Shoemaker, M. O., Lau, W., Shattuck, R. L., Kwiatkowski, A. P., Matrisian, P. E., Guerra-Santas, L., Wilson, E., Lukas, T. J., Van Eldik, L., & Watterson, D. M. (1990) *J. Cell Biol. 111*, 1107-1125.
Stoclet, J. C., Gerard, D., Kilhoffer, M. C., Lugnier, C., Miller, R., & Schaeffer, P. (1987) *Prog. Neurobiol. 29*, 321-364.
Tanaka, T., & Hidaka, H. (1980) *J. Biol. Chem. 255*, 11078-11080.
Teleman, O., & Ahlstrom, P. (1986) *J. Am. Chem. Soc. 108*, 4333-4341.
Trewhella, J., Blumenthal, D. K., Rokop, S. E., & Seeger, P. A. (1990) *Biochemistry 29*, 9316-9324.
Wahl, P., & Auchet, J. C. (1972) *Biochim. Biophys. Acta 285*, 99-117.
Weber, P. C., Lukas, T. J., Craig, T. A., Wilson, E., King, M. M., Kwiatkowski, A. P., & Watterson, D. M. (1989) *Proteins: Struct., Funct., Genet. 6*, 70-85.
Werner, T. C., & Forster, L. S. (1979) *Photochem. Photobiol. 29*, 905-914.
Yazawa, M., Ikura, M., Hikichi, K., Ying, L., & Yagi, K. (1987) *J. Biol. Chem. 262*, 10951-10954.
Zimmer, W. E., Schoss, J. A., Silslow, C. D., Youngblom, G., & Watterson, D. M. (1988) *J. Biol. Chem. 263*, 19370-19383.

Characterization of the Binding of Plasminogen to Fibrin Surfaces: The Role of Carboxy-Terminal Lysines†

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ABSTRACT: In the present study we have quantitatively characterized the interaction of purified human Glu- and Lys-plasminogen with intact and degraded fibrin by ligand-binding experiments using a radioisotopic dilution method and antibodies against human plasminogen. A fibrinogen monolayer was covalently linked to a solid support with polyglutaraldehyde and was treated with thrombin or with thrombin and then plasmin to respectively obtain intact and degraded fibrin surfaces. Under these conditions, a well-defined surface of fibrin is obtained (410 ± 4 fmol/cm^2) and, except for a 39-kDa fragment, most of the fibrin degradation products remain bound to the support. New binding sites for plasminogen were detected on the degraded surface of fibrin. These sites were identified as carboxy-terminal lysine residues both by inhibition of the binding by the lysine analogue 6-aminohexanoic acid and by carboxy-terminal end-group digestion with carboxypeptidase B. The binding curves exhibited a characteristic Langmuir adsorption isotherm saturation profile. The data were therefore analyzed accordingly, assuming a single-site binding model to simplify the analysis. Equilibrium dissociation constants (K_d) and the maximum number of binding sites (B_{max}) were derived from linearized expression of the Langmuir isotherm equation. The K_d for the binding of Glu-plasminogen to intact fibrin was 0.99 ± 0.17 μM and for degraded fibrin was 0.66 ± 0.22 μM. The K_d for the binding of Lys-plasminogen to intact fibrin was 0.41 ± 0.22 μM and for degraded fibrin was 0.51 ± 0.12 μM. The K_i for the inhibition of the binding by 6-aminohexanoic acid was 130 ± 15 μM on intact fibrin and 109 ± 18 μM on degraded fibrin. The total number of binding sites markedly increased upon degradation of the fibrin surface by plasmin: ~0.1 pmol of plasminogen was bound to intact fibrin while ~1 pmol was detected on the degraded surface. The plasminogen/fibrin ratio increased from 0.3 to 2.7 upon plasmin degradation. These data provide quantitative evidence of the amplification of fibrinolysis by carboxy-terminal lysine residues unveiled by plasmin on the surface of fibrin and support the concept of fibrinolysis as a surface-controlled process.

Physiological fibrinolysis involves heterogeneously catalyzed reactions that proceed at the fibrin/plasma interface, where fibrin provides a surface to which tissue plasminogen activator (t-PA)[1] [EC 3.4.21.31] and plasminogen adsorb in a sequential and ordered manner (Wiman & Collen, 1978; Hoylaerts et al., 1982). Molecular assembly of these proteins results in a ternary complex that efficiently generates plasmin [EC 3.4.21.7] on the surface of fibrin and thereby triggers the dissolution of a clot. It has been recently suggested that early fibrin degradation accelerates fibrinolysis by increasing the binding of both t-PA and plasminogen to new binding sites created by plasmin on the surface of fibrin (Suenson et al., 1984; Tran-Thang et al., 1984; Harpel et al., 1985; Higgins & Vehar, 1987). This mechanism is mediated by specific interactions of lysine residues in fibrin with the kringle domains of plasminogen (Varadi & Patthy, 1983; Nieuwenhuizen et

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[1] Abbreviations: Glu-plasminogen, native human plasminogen with N-terminal glutamic acid; Lys-plasminogen, plasmin-modified forms of Glu-plasminogen with N-terminal lysine, valine, or methionine (mostly lysine) obtained by hydrolysis of the Arg-68-Met-69, Lys-77-Lys-78, or Lys-78-Val-79 peptide bonds; t-PA, tissue-type plasminogen activator; CBS 1065, chromogenic substrate (methylmalonyl)hydroxyprolyl-arginine-*p*-nitroanilide; S-2366, chromogenic substrate L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroanilide; 6-AHA, 6-aminohexanoic acid; AMCHA, *trans*-4-(aminomethyl)cyclohexanecarboxylic acid; VPL, dansyl-valyl-L-phenylalanyl-L-lysine chloromethyl ketone; PPACK, dansyl-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; DFP, diisopropyl fluorophosphate; SDS, sodium dodecyl sulfate.

