Klapper, M. H., & Faraggi, M. (1979) *Q. Rev. Biophys. 12*, 465–519.
Klapper, M. H., & Faraggi, M. (1983) *Biochemistry 22*, 4067–4071.
Land, E. J., & Swallow, A. J. (1969) *Biochemistry 8*, 2117–2125.
Mayhew, S. G., & Ludwig, M. L. (1975) *Enzymes (3rd Ed.) 12*, 57–118.
Meites, L., & Meites, T. (1948) *Anal. Chem. 20*, 984–985.
Prütz, W. A., Butler, J., Land, E. J., & Swallow, A. J. (1980) *Biochem. Biophys. Res. Commun. 96*, 408–414.
Prütz, W. A., Land, E. J., & Sloper, R. W. (1981) *J. Chem. Soc., Faraday Trans. 1, 77*, 281–292.
Steiner, J. P., Faraggi, M., Klapper, M. H., & Dorfman, L. M. (1985) *Biochemistry 24*, 2139–2146.
Winfield, M. E. (1965) *J. Mol. Biol. 12*, 600–611.
Winkler, J. R., Nocera, D. G., Yocum, K. M., Bordignon, E., & Gray, H. B. (1982) *J. Am. Chem. Soc. 104*, 5798–5800.

Quantitative Characterization of the Binding of Plasminogen to Intact Fibrin Clots, Lysine–Sepharose, and Fibrin Cleaved by Plasmin†

Robert A. Bok and Walter F. Mangel*,‡
Department of Biochemistry, University of Illinois, Urbana, Illinois 61801
Received July 19, 1984

ABSTRACT: The binding of human Glu- and Lys-plasminogens to intact fibrin clots, to lysine–Sepharose, and to fibrin cleaved by plasmin was quantitatively characterized. On intact fibrin clots, there was one strong binding site for Glu-plasminogen with a dissociation constant, K_d, of 25 μM and one strong binding site for Lys-plasminogen with a K_d of 7.9 μM. In both cases, the number of plasminogen binding sites per fibrin monomer was 1. Also, a much weaker binding site for Glu-plasminogen was observed with a K_d of about 350 μM. Limited digestion of fibrin by plasmin created additional binding sites for plasminogen with K_d values similar to the binding of plasminogen to lysine–Sepharose. This was predictable given the observations that plasminogen binds to lysine–Sepharose and can be eluted with ϵ-aminocaproic acid [Deutsch, D. G., & Mertz, E. T. (1970) *Science (Washington, D.C.) 170*, 1095–1096] and that plasmin preferentially cleaves fibrin at the carboxy side of lysyl residues [Weinstein, M. J., & Doolittle, R. F. (1972) *Biochim. Biophys. Acta 258*, 577–590], because the structures of the lysyl moiety in lysine–Sepharose and of ϵ-aminocaproic acid are identical with the structure of a COOH-terminal lysyl residue created by plasmin cleavage of fibrin. The K_d for the binding of Glu-plasminogen to lysine–Sepharose was 43 μM and for fibrin partially cleaved by plasmin 48 μM. The K_d for the binding of Lys-plasminogen to lysine–Sepharose was 30 μM. With fibrin partially cleaved by plasmin, there were two types of binding sites for Lys-plasminogen, one with a K_d of 7.6 μM and the other with a K_d of 44 μM. Plasmin-created plasminogen binding sites may be physiologically relevant as a mechanism for accelerating clot destruction and because fragments of fibrin and other proteins created by plasmin cleavage at lysyl residues would be targeted for destruction anywhere in the circulatory system. Fibrin was shown to be a positive regulator of the activation of plasminogen by human urokinase. After a lag period, presumably due to impedance of diffusion by the dense, fibrous matrix of the clot, the rate of activation of Glu-plasminogen rapidly accelerated in the presence of fibrin, compared to in its absence or in the presence of fibrinogen.

The role of fibrin in regulating its own destruction is not clear. The enzyme system involved consists of the serum zymogen plasminogen which upon cleavage of a single arginyl–valyl bond by a plasminogen activator becomes the potent protease plasmin that dissolves fibrin clots. Some theories for the regulation of fibrinolytic activity by fibrin stress the importance of plasminogen binding to fibrin (Alkjaersig et al., 1959), of plasminogen activators binding to fibrin (Chesterman et al., 1972), and of the protective effect of fibrin on the fibrinolytic enzymes from attack by protease inhibitors (Wiman & Collen, 1978).

Quantitative studies of the binding of plasminogen to fibrin were first reported by Thorsen (1975). Native or Glu-plasminogen,[1] which is a single polypeptide chain comprised of 790 amino acids including an NH_2-terminal glutamic acid (Wallén & Wiman, 1972), exhibited a modest affinity for fibrin. Lys-plasminogen, a 714 amino acid variant with an NH_2-terminal lysine created by plasmin cleavage of the lysyl–lysyl bond at position 76–77 from the NH_2-terminus of Glu-plasminogen (Robbins et al., 1967; Wallén & Wiman, 1972), exhibited a stronger affinity for fibrin. The interaction between these two forms of plasminogen and fibrin is mediated through lysine binding sites on plasminogen, because only fragments of plasminogen that bind to lysine–Sepharose bind to fibrin, and because binding of plasminogen to fibrin can be abolished by the presence of certain α,ω-amino acids (Wiman & Wallén, 1977).

† This work was supported by Grant CA-25633 from the National Institutes of Health.
‡ Present address: Biology Department, Brookhaven National Laboratory, Upton, NY 11973.

[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; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

0006-2960/85/0424-3279$01.50/0

Fibrin has been postulated to play a positive role in regulating the activation of plasminogen (Wiman & Wallén, 1977; Peltz et al., 1982). Glu-plasminogen in the presence of certain α,ω-amino acids or Lys-plasminogen in their presence or absence are much more activatable by urokinase than is Glu-plasminogen in the absence of these α,ω-amino acids (Wallén & Wiman, 1972; Claeys & Vermylen, 1974; Walther et al., 1975; Christensen, 1977; Christensen & Mullertz, 1977; Markus et al., 1978; Peltz et al., 1982; Lucas et al., 1983b). This enhancement of the activation of plasminogen has been postulated to occur through a lowering of the K_m (Peltz et al., 1982) or a raising of the k_{cat} (Christensen, 1977; Lucas et al., 1983b). In order to determine if fibrin positively regulates the activation of plasminogen and the kinetic origin of this regulation, the binding of plasminogen to fibrin must be quantitatively characterized.

The objective of this investigation was to characterize quantitatively the binding of plasminogen to fibrin clots. This has been difficult to do experimentally. Clots formed from the interaction of thrombin with fibrinogen are insoluble gels. They are not easy to manipulate physically and present problems with diffusion (Lorand & Middlebrook, 1952) and trapping when trying to assess the rate and extent of binding of plasminogen. Plasminogen itself absorbs strongly to artificial surfaces such as glass and plastic (Suenson & Thorsen, 1981). To obviate these and additional problems, others have studied the binding of plasminogen to altered forms of fibrin. These include threads of fibrin created by incubating radiolabeled plasminogen with fibrinogen and thrombin and spooling the resultant fibrin on glass rods (Thorsen, 1975; Rákóczi et al., 1978; Whitaker et al., 1980), fibrin monomers attached to Sepharose (Wiman & Wallén, 1977), short, soluble fibrin polymers precipitated by plasminogen (Garman & Smith, 1982), and fibrin suspensions generated by sonication (Lucas et al., 1983a). Here we have characterized the binding of plasminogen to intact fibrin clots.

