl that contained either 7 mg/mL Glu-plasminogen or 6 mg/mL Lys-plasminogen, either 0.1 M lysine or no lysine, and either 1000 kallikrein inactivator units of aprotinin/mg of plasminogen or no aprotinin. Each lane contains 10 μg of protein. Gel A: the activation of Glu-plasminogen by 8960 Plough units/mL urokinase in the presence of aprotinin for 0 (a and j), 30 (b), 60 (c), 90 (d), and 120 min (e) and the activation of Lys-plasminogen by 4480 Plough units/mL urokinase in the presence of aprotinin for 30 (f), 20 (g), 10 (h), and 0 min (i). Gel B: the activation of Glu-plasminogen for 20 min by 4480 Plough units/mL urokinase in the presence of lysine (b) or in the presence of lysine and aprotinin (c), the activation of Lys-plasminogen for 20 min by 4480 Plough units/mL urokinase in the presence of lysine (d) or in the presence of lysine and aprotinin (e), the activation of Glu-plasminogen by 4480 Plough units/mL urokinase in the presence of lysine and aprotinin for 30 (f), 20 (g), 10 (h), and 0 min (a and j), and the activation of Lys-plasminogen for 10 min by 4480 Plough units/mL urokinase in the presence of aprotinin (i). Pmg denotes plasminogen; Pmn-A denotes the plasmin A chain; Pmn-B denotes the plasmin B chain.

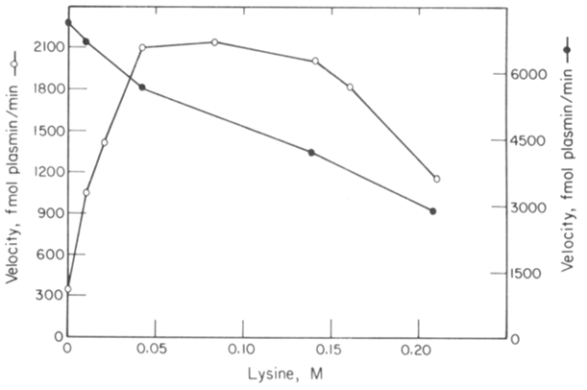

FIGURE 4: Effect of lysine on initial rates of activation of Glu- and Lys-plasminogens by urokinase. Activation reactions were performed at room temperature in 0.03 mL of P_i/NaCl containing 10 μM Glu-plasminogen (O) or 10 μM Lys-plasminogen (●), 22 Plough units of urokinase, and the indicated concentrations of lysine. After 7 min, 0.02-mL aliquots were removed and the amount of plasmin present was titrated with FMGB.

Effect of Lysine on Initial Rates of Activation of Glu- and Lys-plasminogens by Urokinase. The effects of lysine on the initial rates of activation of Glu- and Lys-plasminogens by urokinase are shown in Figure 4. The experiments were

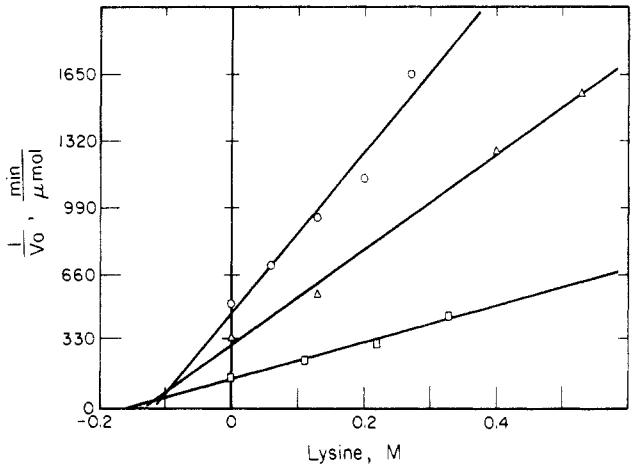

FIGURE 5: Determination of mode of inhibition and inhibitor constant for inhibition by lysine of activation of Lys-plasminogen by urokinase. Activation reactions of 0.03 mL in P_i/NaCl were prepared containing 22 Plough units of urokinase, the indicated concentrations of lysine, and either 11 (□), 7.33 (△), or 4.88 μM (○) Lys-plasminogen. After incubation at room temperature for 6 min, 0.02-mL aliquots were removed and the amount of plasmin present was titrated with FMGB. The data are present in the form of a Dixon plot.

performed by incubating the plasminogens with urokinase and the indicated concentrations of lysine at room temperature for 7 min 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 for the activation of Glu-plasminogen by urokinase. A plateau is 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.