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al., 1988; Thorsen, 1975; Wiman & Wallen, 1977). Native Glu-plasminogen and Lys-plasminogen possess high- and low-affinity lysine-binding sites. The high-affinity binding sites have been localized on the elastase fragments of plasminogen, kringle 1 + 2 + 3 and kringle 4, which do not bind to fibrin (Sottrup-Jensen et al., 1978; Thorsen et al., 1981). In contrast, the elastase fragment miniplasminogen Val^{443}–Asn^{791} (kringle 5 + the light chain) possesses a weak binding site through which it binds to fibrin and to aminohexyl-Sepharose and has been therefore termed the aminohexyl-binding site (Christensen, 1984, 1985). Christensen (1984) has shown that the aminohexyl-binding site is available in Glu-plasminogen and prefers ligands not carrying a free carboxylate function. It may therefore interact with the side chain of lysine in fibrin. In contrast, the strong lysine-binding site of plasminogen interacts particularly with carboxy-terminal lysine residues (Christensen, 1985). Thus, the interactions of plasminogen with the corresponding lysine residues present in the intact or the degraded surface of fibrin may be mediated via specific sites that have been identified in the plasminogen elastase-derived fragments and be of most importance in the regulation of fibrinolysis (Suenson et al., 1984; Norrman et al., 1985). The characteristics of the binding of plasmin(ogen) to fibrin and to plasmin-degraded fibrin(ogen) have been therefore studied by several groups (Suenson & Thorsen, 1981; Lucas et al., 1983; Bok & Mangel, 1985; Harpel et al., 1985; Tran-Thang et al., 1986). However, there is no consensus in the values reported for dissociation constants and binding stoichiometry. Moreover, binding of plasminogen to fibrin and to plasmin-degraded fibrin has not been fully analyzed comparatively. The progressive modification of the fibrin surface by plasmin is actually an intermediate short-lived state in the biochemical transformation of fibrin, and interactions evolving on this virtual surface escape direct detection. Preparation of a solid-phase fibrin surface (Anglés-Cano, 1986) progressively degradable by plasmin is feasible and is a stable model of such a transient state. To further analyze and quantitate the effect of plasmin degradation on the binding of Glu- and Lys-plasminogen to fibrin, experiments with both types of surfaces were performed. Our results indicate that exposure of carboxy-terminal lysine residues on the surface of fibrin markedly enhances the number of binding sites for plasminogen but does not significantly change its affinity for fibrin.[2]

Materials and Methods

Chemicals and Reagents. Materials were purchased from the following sources: ethylenediaminetetraacetic acid (EDTA), bovine serum albumin, poly(ethylene glycol) $M_r \sim 20\,000$, and Tween 20 from Serva (Heidelberg, Germany); Ultrogel AcA 44, gelatin–Ultrogel, and DEAE-Trisacryl from IBF (Villeneuve-La-Garenne, France); lysine- and CH-Sepharose 4B and PD-10 Sephadex G-25 (medium grade) columns from Pharmacia (Uppsala, Sweden); Affi-Gel 501, acrylamide, bisacrylamide, ammonium persulfate, and *N,N,N′,N′*-tetramethylethylenediamine from Bio-Rad Laboratories (Richmond, CA); poly(vinyl chloride) U-shaped microtitration plates and plate sealers from Dynatech (Nanterre, France); glutaraldehyde, 25% (v/v) aqueous solution, from TAAB Laboratories (Reading, Berkshire, U.K.); $Na^{125}I$ from Amersham International (Amersham, Buckinghamshire, U.K.); chromogenic substrate (methylmalonyl)hydroxyprolylarginine-*p*-nitroanilide (CBS 1065) and aprotinin from Diagnostica Stago (Asnières, France); chromogenic substrate L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroanilide (S-2366) from Kabi Vitrum (Mölndal, Sweden). Benzamidine, 6-aminohexanoic acid (6-AHA), and diisopropyl fluorophosphate (DFP) were purchased from Aldrich (Beerse, Belgium); *trans*-4-(aminomethyl)cyclohexanecarboxylic acid (AMCHA) and *p*-nitrophenyl *p′*-guanidinobenzoate were from Sigma (St. Louis, MO); dansylvalyl-L-phenylalanyl-L-lysine chloromethyl ketone (VPL) and dansylphenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) were from France Biochem (Meudon, France). Bovine thrombin [EC 3.4.21.5] was obtained from Hoffmann-La Roche (Basel, Switzerland); DFP-treated carboxypeptidase B [EC 3.4.17.2] was from Boehringer (Mannheim, Germany), and urokinase [EC 3.4.21.31] was from the Institut Choay (Paris, France). All other chemicals were of the best reagent grade commercially available.