Materials and Methods

Reagents. FMGB, 3′-(4-guanidinobenzoyloxy)-6′-hydroxyspiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one, was synthesized as described in 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 7.4. Soybean trypsin inhibitor, bovine serum albumin, human fibrinogen, ϵ-aminocaproic acid, and Sepharose 4B were purchased from Sigma Chemical Co. Lysine-Sepharose 4B was purchased from Pharmacia and Sigma Chemical Co. Lactoperoxidase and glucose oxidase coimmobilized on a polyacrylamide resin were purchased as Enzymobeads from Bio-Rad, as were all electrophoresis reagents. Bovine thrombin was purchased from Miles Laboratories. Glycine was purchased from Fisher Scientific Co. Porous Teflon filters, Zitex filter membranes, were purchased from Chemplast, Inc. Carrier-free $Na^{125}I$ (16 mCi/μg) was purchased from Amersham.

Purification and Characterization of Human Glu- and Lys-plasminogens. Human Glu-plasminogen was purified from plasma that had been obtained no more than 4 h prior to the addition of aprotinin to a concentration of 100 kallikrein inactivator units (KIU)/mL. All operations were performed at 4 °C. After passage through a cheesecloth filter and centrifugation for 30 min at 27000*g*, the supernatant (1000 mL) was applied to a 2.6 × 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 ϵ-aminocaproic 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 the plasminogen was precipitated by adding 0.31 g of $(NH_4)_2SO_4$/mL of solution, stirring for 30 min, and then centrifuging for 30 min at 30000*g*. The precipitate was dissolved in less than 4 mL of P_i/NaCl, and that solution was applied at a flow rate of 20 mL/h to a 2.6 × 70 cm column of Sephadex G-200. The peak fractions were pooled and concentrated by filtration under nitrogen pressure with an Amicon ultrafiltration membrane. The plasminogen, at concentrations greater than 20 mg/mL, was stored in aliquots at −20 °C. The yield from 100 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 15 mg/mL in P_i/NaCl was incubated with 0.48 μM plasmin. After 48 h at 4 °C, 0.5 mL of aprotinin–Sepharose beads was added to the reaction mixture to remove the plasmin. After 3 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, $E^{1\%}_{280nm}$, of 16.9.

The purity of human Glu- and Lys-plasminogen preparations was assessed by $NaDodSO_4$–polyacrylamide gel electrophoresis as described by Laemmli (1972), by polyacrylamide gel electrophoresis at pH 3.2 in the presence of urea, as described by Panyim & Chalkley (1969), and by NH_2-terminal amino acid analysis as described in Peltz et al. (1982). The plasminogen preparations were judged to be greater than 95% pure by these criteria and at least 95% activatable to plasmin in the presence of urokinase.

Radioiodination of Plasminogen. Glu- and Lys-plasminogens were radiolabeled by using the solid-phase lactoperoxidase–glucose oxidase method (Thorell & Johansson, 1971; Haebner et al., 1979). Reaction mixtures of 100 μL in P_i/NaCl contained 0.1–1.0 mg of plasminogen, 10–20 μL of Enzymobeads, 1–2 mCi of $Na^{125}I$, and 0.2% α-D-glucose that had been allowed to mutarotate overnight at 25 °C. After 1–2 h at 25 °C, the reaction vial was centrifuged for 1 min at 14000*g* and the supernatant applied to a 0.5 × 10 cm column of Sephadex G-25 that had been equilibrated in P_i/NaCl. The radioactivity in the void volume was collected and stored in aliquots at −20 °C. The specific activity varied from 0.5 to 1.5 mCi/mg.

The labeled plasminogens were characterized by $NaDodSO_4$–polyacrylamide gel electrophoresis (Laemmli, 1972) and by polyacrylamide gel electrophoresis at pH 3.2 in the presence of urea (Panyim & Chalkley, 1969), followed by Coomassie Blue staining and autoradiography. No differences were observed in the electrophoretic behavior of the radiolabeled plasminogens compared to the corresponding well-characterized unlabeled plasminogens. Radiolabeled Glu- and Lys-plasminogens were also activated by urokinase in the presence and absence of 0.1 M lysine (Peltz et al., 1982), and the resultant plasmin was quantitated after $NaDodSO_4$–polyacrylamide gel electrophoresis by Coomassie Blue staining and by autoradiography. The activation of ^{125}I-Glu-plasminogen was stimulated by the presence of 0.1 M lysine whereas the activation of ^{125}I-Lys-plasminogen was not. Greater than 92%

of the radiolabeled Glu- and Lys-plasminogens bound to lysine–Sepharose.

Fibrin. Human fibrinogen was passed through a column of lysine–Sepharose equilibrated in P_i/NaCl minus calcium and magnesium to remove any contaminating plasminogen. Fibrin clots were formed on Teflon filters (1-cm diameter disks) by placing the indicated volume of 6.5 mg/mL fibrinogen in P_i/NaCl minus calcium and magnesium on the disk and adding to it the indicated volume of 100 units/mL bovine thrombin in P_i/NaCl. After 1 h at 25 °C, the filters were placed on a Büchner funnel, and the liquid in the clots was removed by vacuum filtration. The clots were then washed with 5 mL of distilled water which was also removed by vacuum filtration. The amount of fibrin in a clot was determined by dissolving it in 1–2 mL of 2.5 N NaOH, incubating the solution in a boiling water bath for 15 min, and measuring the absorbance at 280 nm. The moles of fibrin per clot was calculated by using an extinction coefficient, $E^{1\%}_{280nm}$, of 16.17 and a molecular weight of 340 000 (Thorsen, 1975).

Binding Assays. Radiolabeled plasminogens in binding buffer were incubated at 37 °C with fibrin in polypropylene tubes. Binding buffer was P_i/NaCl with 10 mg/mL bovine serum albumin, 0.27 mg/mL soybean trypsin inhibitor, and 0.02% NaN_3. In most experiments, the extent of binding was determined either by counting the radioactivity in an aliquot removed from the solution above the clot or by placing the clot on a GF/C filter (Whatman), washing with P_i/NaCl, and then counting the radioactivity associated with the clot. Controls were performed in an identical manner but in the absence of fibrin or in the presence of fibrin plus 0.05 M ϵ-aminocaproic acid. Radioactivity was determined with a Beckman Gamma-8000 counter.

Kinetics of Activation by Urokinase. Activation reactions in 0.1 mL of P_i/NaCl contained 1 μM Glu-plasminogen, 1.7 Plough units of human urokinase, and in some cases 1 nmol of fibrin or fibrinogen. After the indicated time intervals at 25 °C, 0.9 mL of 2 μM FMGB in P_i/NaCl was added and 2 min later the increase in fluorescence measured. The difference between this increase in fluorescence and that of an identical solution but lacking urokinase is directly proportional to the concentration of plasmin that had formed (Melhado et al., 1982).