Determination of Mode of Inhibition and Inhibitor Constant for Inhibition by Lysine of 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 various concentrations of lysine. After 6 min at room temperature, the amount of plasmin formed was measured by an active site titration with FMGB. The data are presented in Figure 5 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 (Table I). Thus, the inhibition of the activation of both Glu- and Lys-plasminogens by lysine occurs because both plasminogen and lysine compete for binding to the active site of urokinase. This is consistent with the conclusions of Lorand & Condit (1965), who showed that 6-aminohexanoic acid inhibits urokinase competitively with a K_d of 0.01 M.

Determination of K_m and k_{cat} for Activation of Glu- and Lys-plasminogens by Urokinase and Effect of Lysine on These Parameters. Clearly the initial rates of activation of Glu- and Lys-plasminogens 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} or both? To measure these macroscopic kinetic constants, we incubated at room temperature solutions of P_i/NaCl containing various concentrations of Glu- and Lys-plasminogens, urokinase, and either 0.1 M lysine or no lysine. After 5 min, the amount of plasmin formed was determined by an active site titration with FMGB. The urokinase concentration was low and the time for activation short so that high concentrations of plasmin could not build up that would result in the conversion of some of the Glu-plasminogen to Lys-plasminogen. The data are presented in Figure 6 in the form of a Hanes–Woolf linear transformation of the Michaelis–Menten rate equation.

The data indicate the activation of Glu- and Lys-plasmi-

Table I: Kinetic Constants

Kinetic Constants for Activation of Plasminogen by Urokinase	K_m (μM)	k_{cat} (s^{-1})
Glu-plasminogen		
minus lysine	200	1.48
plus 0.1 M lysine	24	1.78
Lys-plasminogen		
minus lysine	13	1.89
plus 0.1 M lysine	8	1.62