Buffers. Buffer A was 0.05 M sodium phosphate buffer, pH 7.4, containing 0.08 M NaCl. Buffer B was 0.05 M sodium phosphate buffer, pH 6.8, containing 0.08 M NaCl. Assay buffer was buffer A containing 2 mg of bovine serum albumin/mL and 0.01% (v/v) Tween 20. Binding buffer was buffer B containing 4 mg of bovine serum albumin/mL, 0.01% (v/v) Tween 20, and 2 mM EDTA. Mass buffer, a buffer that provided the mass action effects on the competitive nonspecific adsorption of plasminogen to the fibrin surfaces, was binding buffer containing 40 mg of bovine serum albumin/mL. Electrophoresis sample buffer was 0.125 M Tris-HCl, pH 6.8, containing 2% (w/v) sodium dodecyl sulfate (SDS) and 2.5% (v/v) glycerol. All other buffers were prepared as described in the text.

Purified Proteins. Glu-plasminogen was purified from DFP-treated fresh-frozen human plasma by affinity chromatography on lysine-Sepharose 4B (Deutsch & Mertz, 1970), ion-exchange chromatography on DEAE-Trisacryl, and gel filtration on Ultrogel AcA 44. All procedures were performed at 4 °C in the presence of aprotinin. No contaminant plasmin activity was detected by incubating the plasminogen with the chromogenic substrate CBS 1065 (1.5 mM final concentration) for 48 h at 37 °C. Lys-plasminogen was prepared from Glu-plasminogen as described (Lucas et al., 1983). In brief, 40 μM Glu-plasminogen in 50 mM Tris-HCl, pH 8.6, was incubated with 0.4 μM urokinase-free plasmin (enzyme/substrate molar ratio 1/100) for 4 h at 22 °C. Aprotinin–Sepharose was added to remove plasmin, and the suspension was gently shaken overnight at 4 °C. After centrifugation, less than 0.03% of residual plasmin amidolytic activity was detected. The solution of Lys-plasminogen was treated twice with 15 mM DFP (final concentration) and extensively dialyzed against buffer A. The plasminogen preparations were considered to be more than 99% pure and the Glu- to Lys-plasminogen transformation was considered more than 95% efficient as assessed by SDS–polyacrylamide gel electrophoresis and autoradiography of the ^{125}I-labeled products and by amino-terminal analysis (Edman & Henschen, 1975). The concentration of the plasminogen preparations was measured spectrophotometrically in buffer A using $E(1\%, 1\text{ cm}) = 16.8$ at 280 nm (Wallen & Wiman, 1972).

Fibrinogen was purified from fresh-frozen human plasma supplemented with proteinase inhibitors (100 kallikrein inhibitory units/mL aprotinin, 2 mM DFP, 1 μM PPACK, 0.2 M 6-AHA, 10 μM *p*-nitrophenyl *p′*-guanidinobenzoate, 4 mM benzamidine and 0.5 unit/mL hirudin, final concentrations) by glycine precipitation according to Kazal et al. (1963) with modifications (P. Grailhe, personal communication). It was then chromatographed on a Sepharose 6B column in 50 mM

[2] This work was presented in part at the 10th International Congress on Fibrinolysis (Indianapolis, IN) (Fleury & Anglés-Cano, 1990).

phosphate buffer, pH 7.4, containing 0.5 M NaCl, 2 mM EDTA, and all inhibitors except 6-AHA. Further purification was obtained by affinity chromatography on lysine–Sepharose 4B, gelatin–Ultrogel, and organomercurial agarose with use of the same buffer. Fibrinogen was concentrated by precipitation with 45% ammonium sulfate, extensively dialyzed against 0.1 M phosphate buffer, pH 7.4, containing 0.3 M NaCl, separated into aliquots (12 mg/mL), and stored at −70 °C. The purified fibrinogen was free of von Willebrand Factor, plasminogen, fibronectin, and factor XIII as determined by an enzyme-linked immunosorbent assay specific for these proteins; the absence of plasminogen or plasmin was confirmed by incubation for 72 h at 37 °C of the fibrin–agar plates prepared with the purified products supplemented or not with t-PA. Fibrinogen was more than 98% clottable and appeared homogeneous by SDS–polyacrylamide gel electrophoresis and autoradiography of the ^{125}I-labeled product. Protein concentration was determined by measuring the absorbance in buffer A at 280 nm using $E(1\%, 1\text{ cm}) = 15.1$ (Blombäck & Blombäck, 1956).

Plasmin was prepared as described (Robbins et al., 1981). Briefly, 43 μM plasminogen was incubated with 500 international units/mL urokinase in 0.05 M Tris-HCl, pH 9, 0.1 M lysine, 0.08 M NaCl, glycerol 25% (v/v) for 30 min at 37 °C. The activation mixture was then diluted 15-fold with 0.1 M sodium phosphate, pH 8.0, and plasmin was separated from urokinase by affinity chromatography at 4 °C on a lysine–Sepharose column equilibrated with the same buffer. The column was extensively washed with 0.3 M sodium phosphate, pH 8.0, containing 0.5 M NaCl, and plasmin was eluted with 15 mM 6-AHA in 0.1 M sodium phosphate, pH 8.0. The urokinase-free plasmin was concentrated by dry dialysis against poly(ethylene glycol) $M_r \sim 20000$ at 4 °C to a final concentration of 13.5 μM in 25% glycerol and stored at −70 °C.