Fluorescence was measured in a Perkin-Elmer MPF-44A fluorescence spectrofluorometer with a Universal digital readout. The excitation and emission wavelengths were 491 and 514 nm, respectively, both set with a bandwidth of 5 nm. The fluorometer was standardized with rhodamine B embedded in a polymethacrylate block so that the relative fluorescence in different experiments was comparable.

Results

Kinetics of Binding of Glu-plasminogen to Fibrin. The kinetics of binding of Glu-plasminogen to fibrin and to fibrin attached to Teflon filters are shown in Figure 1. The clots contained 2 nmol of fibrin, and in the presence of 22 μM Glu-plasminogen, 12% of the Glu-plasminogen was bound at equilibrium. The presence of Teflon filters as a support for fibrin did not affect either the rate or the extent of binding. The kinetics of binding were quite slow, the time to reach equilibrium being about 15 h. Incubation of the dried fibrin clots in P_i/NaCl for several hours prior to the addition of Glu-plasminogen did not alter the time required to reach maximal binding (data not shown). Thus, the rate-limiting step was not a slow rehydration of the dried clots.

Affinity of Glu- and Lys-plasminogens for Fibrin. The binding of Glu-plasminogen to fibrin is quantitatively characterized in Figure 2. ^{125}I-Glu-plasminogen ranging in concentration from 0.1 to 65 μM was incubated for 15 h at 37 °C with 2 nmol of fibrin on Teflon filters. The unbound plasminogen was then removed by suction, and the amount of plasminogen bound to fibrin was calculated after counting the radioactivity remaining on the filters. The data are presented in Figure 2A in the form of a Scatchard plot. There appear to be two classes of binding sites on fibrin for Glu-plasminogen, one with a K_d of 25 μM and the other, a much weaker binding site, with a K_d greater than 350 μM. The number of strong Glu-plasminogen binding sites per fibrin monomer was about 0.7. The strong Glu-plasminogen binding site was also characterized in binding assays in which the fibrin content was varied, and the ^{125}I-Glu-plasminogen concentration was held constant. The data are presented in Figure 2B in the form of a Bjerrum plot. A binding stoichiometry of one Glu-plasminogen per fibrin monomer was assumed, and a K_d of 25 μM was obtained.

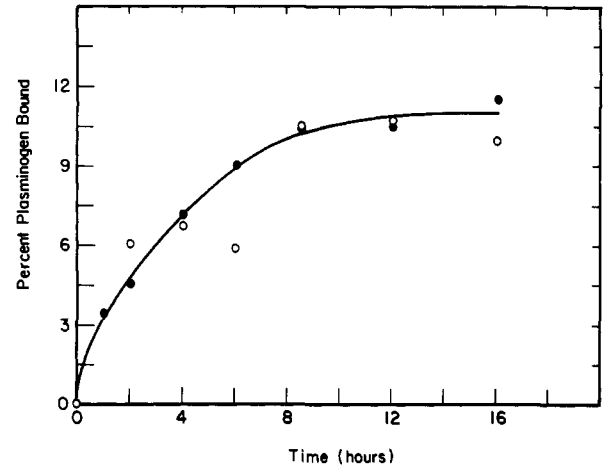

FIGURE 1: Kinetics of binding of Glu-plasminogen to fibrin clots. Fibrin clots, formed from 2 nmol of fibrinogen on Teflon filters, were dried and washed with water. Then, either filters with clots (●) or clots removed from filters (○) were placed in vials and overlayed with 0.5-mL aliquots of 2.2 μM ^{125}I-Glu-plasminogen in binding buffer. After the indicated time intervals at 37 °C, 10-μL aliquots were removed and counted for radioactivity. The percent Glu-plasminogen bound was defined as 100[(decrease in soluble Glu-plasminogen in the presence of fibrin − decrease in soluble Glu-plasminogen in the absence of fibrin)/total amount of Glu-plasminogen].

The binding of Lys-plasminogen to fibrin is quantitatively characterized in Figure 3. ^{125}I-Lys-plasminogen ranging in concentration from 0.95 to 30 μM was incubated for 15 h at 37 °C with clots formed from 0.37 or 0.92 nmol of fibrinogen. The data are presented in Figure 3A in the form of a Hanes–Woolf linear transformation. Both lines intersected the abscissa at about the same point, yielding a K_d of 10 μM. The intercepts on the ordinate, which reflect maximal binding, indicated the number of Lys-plasminogen binding sites per fibrin monomer was 1.1 from the binding assays with clots formed from 0.37 nmol of fibrinogen and was 0.90 from the binding assays with clots formed from 0.92 nmol of fibrinogen. The binding of Lys-plasminogen to fibrin was also characterized in assays in which the fibrin content was varied, and the ^{125}I-Lys-plasminogen concentration was held constant. The data are presented in Figure 3B in the form of a direct plot with the line representing the best-fit rectangular hyperbola as determined by using the iterative method of Cleland (1967). A binding stoichiometry of one Lys-plasminogen per fibrin monomer was assumed, and a K_d of 5.8 μM was obtained.

Affinity of Glu- and Lys-plasminogens for Lysine–Sepharose. The binding of Glu-plasminogen to lysine–Sepharose

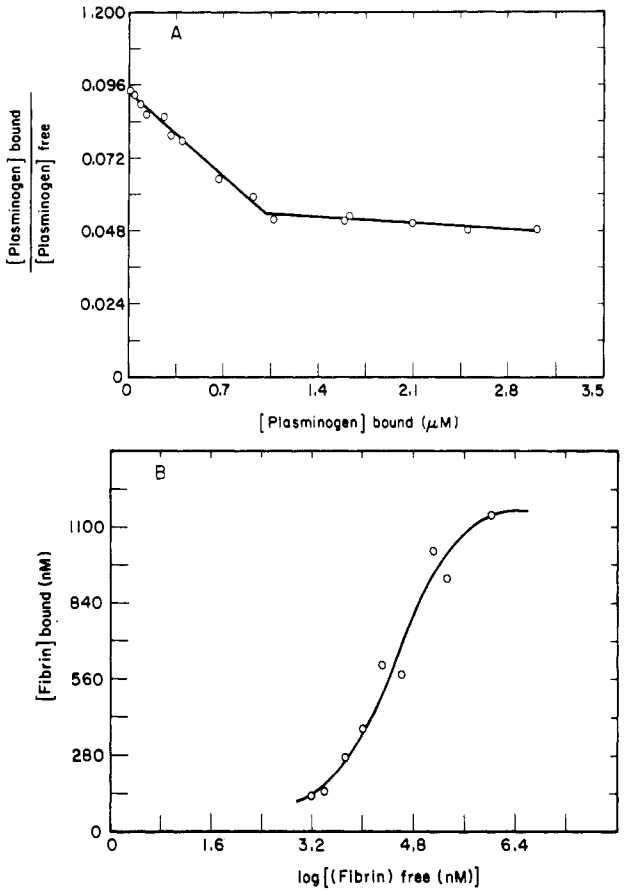

FIGURE 2: Binding of Glu-plasminogen to fibrin: (A) variation of Glu-plasminogen concentration; (B) variation of fibrin concentration. (A) Clots, formed with 2 nmol of fibrinogen on Teflon filters, were incubated with 0.1-mL aliquots of ^{125}I-Glu-plasminogen ranging in concentration from 0.1 to 65 μM in binding buffer. After 16 h at 37 °C, the Teflon filters were placed on a filtration block, and the unbound Glu-plasminogen was removed by suction. The amount of Glu-plasminogen specifically bound to fibrin was determined by counting the radioactivity bound to the filters and subtracting from that the radioactivity bound to filters in an analogous experiment but without fibrin. The data are presented in a Scatchard plot. (B) Clots were formed on Teflon filters with 0.16–96 nmol of fibrinogen, removed from the filters, and then incubated with 0.1-mL aliquots of 1.09 μM ^{125}I-Glu-plasminogen in binding buffer. After 16 h at 37 °C, the clots were placed on GF/C filters and washed with 2.0 mL of P_i/NaCl minus calcium and magnesium. The amount of Glu-plasminogen specifically bound to fibrin was determined by counting the radioactivity bound to the clots. The data are presented in a Bjerrum plot.