Kinetic Constant for Inhibition of Urokinase by Lysine	K_i (mM)
urokinase	100

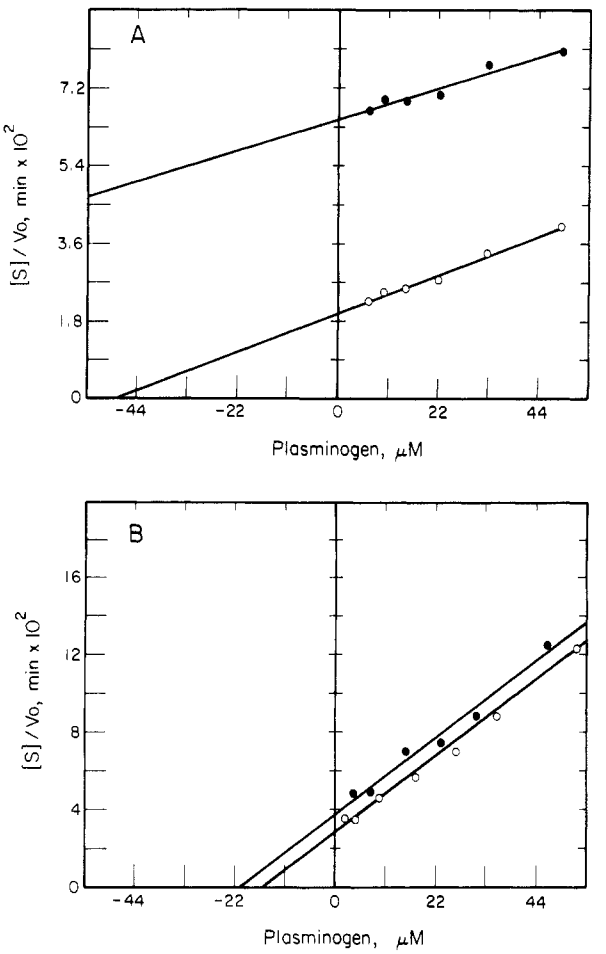

FIGURE 6: Determination of K_m and k_{cat} for activation of (A) Glu-plasminogen and (B) Lys-plasminogen by urokinase and effect of lysine on these parameters. Activation reactions were performed at room temperature in 0.05 mL of P_i/NaCl containing the indicated concentrations of plasminogen, 11.2 Plough units of urokinase, and either 0.1 M lysine (○) or no lysine (●). After 5 min, the reactions were diluted to 1.0 mL with P_i/NaCl containing 2 μM FMGB to titrate the amount of plasmin formed. The data are presented in the form of a Hanes–Woolf linear transformation.

nogens by urokinase in the presence and absence of lysine follows Michaelis–Menten kinetics. The K_m for the activation of Glu-plasminogen is 200 μM, and the k_{cat} is 1.48 s^{-1}, while the K_m for the activation of Lys-plasminogen is 12 μM and the k_{cat} is 1.89 s^{-1} (Table I). In the presence of 0.1 M lysine, the K_m for the activation of Glu-plasminogen decreases to 24 μM, and the k_{cat} is 1.78 s^{-1}, while the K_m for the activation of Lys-plasminogen is 7.5 μM and the k_{cat} is 1.62 s^{-1}. The K_m values in the presence of 0.1 M lysine were calculated by dividing the apparent K_m values by $1 + I/K_i$, where I is the lysine concentration, to compensate for the competitive inhibition by lysine.

An important control for these experiments is to show that the change in K_m from 200 to 24 μM by the presence of 0.1 M lysine is not the result of lysine increasing the solubility of Glu-plasminogen. This was demonstrated by adding an aliquot of Glu-plasminogen to a solution of P_i/NaCl and to a solution of P_i/NaCl containing 0.1 M lysine. The final concentration of Glu-plasminogen was equivalent to the highest concentration of Glu-plasminogen used in the experiments in Figure 6. After 5 min at room temperature, both solutions were centrifuged and the optical densities of the supernatants determined. The optical densities were the same. Indirect evidence also suggests that lysine did not affect the solubility of the Glu-plasminogen used in the experiments in Figure 5. The different concentrations of Glu-plasminogen were prepared by serial dilution. If, at the higher concentrations, some of the Glu-plasminogen was not in solution, the initial velocities measured in those solutions would not have fit the Hanes–Woolf linear transformation of the Michaelis–Menten rate equation.

Discussion

The major conclusions from these experiments are summarized in Table I. Lysine is a competitive inhibitor of urokinase. The k_{cat} values for the activation of Glu- or Lys-plasminogen by urokinase in the presence or absence of 0.1 M lysine are the same. The K_m for the activation of Glu-plasminogen by urokinase is 10-fold higher than that for the activation of Lys-plasminogen by urokinase. In the presence of 0.1 M lysine, the K_m for the activation of Glu-plasminogen by urokinase decreases 10-fold to that for the activation of Lys-plasminogen by urokinase, which is not affected by lysine.

These conclusions are consistent with the data previously obtained on the binding of 6-aminohexanoic acid or lysine to Glu- or Lys-plasminogen (Wallén & Wiman, 1972; Brockway & Castellino, 1972; Violand et al., 1978; Sjöholm et al., 1973; Castellino et al., 1973; Claeys & Vermylen, 1974). The conformation of Glu-plasminogen is different from that of Lys-plasminogen whereas that of Lys-plasminogen is similar to that of plasmin. The binding of lysine to Glu-plasminogen changes its conformation to that of Lys-plasminogen. Lysine does not affect the conformation of Lys-plasminogen. Our data imply that the binding of plasminogen to urokinase is dependent upon the conformation of plasminogen but that once bound to the active site, the rate of cleavage of the arginyl–valyl bond is independent of the plasminogen conformation. Thus, the K_m for the binding of Glu-plasminogen to urokinase is 10-fold higher than the binding of Glu- or Lys-plasminogen in the presence of lysine or of Lys-plasminogen in the absence of lysine to urokinase. However, in all four cases, the k_{cat} is essentially the same.