Miscellaneous. The IgG fraction of a goat antiserum (Biopool, Umeå, Sweden) against human plasminogen was purified by ammonium sulfate precipitation and ion-exchange chromatography on a DEAE-Trisacryl column.

Glu- and Lys-plasminogen, fibrinogen, and the IgG against plasminogen were radiolabeled with Na^{125}I according to the Iodogen method of Fraker and Speck (1978) with the following modifications. Protein (10 μg in 0.2 M phosphate buffer, pH 7.4) and 1 mCi of the radioisotope were added (20-μL final volume) to a conical plastic centrifuge vial (Eppendorf) precoated with Iodogen (10 μg) according to the manufacturer's instructions. After an iodination time of 4 min at 4 °C, the labeled protein was separated from free Na^{125}I by gel filtration on a PD-10 Sephadex G-25 column (medium grade) column. The specific activities obtained were 15–20 nCi/ng of protein for the Glu-plasminogen, 26 nCi/ng for the Lys-plasminogen, 16.5 nCi/ng for the fibrinogen, and 5 nCi/ng for the IgG. The labeled plasminogen was identical with the unlabeled product as assessed by SDS–polyacrylamide gel electrophoresis and autoradiography performed before and after (i) activation with fibrin-bound t-PA and (ii) proteolysis by plasmin.

SDS–polyacrylamide gel electrophoresis of radioiodinated proteins was performed under reducing conditions [2-mercaptoethanol, 5% (v/v) final concentration], on 0.75-mm-thick, 4% stacking and 7.5% (w/v) polyacrylamide separating slab gels according to Laemmli (1970). After electrophoresis, the gels were dried and autoradiographed on Kodak XS-1 films for 24–48 h at −70 °C, with a Kodak X-Omatic intensifying screen.

Aprotinin–Sepharose was prepared by coupling 10 mg of purified aprotinin to 9 mL (3 g) of activated CH-Sepharose according to the instructions of the manufacturer.

Preparation of the Fibrin Surfaces. Solid-phase fibrin was prepared as previously described (Anglés-Cano, 1986; Anglés-Cano et al., 1988). In brief, poly(vinyl chloride)-bound stable polyglutaraldehyde derivatives were first produced by treating U-microtitration poly(vinyl chloride) plates with 100 μL/well of 2.5% (v/v) glutaraldehyde in 0.1 M sodium bicarbonate buffer, pH 9.5, for 2 h at 22 °C. Fibrinogen (100 μL/well, 0.3 μM in 0.1 M sodium phosphate buffer, pH 7.4, containing 1 mM $CaCl_2$) was then covalently fixed for 18 h at 4 °C. Unreacted fibrinogen was discarded, the plate was washed with assay buffer, and the fibrinogen monolayer was transformed in a fibrin surface by treatment with 100 μL/well of thrombin (20 NIH units/mL in assay buffer containing 1 mM $CaCl_2$) at 37 °C for 1 h. The excess thrombin was eluted by three washes with a high ionic strength solution [0.5 M NaCl, 8 mM $CaCl_2$, and 0.05% (v/v) Tween 20]. A final wash with 5 mM sodium phosphate buffer [pH 6.8, containing 0.05% (v/v) Tween 20] was done, and 100 μL/well of buffer B containing 0.02 M lysine, 0.2% (w/v) bovine serum albumin, and 0.01% (v/v) NaN_3, was added. The plate was sealed and stored at 4 °C until further use. The absence of thrombin amidolytic activity was verified by adding 100 μL/well of 1 mM chromogenic substrate S-2366 in 0.05 M Tris, pH 8.0, 0.08 M NaCl, 4 mM $CaCl_2$. The ability of thrombin to cleave fibrinogen was monitored by use of a horseradish peroxidase labeled mouse monoclonal antibody (Y18-HRP) kindly provided by Organon Teknika (Fresnes, France). The immunoreactivity of this antibody with the Aα stretch 1–51 of human fibrinogen disappeared upon treatment with thrombin (Koppert et al., 1985).