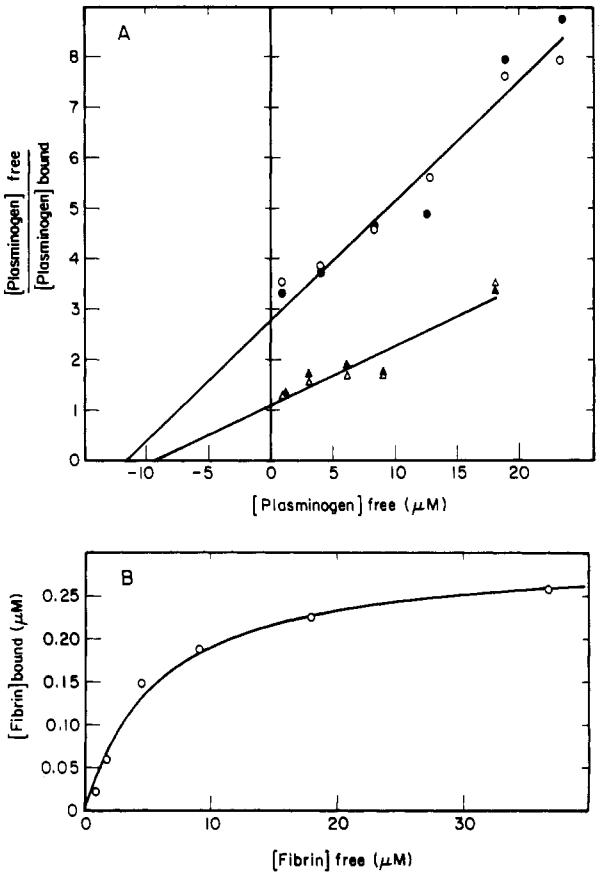

FIGURE 3: Binding of Lys-plasminogen to fibrin: (A) variation of Lys-plasminogen concentration; (B) variation of fibrin concentration. (A) Clots, formed from 0.37 nmol of fibrinogen (circles) or 0.92 nmol of fibrinogen (triangles) on Teflon filters, were dried, washed with distilled water, and transferred from filters to assay tubes. The clots were then incubated with 0.1-mL aliquots of ^{125}I-Lys-plasminogen ranging in concentration from 0.95 to 30 μM in binding buffer. After 15 h at 37 °C, 0.05-mL aliquots were removed, and the radioactivity in the aliquots and that remaining in the assay tubes were counted. The amount of Lys-plasminogen specifically bound to fibrin was defined as the decrease in soluble Lys-plasminogen observed in the presence of fibrin minus the decrease in soluble Lys-plasminogen observed in the absence of fibrin (closed symbols) or as the increase in Lys-plasminogen remaining in the assay tubes with fibrin minus that remaining in assay tubes without fibrin (open symbols). The data are presented in the form of a Hanes–Woolf linear transformation. (B) Clots were formed on Teflon filters with from 0.092 to 3.7 nmol of fibrinogen and then incubated with 0.1-mL aliquots of 0.5 μM ^{125}I-Lys-plasminogen in binding buffer. After 15 h at 37 °C, the clots were placed on GF/A filters and washed with 2 mL of P_i/NaCl minus calcium and magnesium. The amount of Lys-plasminogen specifically bound to fibrin was determined by counting the radioactivity bound to the clots. The data are presented in a direct plot.

is quantitatively characterized in Figure 4. ^{125}I-Glu-plasminogen ranging in concentration from 2.3 to 200 μM was incubated for 1 h at 37 °C with different densities of lysine–Sepharose. The concentration of unbound ^{125}I-Glu-plasminogen was determined after counting the radioactivity in an aliquot from the supernatant of the reaction mixture after centrifugation. The data are shown in Figure 4A in the form of a direct plot. Half of the lysine residues on the lysine–Sepharose were saturated at a free ^{125}I-Glu-plasminogen concentration of 43 μM. At the higher ^{125}I-Glu-plasminogen concentrations, the ratio $[\text{plasminogen}]_{\text{bound}}/[\text{lysine residues}]$ approached a maximum value of 1.0. The binding of ^{125}I-Glu-plasminogen to lysine–Sepharose was also characterized in assays in which the amount of lysine–Sepharose was varied 1000-fold, and the ^{125}I-Glu-plasminogen concentration was held constant. The data are presented in Figure 4B in the form of a Bjerrum plot. A binding stoichiometry of one ^{125}I-Glu-plasminogen per lysine residue on lysine–Sepharose was assumed, and a K_d of 32 μM was obtained.

The binding of Lys-plasminogen to lysine–Sepharose was also quantitatively characterized (data not shown). Binding assays were performed in which the amount of lysine–Sepharose was varied 100-fold and the concentration of ^{125}I-Lys-plasminogen was held constant at 2 μM. A binding stoichiometry of one ^{125}I-Lys-plasminogen per lysine residue on the lysine–Sepharose was assumed, and a K_d of 30 μM was obtained.

Effect of Cleavage of Fibrin by Plasmin on the Extent of Binding of Glu-plasminogen. To determine whether cleavage of fibrin by plasmin creates additional binding sites for Glu-plasminogen, we incubated fibrin clots attached to Teflon filters with 90 nM plasmin for varying periods of time up to 50 min. The activity of plasmin was then inhibited by the addition of aprotinin. Next, ^{125}I-Glu-plasminogen was added to a concentration of 2.3 μM and the reaction mixture incu-