Our data also explain some of the paradoxical observations that have been made on the activation of Glu- and Lys-plasminogens by urokinase and on the effects of lysine on activation. Glu-plasminogen does not appear as activatable by urokinase as does Lys-plasminogen (Figure 2) because its K_m is 10-fold higher. In the presence of lysine, however, Glu-plasminogen is as activatable by urokinase as is Lys-plasminogen in the absence of lysine because the K_m values are the same and the k_{cat} values are the same. At low concentrations of lysine, the initial rate of activation of Glu-plasminogen by urokinase is stimulated (Figure 4) because the K_m is decreased. This does not occur with Lys-plasminogen, because its K_m for urokinase is not significantly altered by lysine. At higher concentrations of lysine, the activation of both Glu- and Lys-plasminogens is inhibited, because at those concentrations lysine becomes a competitive inhibitor of urokinase.

The kinetics of activation of human Glu- and Lys-plasminogens by human urokinase have been studied by Christensen & Mullertz (1977), Christensen (1977), and Wohl et al. (1980). A double rate assay was used in which plasminogen is converted to plasmin by urokinase and the plasmin then reacts with a synthetic substrate whose hydrolysis is detected spectrophotometrically. The kinetic profiles show an acceleration of synthetic substrate hydrolyzed that must be converted to an initial velocity before the kinetic parameters can be calculated. For the activation of Glu-plasminogen by urokinase Christensen (1977) calculated a K_m of 32 μM and a k_{cat} of 0.26 s^{-1} in the absence of lysine and a K_m of 35 μM and a k_{cat} of 1.5 s^{-1} in the presence of 0.1 M lysine. Although these values for k_{cat} and K_m differ from ours, the values for the ratio k_{cat}/K_m do agree with those obtained by us. For the activation of Lys-plasminogen by urokinase, Christensen & Mullertz (1977) calculated a K_m of 40.7 μM and a k_{cat} of 2.59 s^{-1}. In contrast, Wohl et al. (1980) calculated for the activation of Glu- and Lys-plasminogens by the low and high molecular weight forms of urokinase K_m values about 1.98 μM and k_{cat} values about 0.76 s^{-1}. We do not know why the results of Christensen (1977) and Christensen & Mullertz (1977) differ from those of Wohl et al. (1980) nor why both of their results differ from ours. Our assay is simple and direct whereas their assays are much more complex and their data require extensive analysis.

Our data have implications for the use of certain α,ω-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. They are antifibrinolytic in that occupation of the lysine binding sites prevents plasminogen from binding to fibrin. Thus, 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 positive regulation of fibrinolysis 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 (Sherry, 1968; Rabiner et al., 1969), is much lower than the K_m of 200 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 10-fold so that it is now activatable. The plasmin formed begins to degrade the fibrin. It also converts Glu-plasminogen to Lys-plasminogen. This also results in a lowering of the K_m by 10-fold and accelerates the production of plasmin. Once the clot is dissolved, the K_m for the activation of Glu-plasminogen is increased 10-fold 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 left it only at the site of the clot for the short period of time required to dissolve it. This model for the positive control of fibrinolysis and models for the negative control of fibrinolysis based upon the activity of antiplasmin (Wiman & Collen, 1978) and of a histidine-rich plasma glycoprotein (Linjen et al., 1980) all indicate that the formation of active plasmin is highly regulated.

Our model for the positive regulation of fibrinolysis may also be relevant to the other physiological processes mediated by the activation of plasminogen, e.g., cell migration (Ossowski et al., 1975) and tissue remodeling (Ossowski et al., 1979). There are two points of control, regulation of the synthesis and secretion of plasminogen activators and regulation of the presence of plasmin substrates to which plasminogen must bind before being activatable. By having either plasminogen activators or plasmin substrates, but not both, present continuously ensures that the activation of plasminogen can occur quickly upon the appearance of both components. Requiring that both plasminogen activators and plasmin substrates be present simultaneously ensures that formation of plasmin occurs only where and when it is needed. We shall show in a subsequent article[2] that the two major forms of cell-associated plasminogen activators, the tissue activator of a human melanoma cell line and the activator of a human lung cell line with molecular weights identical with those of human urokinase, respond to Glu- and Lys-plasminogens in a manner similar to that described for urokinase in this paper.

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