Preparation and Carboxy-Terminal Analysis of Degraded Fibrin. A microtitration plate containing the solid-phase fibrin surface prepared as described above was washed three times with assay buffer. Solutions of 0–50 nM plasmin in assay buffer (50 μL/well) were then incubated with the fibrin surface for 0–120 min at 37 °C. In the next step the fluid phase with the plasmin was discarded, the surface was washed three times with assay buffer and finally incubated for 24 h at 22 °C with assay buffer containing 0.2 M AMCHA, 1 mM benzamidine, and 10 μM VPL. After three further washes of the surface with assay buffer, 1.5 mM CBS 1065 was added to possibly detect residual plasmin activity. No significant amidolytic activity was detected even after 48 h of incubation at 37 °C. The degradation of the fibrin surface by plasmin was verified by the direct binding of a monoclonal antibody directed against fibrin degradation products (Koppert et al., 1986), antibody FDP-14 (kindly provided by Dr. Willem Nieuwenhuizen), and by the indirect quantitation of the appearance of new carboxy-terminal lysine residues as measured by the binding of plasminogen before and after digestion of the surface with carboxypeptidase B that has been previously treated with 2 mM DFP. Optimal conditions of enzyme and time of digestion were determined by incubating for 2 h at 37 °C 50 μL/well of 0–100 μg/mL of the DFP-treated carboxypeptidase B in 0.05 M HEPES, pH 7.5, containing 0.5 M NaCl and 10 μM $ZnCl_2$. The surface was then washed with binding buffer and incubated for 18 h at 4 °C with 50 μL/well of mass buffer containing 500 nM ^{125}I-Glu-plasminogen (specific radioactivity 80 pCi/ng). The supernatants were then collected, the plate was washed three times with binding buffer, and the radioactivity in the wells was counted in a γ-radiation counter. The amount of plasminogen bound to the fibrin surface was calculated by dividing the radioactivity of each well by the molar radioactivity of plasminogen in the radioactive probe. In

parallel experiments the ^{125}I-Glu-plasminogen was eluted from the surface of degraded fibrin by incubation for 18 h at 22 °C with 50 μL/well of 0.2 M 6-AHA in electrophoresis sample buffer. The nature of the eluted plasminogen was verified by SDS–polyacrylamide gel electrophoresis and autoradiography.

Binding of Plasminogen to the Fibrin Surfaces. Solutions of either Glu- or Lys-plasminogen (0–10 μM) with different specific radioactivities were prepared in mass buffer containing 1 mM benzamidine by addition of a trace amount (6 nM Glu- and 3 nM Lys-plasminogen) of the respective form of ^{125}I-plasminogen. In parallel experiments, varying amounts of a competitive ligand, 6-AHA, were added. These mixtures (50 μL/well) were incubated for 18 h at 4 °C with the intact or degraded fibrin surfaces. The supernatant was collected, the surface was washed three times with binding buffer, and the extent of binding was determined by counting the radioactivity of the wells in a γ-radiation counter. The amount of plasminogen bound to the surface of intact or degraded fibrin was calculated by dividing the radioactivity of each well by the molar radioactivity (molar concentration of plasminogen/total disintegrations per minute) of plasminogen in each of the solutions. Nonspecific binding was determined from the amount of radioactivity bound in the presence of an excess of cold ligand (ratio 1/500). Experiments were performed without the radiolabeled probe, and the amount of plasminogen bound was detected by incubating the surface with radiolabeled IgG against plasminogen in mass buffer (50 μL/well = ~500 000 dpm). The extent of binding was determined by counting the radioactivity bound per well.

Analysis of Binding Data. At least three of the five kringle domains of human plasminogen, kringles 1, 4, and 5, can potentially interact with fibrin. Their binding sites share common structural features (Thewes et al., 1990; Tulinsky et al., 1988) and have variable affinity for lysine and related ω-amino acid analogues (Lerch et al., 1980; Vali & Patthy, 1982; Markus et al., 1981). Therefore, in the analysis of binding data we assume, for simplicity, that the interaction of the plasminogen molecule with the fibrin surface can be described by single-site binding and a single association–dissociation mechanism according to the equation

$$[\mathrm{Fn}] + [\mathrm{Pg}] \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} [\mathrm{Fn{\cdot}Pg}] \quad (1)$$

According to the law of mass action, the following equilibrium equation can be derived:

$$[\mathrm{Fn}] = [\mathrm{Fn_o}] - [\mathrm{Fn{\cdot}Pg}] \quad (2)$$

where [Fn] is the equilibrium number of fibrin free binding sites, [Pg] is the equilibrium concentration of plasminogen, [Fn·Pg] represents the number of molecules of plasminogen adsorbed on fibrin, and [Fn_o] is the total number of binding sites on fibrin (v = 50 μL).

Then, the affinity of the interaction is given by the equilibrium constant K.

$$K = \frac{k_{on}}{k_{off}} = \frac{[\mathrm{Fn{\cdot}Pg}]}{[\mathrm{Fn}][\mathrm{Pg}]} \quad (3)$$

At constant temperature and fixed [Fn_o], the Langmuir adsorption isotherm can be derived (Adamson, 1990) from the mass action eqs 2 and 3.

$$[\mathrm{Fn{\cdot}Pg}] = [\mathrm{Fn_o}]\frac{K[\mathrm{Pg}]}{(1 + K[\mathrm{Pg}])} \quad (4)$$

Therefore, the fraction α of the fibrin surface sites occupied by plasminogen molecules, [Fn·Pg], relative to [Fn_o], the total number of available sites on the surface, is given by

$$\alpha = \frac{[\mathrm{Fn{\cdot}Pg}]}{[\mathrm{Fn_o}]} = \frac{K[\mathrm{Pg}]}{(1 + K[\mathrm{Pg}])} \quad (5)$$

Equations 4 and 5 are classical representations of the Langmuir adsorption isotherm that have been previously applied to the analysis of ligand-binding data (De Marco et al., 1982; Thewes et al., 1990). A plot of either [Fn·Pg] or α as a function of [Pg] yields the characteristic hyperbolic saturation profile.