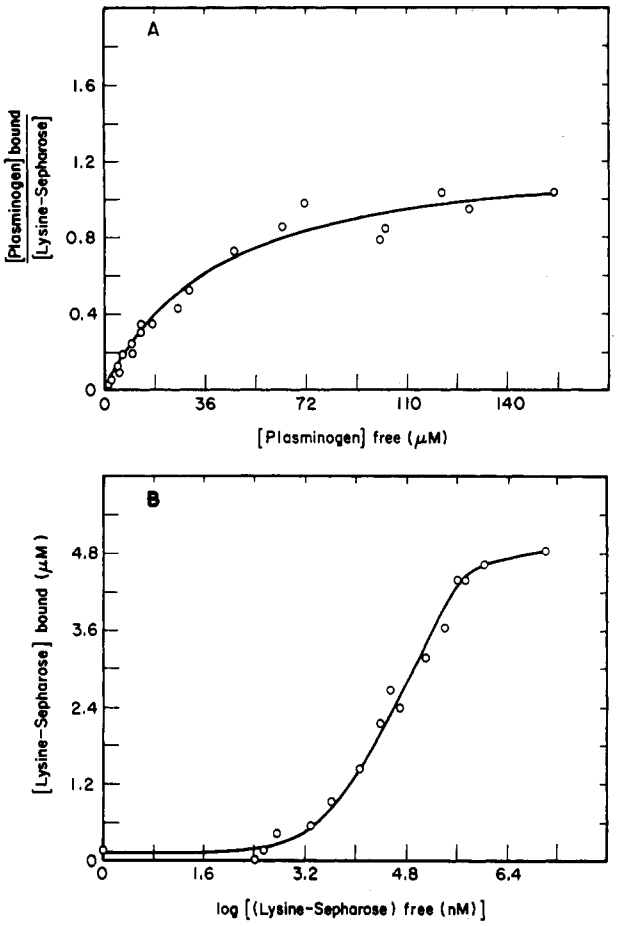

FIGURE 4: Binding of Glu-plasminogen to lysine–Sepharose: (A) variation of Glu-plasminogen concentration; (B) variation of lysine–Sepharose concentration. (A) Aliquots of ^{125}I-Glu-plasminogen ranging in concentration from 2.3 to 199 μM in binding buffer were incubated with different densities of lysine–Sepharose. After 1 h of continuous rotation at 37 °C, the assay tubes were centrifuged at 14000*g* for 2 min, and 0.01-mL aliquots of the supernatant were removed and counted for radioactivity. The amount of Glu-plasminogen specifically bound to lysine–Sepharose was defined from the decrease in soluble Glu-plasminogen observed in the presence of lysine–Sepharose minus the decrease in soluble Glu-plasminogen observed in an analogous experiment but with Sepharose. (B) Suspensions of lysine–Sepharose in binding buffer, whose immobilized lysine content ranged from 0.004 to 200 nmol, were incubated with 5 μM ^{125}I-Glu-plasminogen in a volume of 0.1 mL. After 1 h of continuous rotation at 37 °C, the reaction mixtures were processed as described in (A).

bated at 37 °C for 15 h. Control experiments were performed in an identical manner except without plasmin. The data are presented in Figure 5 and indicate that progressive cleavage of fibrin by plasmin results in a progressive increase in the amount of Glu-plasminogen bound to fibrin. The highest percent increase in plasminogen bound was 60%, and that was exhibited by fibrin that had been incubated with plasmin for 50 min. Longer incubation times with plasmin resulted in a decrease in the binding of ^{125}I-Glu-plasminogen, presumably because extensive amounts of fibrin were being solubilized.

Affinity of Glu- and Lys-plasminogens for Fibrin Cleaved by Plasmin. To determine the affinity of Glu-plasminogen for fibrin cleaved by plasmin, we incubated fibrin clots formed from 2 nmol of fibrinogen on Teflon filters with 90 nM plasmin for 60 min, inactivated the plasmin with aprotinin, and then incubated the clots with ^{125}I-Glu-plasminogen ranging in concentration from 1 to 143 μM. After 15 h at 37 °C, the amount of bound ^{125}I-Glu-plasminogen was determined. The data are presented in Figure 6A in the form of a direct plot.

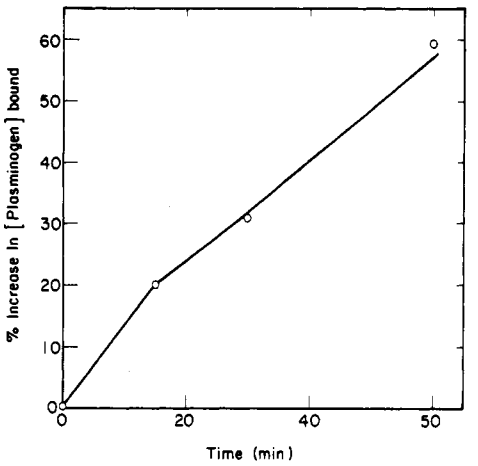

FIGURE 5: Progressive increase in the extent of binding of Glu-plasminogen to fibrin after progressive cleavage of fibrin by plasmin. Clots, formed with 2 nmol of fibrinogen on Teflon filters, were incubated in 0.3-mL aliquots of 90 nM plasmin in P_i/NaCl. After the indicated time intervals at 37 °C, the soluble plasmin was removed, and the clots were incubated with 0.25-mL aliquots of P_i/NaCl containing 8000 KIU/mL aprotinin and 0.2 M ϵ-aminocaproic acid. After 15 min at 22 °C, the clots were washed with 4 mL of P_i/NaCl minus calcium and magnesium, incubated with 0.2 mL of a solution containing 10 000 KIU/mL aprotinin, and washed again with 4 mL of P_i/NaCl minus calcium and magnesium. The clots were then overlayed with 0.5-mL aliquots of 2.3 μM ^{125}I-Glu-plasminogen in binding buffer. After incubation at 37 °C for 15 h, 0.025-mL aliquots were removed from the supernatant and counted for radioactivity. The amount of Glu-plasminogen specifically bound to fibrin was defined as the decrease in soluble Glu-plasminogen observed in the presence of fibrin minus the decrease in soluble Glu-plasminogen observed in an analogous experiment but in the absence of fibrin. The percent increase in Glu-plasminogen bound was calculated as 100[(amount of Glu-plasminogen bound to fibrin cleaved by plasmin − amount of Glu-plasminogen bound to fibrin)/amount of Glu-plasminogen bound to fibrin].

The K_d was 48 μM, and the number of Glu-plasminogen binding sites per fibrin monomer was 1.1, a value greater than that obtained with intact clots.

The affinity of Lys-plasminogen for fibrin cleaved by plasmin is shown in Figure 6B in the form of a Scatchard plot. The curvilinear distribution of the points suggested the existence of two classes of binding sites, and the data were fitted to two straight lines by linear regressions. The slopes of the two lines indicated a high-affinity site with a K_d of 7.6 μM and a lower affinity site with a K_d of 44 μM. The number of sites per fibrin monomer were 0.85 and 1.3, respectively.