In the presence of a competitive inhibitor, the Langmuir equation becomes

$$\alpha = \frac{K[\mathrm{Pg}]}{1 + [\mathrm{I}]/K_i + K[\mathrm{Pg}]} \quad (6)$$

where [I] is the equilibrium amount of inhibitor and K_i is the inhibition constant.

The total binding sites in the fibrin surface and the affinity of the adsorption can be conveniently evaluated by transforming eqs 4 and 5 into the linearized expressions

$$\frac{[\mathrm{Pg}]}{[\mathrm{Fn{\cdot}Pg}]} = \frac{1}{K[\mathrm{Fn_o}]} + \frac{[\mathrm{Pg}]}{[\mathrm{Fn_o}]} \quad (7)$$

$$\frac{[\mathrm{Fn{\cdot}Pg}]/[\mathrm{Fn_o}]}{[\mathrm{Pg}]} = K - K\frac{[\mathrm{Fn{\cdot}Pg}]}{[\mathrm{Fn_o}]} \quad (8)$$

$$\frac{1}{\alpha} = 1 + \frac{1}{K[\mathrm{Pg}]} \quad (9)$$

A plot of [Pg]/[Fn·Pg] vs [Pg] gives a straight line of slope 1/[Fn_o]. A plot of 1/α vs 1/[Pg] yields a straight line with slope 1/K.

Results

Characteristics of the Fibrin Surface. The transformation of the solid-phase monolayer of fibrinogen into fibrin by thrombin-catalyzed fibrinopeptide cleavage was verified by measuring the disappearance of immunoreactivity of the monoclonal antibody Y18-HRP with solid-phase fibrinogen upon treatment with thrombin (data not shown). The Arg residue Aα16, i.e., the thrombin cleavage site, is essential for a conformational epitope mapped within fibrinogen Aα 1–51 that is recognized by the antibody Y18-HRP (Koppert et al., 1985). Thrombin was efficiently desorbed from the fibrin surface as indicated by the disappearance of surface-bound clotting and amidolytic activities after addition of fibrinogen and the thrombin-selective substrate S-2366 (1 mM), respectively. The fibrin surface has been characterized and suggested as a useful model for equilibrium binding studies and plasminogen activation experiments (Rouy & Anglés-Cano, 1990). Its capacity to bind t-PA specifically and to stimulate its plasminogen activating activity reflects its similarity to a fibrin clot surface (Anglés-Cano, 1986; Masson & Anglés-Cano, 1988). The maximal fibrin surface density (moles per unit of area) that could be obtained, i.e., 410 ± 4 fmol/cm^2, was calculated from the amount of fibrinogen adsorbed to the solid phase (Anglés-Cano & Sultan, 1984). The plasminogen binding experiments were performed by use of 365 ± 4 fmol of fibrin/88 mm^2, the surface covered by a volume of 50 μL.

Characterization of the Plasmin-Degraded Fibrin Surface. Prior to the quantitative analysis of the binding of plasminogen to degraded fibrin, experiments were performed to establish the optimal conditions of plasmin degradation. At the end of the degradation the desorption of plasmin with AMCHA was verified by the absence of hydrolysis of the chromogenic substrate CBS 1065. The progressive proteolysis of covalently

bound fibrin by plasmin was followed both by immunological means and by carboxy-terminal end-group digestion with carboxypeptidase B. The degraded fibrin surface was recognized by FDP-14, a monoclonal antibody that specifically reacts with fibrin degradation products but not with intact fibrin (Koppert et al., 1986). Fibrin was digested by plasmin in a time- and concentration-dependent manner as assessed by the progressive increase in the binding of Glu-plasminogen. With 25 nM plasmin, the degradation time necessary to obtain 50% of the maximal binding was 3.7 min and a plateau was reached within 30 min (Figure 1A). The increase in the binding of plasminogen was not related to the plasmin-induced Glu- to Lys-transition but to the appearance of new binding sites as indicated by the absence of Lys forms after elution of plasminogen with 0.2 M 6-AHA (Figure 1A, inset). Moreover, digestion of the surface of degraded fibrin with carboxypeptidase B abolished the binding of Glu-plasminogen, suggesting that the binding was mediated by carboxy-terminal lysine residues removed from the surface upon treatment with the enzyme. The effect of carboxypeptidase B was a function of the time of digestion (Figure 1B) and of the concentration of the enzyme (not shown). In contrast, carboxypeptidase B had no effect on the binding of plasminogen to intact fibrin. The digestion of fibrin by plasmin can generate lysine as well as arginine carboxy-terminal residues, and carboxypeptidase B might cleave both of them. Therefore, the specificity of the interaction of plasminogen with carboxy-terminal lysines was further verified by measuring the effect of the lysine analogue, 6-AHA, on the binding. The competition experiment is represented in the inset of Figure 1B. Plasminogen binding to the degraded surface of fibrin was efficiently inhibited by 6-AHA; ~95% of the binding was abolished at a concentration of 20 mM, and the concentration required to inhibit by 50% the binding of plasminogen (IC_{50}) was 0.196 mM.

Fibrin degradation products released from the surface were identified by SDS–polyacrylamide gel electrophoresis and autoradiography using radiolabeled fibrinogen (not shown). Their relative molecular weight (39 000) and single-chain structure suggest, in agreement with previous reports (Liu et al., 1986), that such a fragment results from proteolysis of a peptide bond, probably at Lys-206, of the Aα chain. From the percentage of radioactivity released from the surface of fibrin upon plasmin degradation [about 23% of the weight of fibrin(ogen)] by reference to the total radioactivity bound, it was found that most of the fragments remained bound to the surface.

Binding of Plasminogen to Intact and to Cleaved Fibrin Surfaces. The amount of plasminogen, either the Glu or the Lys form, adsorbed to the fibrin surface before and after cleavage with 50 nM plasmin for 2 h at 37 °C was measured as a function of plasminogen concentration at constant temperature. Nonspecific binding was substracted from total binding measured in the presence of a 1/500 excess of cold ligand. The binding isotherms of Glu-plasminogen to intact and degraded fibrin surfaces are represented in Figure 2. Computerized, nonlinear, least-squares, regression analysis of untransformed raw data fits the simple hyperbolic expression analogous to that of Langmuir for bimolecular equilibrium adsorption phenomena at heterogeneous interfaces (Adamson, 1990). The amount of plasminogen bound to fibrin as measured with radiolabeled plasminogen and with a radiolabeled antibody against human plasminogen by immunobinding was practically identical (not shown). The labeled plasminogen binds, therefore, like the unlabeled form. Furthermore, similar results (not shown) were obtained with a second monolayer

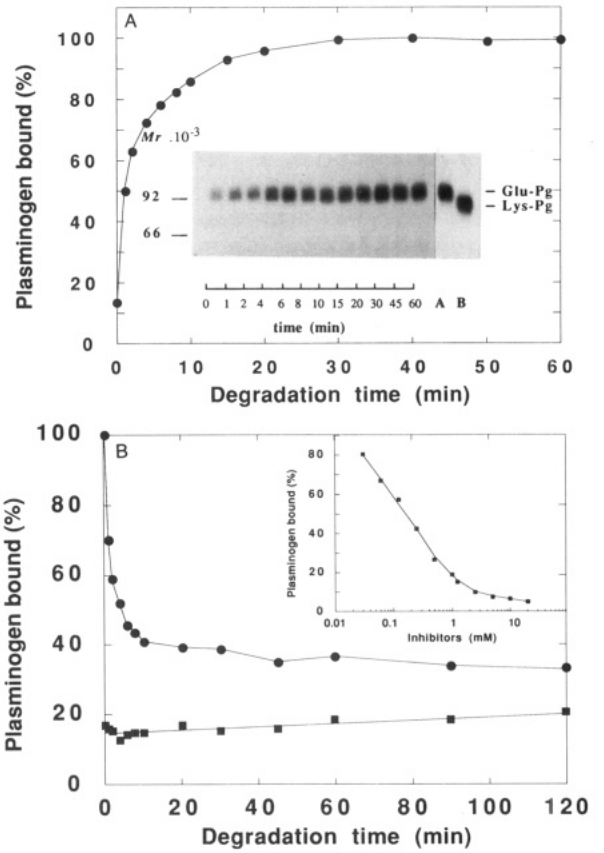

FIGURE 1: Binding of Glu-plasminogen to protease-modified fibrin surfaces. (A) Binding of plasminogen to plasmin-cleaved fibrin. A solid-phase fibrin surface was degraded with 25 nM plasmin as a function of time (0–60 min). At the indicated times, the fluid phase was removed, the wells were washed three times with assay buffer, and bound plasmin was inhibited and eluted from the fibrin surface as described in the Materials and Methods section. A solution of 500 nM Glu-plasminogen containing 2 nM ^{125}I-Glu-plaminogen (final concentration) in mass buffer was then incubated for 18 h at 4 °C. The plasminogen solution was removed, the plate was extensively washed, and the radioactivity of the wells was counted in a γ-radiation counter. Bound plasminogen is expressed as a percentage by reference to the maximum bound at 60 min. (Inset) In parallel experiments, plasminogen bound to the surface was eluted by the addition of 50 μL of SDS–polyacrylamide gel electrophoresis sample buffer containing 0.2 M 6-AHA. The eluate was electrophoresed in 7.5% polyacrylamide slab gels. The gel was dried and exposed to autoradiography. The M_r values of protein standards (phosphorylase *b*, 92 000; bovine serum albumin, 66 000) are shown at the left. Lanes A and B show the mobility of purified Glu-plasminogen (Glu-Pg) and Lys-plasminogen (Lys-Pg), respectively. (B) Binding of Glu-plasminogen to intact or degraded fibrin treated with carboxypeptidase B. Fibrin and plasmin-degraded fibrin, prepared as described in the Materials and Methods section, were digested with 50 μg/mL carboxypeptidase B for 0–120 min. At the indicated times, the carboxypeptidase was removed, the plate was extensively washed, and 500 nM Glu-plasminogen in mass buffer was incubated with fibrin (■) and with degraded fibrin (●) for 18 h at 4 °C. Excess plasminogen was discarded, the plate was washed, and bound plasminogen was detected by adding a solution of ^{125}I-IgG (~500 000 dpm/well) against human plasminogen for 2 h. The plate was washed, and the radioactivity in the wells was counted in a γ-radiation counter. The amount of plasminogen bound is expressed as a percentage relative to the maximum bound at 0 min of treatment with carboxypeptidase B. (Inset) Inhibition of the binding of Glu-plasminogen to degraded fibrin by 6-AHA. Solutions with increasing concentrations, 0–20 mM, of 6-AHA and a constant amount of ^{125}I-Glu-plamsinogen (specific radioactivity ~32 000 dpm/pmol) in mass buffer were incubated for 18 h at 4 °C with a surface of degraded fibrin prepared as described in the Materials and Methods section. Unbound proteins were discarded, the plate was washed, and the radioactivity of the wells was counted in a γ-radiation counter. Bound plasminogen, expressed as a percentage relative to the maximum bound in the absence of 6-AHA, is plotted against 6-AHA concentration in logarithmic scale.