Effect of Fibrinogen and Fibrin on Kinetics of Activation of Glu-plasminogen by Urokinase. The effect of fibrinogen and fibrin on the kinetics of activation of Glu-plasminogen by urokinase is shown in Figure 7. The presence of fibrinogen increased the rate of activation about 2-fold. For the first 30 min, the rate of activation in the presence of fibrin was identical with that in the presence of fibrinogen, but thereafter the rate began to increase dramatically. In a similar experiment but with Lys-plasminogen, the rates of activation in the presence and absence of fibrin were constant and identical and were similar to the rate of activation of Glu-plasminogen in the presence of fibrin after the lag period. The 30-min lag in the stimulation of the activation of Glu-plasminogen by urokinase was probably due to the impedance of diffusion of urokinase and of plasminogen by the dense, fibrous matrix of the clot. If clots were preincubated with either plasminogen or urokinase for up to 1 h and then the other component was added, the kinetics of activation were similar to those in which no preincubation occurred (data not shown). The lag could be shortened if activation were allowed to proceed for 30 min,

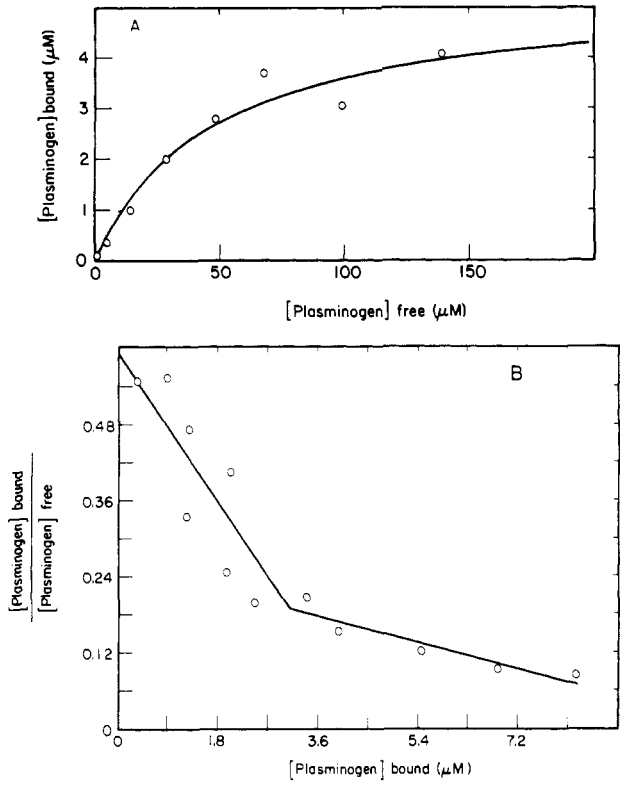

FIGURE 6: Binding of Glu-plasminogen (A) and Lys-plasminogen (B) to fibrin cleaved by plasmin; variation of plasminogen concentration. Clots, formed with 2 nmol of fibrinogen on Teflon filters, were incubated in 0.3-mL aliquots of 90 nM plasmin in P_i/NaCl for 60 min at 37 °C. The soluble plasmin was removed; the clots were washed with 1 mL of P_i/NaCl and then incubated with 0.3-mL aliquots of P_i/NaCl containing 1670 KIU/mL aprotinin. After 30 min at 22 °C, the clots were washed with 1 mL of P_i/NaCl, incubated with 0.3 mL of P_i/NaCl containing 1670 KIU/mL aprotinin for 30 min at 22 °C, and washed again with 3 mL of P_i/NaCl. (A) The clots were then incubated in 0.3-mL aliquots of binding buffer containing ^{125}I-Glu-plasminogen ranging in concentration from 1 to 143 μM and either 0.05 M glycine or 0.05 M ε-aminocaproic acid. (B) The clots were then incubated in 0.3-mL aliquots of binding buffer containing ^{125}I-Lys-plasminogen ranging in concentration from 1 to 105 μM and either 0.05 M glycine or 0.05 M ε-aminocaproic acid. After 15 h at 37 °C, the clots in both experiments were washed with 10 mL of P_i/NaCl minus calcium and magnesium, and the amount of radioactivity bound to the clots was counted. The fraction of plasminogen specifically bound to fibrin was defined as the radioactivity bound in assays with 0.05 M glycine minus the radioactivity bound in assays with 0.05 M ε-aminocaproic acid, divided by the total radioactivity.

the clots washed, and then fresh plasminogen and urokinase added. A partial decrease in the lag was also observed if the clots were preincubated with plasmin.

Discussion

These studies have shown that plasminogen can bind to intact fibrin clots and does so with a physiologically relevant dissociation constant. Since the concentration of plasminogen in plasma is 1–2 μM (Sherry, 1968; Rabiner et al., 1969), about 5% of the fibrin monomers in a clot will have Glu-plasminogen bound to them. However, at least three plasma proteins, α_2-antiplasmin (Moroi & Aoki, 1976), a histidine-rich glycoprotein (Lijnen et al., 1980), and fibrinogen (Wiman & Wallén, 1977), bind to the lysine binding sites on plasminogen. If in plasma a large amount of plasminogen is bound to them, then the concentration of plasminogen potentially able to bind to fibrin would be lower.

Our studies differ from those of others in that we characterized the binding of plasminogen to intact fibrin clots and to plasmin-nicked fibrin clots. Quantitative binding studies

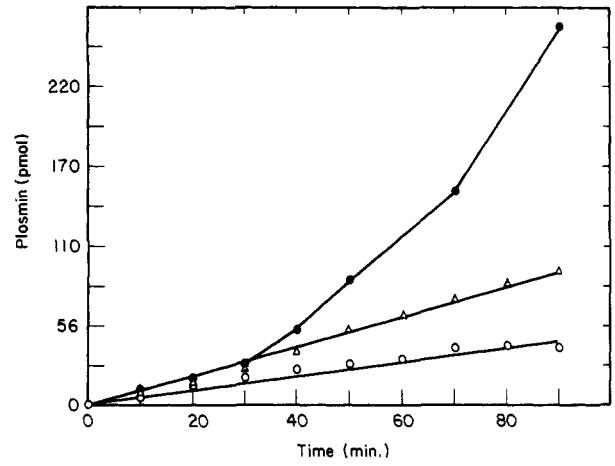

FIGURE 7: Kinetics of activation of Glu-plasminogen by urokinase in the presence of fibrin (●), in the absence of fibrin (○), and in the presence of fibrinogen (△). Activation reactions of 0.1 mL were prepared in P_i/NaCl containing 5.0 μM Glu-plasminogen and 1.7 Plough units of urokinase. The amount of fibrinogen present was 1 nmol, and the fibrin was formed from 1 nmol of fibrinogen. After the indicated time intervals at 22 °C, 0.9 mL of P_i/NaCl containing 2 μM FMGB was added to titrate the amount of plasmin formed.

with these physiologically relevant forms of fibrin were made possible once we realized the time to reach equilibrium was about 15 h at 37 °C. We infer that this long time to reach equilibrium was due to a low rate of diffusion of plasminogen in the dense, fibrous matrix of a clot, because plasminogen binds rapidly to lysine–Sepharose (data not shown), to sonicated fibrin (Lucas et al., 1983a), and to fibrin formed if plasminogen is present during its polymerization (Thorsen, 1975). The long incubation times we had to use to reach equilibrium did not significantly alter the binding properties of plasminogen. Maximal binding of plasminogen to lysine–Sepharose was reached within minutes and then remained constant for 17 h at 37 °C (data not shown). Because of the long incubation periods, sodium azide at 0.02% was present in the binding buffer to prevent microbial growth. Soybean trypsin inhibitor was present in binding buffer to inactivate any contaminating plasmin present. At lower concentrations of soybean trypsin inhibitor, the number of plasminogen binding sites on fibrin increased, implying the plasmin could create new binding sites on fibrin (data not shown). Bovine serum albumin at 10 mg/mL was included in the binding buffer to minimize nonspecific adsorption of plasminogen. At this concentration, which is 4-fold lower than its in vivo concentration (Schultze & Heremans, 1966), albumin had little or no effect on the binding of plasminogen to fibrin, which is in agreement with studies by Moroi & Aoki (1977).

Our study on the binding of plasminogen to intact fibrin clots, summarized in Table I, is in general agreement with several studies on the binding of plasminogen to altered forms of fibrin. In studies of the binding of plasminogen to threads of fibrin created by incubating radiolabeled plasminogen with fibrinogen and thrombin and spooling the resultant fibrin on glass rods, Suenson & Thorsen (1981) obtained K_d values of 42 and 24 μM for Glu-plasminogen and 3.8 and 2.1 μM for Lys-plasminogen. With sonicated fibrin suspensions, Lucas et al. (1983a) obtained a K_d of 38 μM for Glu-plasminogen and 0.32 μM for Lys-plasminogen. Cederholm-Williams (1977) obtained a K_d of 9.3 nM for Lys-plasminogen binding to fibrin during its polymerization on crushed glass, and Garman & Smith (1982) obtained a K_d of 0.18 μM for Lys-plasminogen binding to and precipitating short fibrin polymers. Juhan-Vague et al. (1981) and Shah & Dhall

Table I: Binding of Glu- and Lys-plasminogens to Intact Fibrin Clots, Lysine–Sepharose, and Plasmin-Nicked Fibrin

	Glu-plasminogen				Lys-plasminogen			
	K_d (μM)[a]	n[b]	K_d (μM)[c]	n[d]	K_d (μM)[a]	n[b]	K_d (μM)[c]	n[d]
intact fibrin clots	25	0.7	25	1	10	1.1	5.8	1
	350							
lysine–Sepharose	43	1	32	1			30	1
plasmin-nicked fibrin	48	1.1			7.6	0.85		
					44	1.3		

[a] The dissociation constant was determined by varying the plasminogen concentration. [b] The number of plasminogen binding sites per fibrin monomer or per lysine residue on lysine–Sepharose. [c] The dissociation constant was determined by varying the amount of fibrinogen or lysine–Sepharose. [d] A value of $n = 1$ was assumed and used to calculate the K_d.

(1983) observed no binding of Glu-plasminogen to fibrin. In plasma clots, Rákóczi et al. (1978) found that when added in trace amounts 4% of the radiolabeled Glu-plasminogen and 8% of the radiolabeled Lys-plasminogen associated with the fibrin.

Our data indicated that there are at least two types of plasminogen binding sites on fibrin, one on intact fibrin clots and another on fibrin that has been partially degraded by plasmin. The existence of this latter site was predictable. Plasminogen was shown by Deutsch & Mertz (1970) to bind to lysine–Sepharose and to be eluted by ϵ-aminocaproic acid. Also, plasmin can cleave at the carboxy terminus of lysyl and arginyl residues in fibrin (Weinstein & Doolittle, 1972; Mihalyi, 1983). A comparison of the structure of the lysyl group in lysine–Sepharose, of ϵ-aminocaproic acid, and of a COOH-terminal lysyl residue in fibrin created by plasmin cleavage indicates they are identical. Thus, plasmin-created plasminogen binding sites on fibrin should exhibit K_d values similar to the binding of plasminogen to lysine–Sepharose. Consistent with this was the observation of a new Lys-plasminogen binding site on fibrin nicked by plasmin that had a K_d similar to that of Lys-plasminogen for lysine–Sepharose. Although the K_d values for Glu-plasminogen binding to fibrin, lysine–Sepharose, and plasmin-nicked fibrin were similar, progressive cleavage of fibrin by plasmin resulted in a progressive increase in the number of Glu-plasminogen binding sites. The similarity in K_d values of Glu- and Lys-plasminogen for lysine-Sepharose is consistent with the observation that in a mixture, Glu- and Lys-plasminogens cannot be separated from each other by chromatography on lysine-Sepharose, even with gradient elution by ϵ-aminocaproic acid (Wallén, 1980).

Plasmin-created plasminogen binding sites on fibrin may be physiologically relevant. Once fibrinolysis is initiated, creation of additional plasminogen binding sites would lead to an acceleration of the rate of fibrinolysis. Furthermore, as fibrin is solubilized by plasmin, fragments may escape from the site of the clot. If these fragments contain a COOH-terminal lysine, plasminogen would bind and the fragments would be targeted for destruction anywhere in the circulatory system. Of the 21 identified plasmin cleavage sites in fibrin(ogen), cleavage at 14 of them results in COOH-terminal lysine fragments and at 7 of them COOH-terminal arginine fragments (Doolittle et al., 1978). All of the early cleavages during fibrinolysis are at lysyl bonds; i.e., only lysyl bonds are broken by the time of lysis (Weinstein & Doolittle, 1972). Also, proteins at the site of a clot other than fibrin that are cleaved by plasmin may be similarly targeted for destruction because of newly created plasminogen binding sites. Because plasmin can create plasminogen binding sites in fibrin, studies of fragments of fibrin(ogen) produced by plasmin or trypsin whose objective is to locate the plasminogen binding sites in intact fibrin(ogen) should be viewed with caution.

Fibrin positively regulates the activation of plasminogen. The kinetics of activation of Glu-plasminogen by urokinase were linear for 90 min. In the presence of fibrinogen, a 2-fold increase in the rate of activation was observed. In the presence of fibrin, the kinetics of activation for the first 30 min were identical with those observed in the presence of fibrinogen. After 30 min, however, the rate of activation in the presence of fibrin began to increase, almost exponentially. This dramatic increase in rate was probably not due to the accumulation of simple fibrin(ogen) degradation products or of Lys-plasminogen. If that were true, the rate of activation in the presence of fibrinogen would have increased after 30 min. Rather, the 30-min lag was probably due to the impedance of diffusion of plasminogen and of urokinase by the dense, fibrous matrix of the clot. Studies on the kinetics of activation of Glu-plasminogen by urokinase in the presence of sonicated fibrin showed the rate of activation to be constant and much greater than that in the absence of fibrin (data not shown). Our results are consistent with observations of others (Takada et al., 1979, 1984; Takada & Takada, 1981) who found that the presence of fibrin and fibrinogen enhances the rate of activation of Glu-plasminogen by urokinase and that fibrin enhances the rate of activation more than does fibrinogen. By being able to predict quantitatively how much plasminogen is bound to fibrin, the effect of binding on the kinetics of activation should now be amenable to quantitative analysis.

ACKNOWLEDGMENTS

We gratefully acknowledge the assistance of the Champaign County Blood Bank Division of the Regional Health Resource Center.

Registry No. Plasminogen, 9001-91-6; urokinase, 9039-53-6; lysine, 56-87-1; plasmin, 9001-90-5.

REFERENCES

Alkjaersig, N., Fletcher, A. P., & Sherry, S. (1959) *J. Clin. Invest. 38*, 1086–1095.

Cederholm-Williams, S. A. (1977) *Thromb. Res. 11*, 421–423.

Chesterman, C. N., Allington, M. J., & Sharp, A. A. (1972) *Nature (London), New Biol. 234*, 15–17.

Christensen, U. (1977) *Biochim. Biophys. Acta 481*, 638–647.

Christensen, U., & Mullertz, S. (1977) *Biochim. Biophys. Acta 480*, 275–281.

Claeys, H., & Vermylen, J. (1974) *Biochim. Biophys. Acta 342*, 351–359.

Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol. 29*, 1–32.

Deutsch, D. G., & Mertz, E. T. (1970) *Science (Washington, D.C.) 170*, 1095–1096.

Doolittle, R. F., Watt, K. W. K., Cotrell, B. A., & Takagi, T. (1978) in *Versatility of Proteins* (Li, C. H., Ed.) pp 394–411, Academic Press, New York.

Garman, A. J., & Smith, R. A. G. (1982) *Thromb. Res. 27*, 311–320.

Haebner, J. F., Rosenblatt, M., Dee, D. C., & Potts, J. T. (1979) *J. Biol. Chem. 254*, 10596–10599.

Hoylaerts, M., Rijken, D. C., Lijnen, H. R., & Collen, D. (1982) *J. Biol. Chem. 257*, 2912-2919.
Juhan-Vague, I., Calas, M. F., Roux, F., Juhan, C., Durand-Dessemon, F., deLaforte, C., & Serradimigni, A. (1981) *Thromb. Haemostasis 45*, 154-157.
Laemmli, U. K. (1972) *Nature (London) 227*, 680-685.
Lijnen, H. R., Hoylaerts, M., & Collen, D. (1980) *J. Biol. Chem. 255*, 10214-10222.
Lorand, L., & Middlebrook, W. R. (1952) *Biochem. J. 52*, 196-199.
Lucas, M. A., Fretto, L. J., & McKee, P. A. (1983a) *J. Biol. Chem. 258*, 4249-4256.
Lucas, M. A., Straight, D. L., Fretto, L. J., & McKee, P. A. (1983b) *J. Biol. Chem. 258*, 12171-12177.
Markus, G., Evers, J. L., & Hobika, G. H. (1978) *J. Biol. Chem. 253*, 733-739.
Melhado, L. L., Peltz, S. W., Leytus, S. P., & Mangel, W. F. (1982) *J. Am. Chem. Soc. 104*, 7299-7306.
Mihalyi, E. (1983) *Ann. N.Y. Acad. Sci. 408*, 60-70.
Moroi, M., & Aoki, N. (1976) *J. Biol. Chem. 251*, 5956-5965.
Moroi, M., & Aoki, N. (1977) *Thromb. Res. 10*, 851-856.
Panyim, S., & Chalkley, R. (1969) *Arch. Biochem. Biophys. 130*, 337-346.
Peltz, S. W., Hardt, T. A., & Mangel, W. F. (1982) *Biochemistry 21*, 2798-2804.
Rabiner, S. F., Goldfine, I. D., Hart, A., Summaria, L., & Robbins, K. C. (1969) *J. Lab. Clin. Med. 74*, 265-273.
Rákóczi, I., Wiman, B., & Collen, D. (1978) *Biochim. Biophys. Acta 540*, 295-300.
Robbins, K. C., Summaria, L., Hsieh, B., & Shah, R. (1967) *J. Biol. Chem. 242*, 2333-2342.
Schultze, H. E., & Heremans, J. F. (1966) *Molecular Biology of Human Proteins with Special Reference to Plasma Proteins*, Vol. 1, p 177, Elsevier, Amsterdam.
Shah, G. A., & Dhall, D. P. (1983) *Thromb. Res. 32*, 67-72.
Sherry, S. (1968) *Annu. Rev. Med. 19*, 247-268.
Suenson, E., & Thorsen, S. (1981) *Biochem. J. 197*, 619-628.
Takada, A., & Takada, Y. (1981) *Thromb. Res. 22*, 497-501.
Takada, A., Urano, T., & Takada, Y. (1979) *Thromb. Haemostasis 42*, 901-908.
Takada, A., Takada, Y., & Sugawara, Y. (1984) *Thromb. Res. 33*, 561-569.
Thorell, J. I., & Johansson, B. G. (1971) *Biochim. Biophys. Acta 251*, 363-369.
Thorsen, S. (1975) *Biochim. Biophys. Acta 393*, 55-65.
Wallén, P. (1980) in *Fibrinolysis* (Kline, D. L., & Reddy, K. N. N., Eds.) pp 1-24, CRC Press, Boca Raton, FL.
Wallén, P., & Wiman, B. (1972) *Biochim. Biophys. Acta 257*, 122-142.
Walther, P. J., Hill, R. L., & McKee, P. A. (1975) *J. Biol. Chem. 250*, 5926-5933.
Weinstein, M. J., & Doolittle, R. F. (1972) *Biochim. Biophys. Acta 258*, 577-590.
Whitaker, A. N., Rowe, E. A., Masci, P. P., Joe, F., & Gaffney, P. J. (1980) *Thromb. Res. 19*, 381-391.
Wiman, B., & Wallén, P. (1977) *Thromb. Res. 1*, 213-222.
Wiman, B., & Collen, D. (1978) *Nature (London) 272*, 549-550.

Rates of Hydration of Fatty Acids Bound to Unilamellar Vesicles of Phosphatidylcholine or to Albumin†

Christopher Daniels,‡ Noa Noy, and David Zakim*
Division of Digestive Diseases, Department of Medicine, Cornell University Medical College, New York, New York 10021, and Liver Studies Unit, Veterans Administration Medical Center, San Francisco, California 94121
Received September 24, 1984

ABSTRACT: The rates of hydration of naturally occurring fatty acids bound to unilamellar vesicles of dimyristoylphosphatidylcholine were measured by following the rate of quenching of the inherent fluorescence of albumin. Rates of hydration of fatty acids bound to albumin could be estimated from the same data. The data show that these rates depend on the chain length and unsaturation of the fatty acid. Increasing chain length diminishes the rate of hydration whereas increasing unsaturation increases this rate. Rates of hydration of fatty acids bound to lipid vesicles appear to be rapid enough to account for intracellular movement between compartments in the absence of carrier proteins. It is uncertain whether this is true for hydration of fatty acids bound to albumin. Rates for this process are about 100-300 times slower vs. rates of hydration of fatty acids bound to lipid vesicles.

The pathways by which water-insoluble compounds move between different compartments within cells are uncertain. It has been proposed for several such compounds that they are transported between intracellular compartments as complexes with carrier proteins, the latter having variable specificities for binding of water-insoluble ligands. Some of the putative transport proteins have been shown directly in vitro to catalyze transport of phospholipids, cholesterol, and tocopherol between membranous compartments separated by an aqueous phase (Bloj & Zilversmit, 1977; Catignani & Bieri, 1977; Hellings et al., 1976; Kamp et al., 1973; Noland et al., 1980; Yamada & Sasaki, 1982). It is assumed but unproved, on the other hand, that intracellular transport proteins are essential for movement through water for many other organic, hydrophobic compounds (Ketterer et al., 1976; Miskhin et al., 1972; Ockner et al., 1982; Trulzch & Arias, 1981). There is reason to believe that intracellular movement of hydrophobic compounds will

† This work was supported in part by a grant from the National Institutes of Health (GM 33142). C.D. was a recipient of a Monsanto Fellowship.
* Address correspondence to this author at the Division of Digestive Diseases, Department of Medicine, Cornell University Medical College.
‡ Present address: Division of Cell Biology, Veterans Administration Medical Center, San Francisco, CA.

0006-2960/85/0424-3286$01.50/0