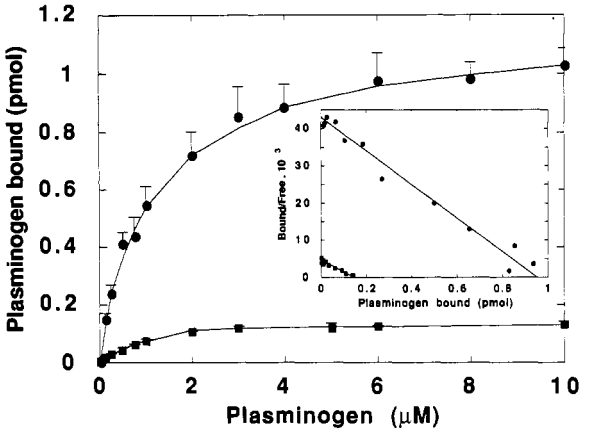

FIGURE 2: Isotherms of the binding of Glu-plasminogen to fibrin surfaces. Amounts of 50 μL of mass buffer containing 1 mM benzamidine, increasing concentrations of Glu-plasminogen, from 0–10 μM, and a constant amount, 2 nM, of ^{125}I-Glu-plasminogen as a tracer were incubated for 18 h at 4 °C with intact fibrin (■) or plasmin-degraded fibrin (●) surfaces. Excess proteins were discarded, the plate was washed, and the radioactivity of the wells was counted in a γ-radiation counter. Bound radioactivity was transformed into picomoles of plasminogen bound per well. The binding obeys the Langmuir equation, eq 4; a plot of bound plasminogen ([Fn·Pg]) vs the equilibrium plasminogen concentration ([Pg]) yields a characteristic hyperbolic curve. The results are mean ± SD of seven independent experiments performed in duplicate. The inset shows the fitting of the data to eq 8, which is the rearrangement proposed by Scatchard. A plot of bound/free vs the amount bound results in straight lines indicating single-site binding. Data were fitted with a linear regression analysis (r = 0.968 for fibrin and 0.988 for degraded fibrin).

of fibrin constructed over the covalently bound fibrin surface, indicating that the covalent bonding of fibrinogen does not involve lysine residues critical for interaction with plasminogen. It can be deduced from data in Figures 1 and 2 that the amount of plasminogen adsorbed is dependent on the number of binding sites present on the surface of intact or degraded fibrin. A similar behavior with the surfaces of fibrin was observed with Lys-plasminogen, but for simplicity only the experiments with Glu-plasminogen are represented. This all together indicates (i) that at equilibrium binding sites are equivalent, (ii) that there is no interaction between molecules of plasminogen adsorbed on adjacent sites, and (iii) that the binding obeys the Langmuir adsorption equation, eq 4. A plot of bound plasminogen ([Fn·Pg]) vs the equilibrium plasminogen concentration ([Pg]) yields the hyperbolic curve shown in Figure 2.

At low concentrations of plasminogen the amount adsorbed is proportional to the concentration, while at high ligand concentration, [Fn·Pg] approaches the limiting [Fn_o] value asymptotically; i.e., the surface saturates, indicating that adsorption is limited to a monolayer. A Bjerrum plot, [Fn·Pg] = f(log [Pg]), of data (not shown) confirmed saturation of the intact fibrin surface at more than 2 μM plasminogen and of the degraded fibrin surface at more than 5 μM plasminogen. Plot of data according to eq 8 shows the expected straight lines (Figure 2, inset). This indicates that all intrinsic binding constants are identical and that the reaction system contains no more than one kind of acceptor site. This site was identified in the cleaved fibrin as a carboxy-terminal lysine. The total number of binding sites and the affinity constant, K, of the interaction were respectively determined from eq 7 and eq 9. The parameters of the binding constants thus obtained for Glu- and Lys-plasminogen are summarized in Table I.

Inhibition of Glu-plasminogen Binding to Intact and Degraded Fibrin by a Lysine Analogue. Competition experiments

Table I: Binding of Glu- and Lys-plasminogen to Fibrin Surfaces

	Glu-plasminogen		Lys-plasminogen	
	fibrin	degraded fibrin	fibrin	degraded fibrin
K_d (μM)	0.99 ± 0.17	0.66 ± 0.22	0.41 ± 0.16	0.51 ± 0.12
B_{max} (pmol)[a]	0.13 ± 0.03	1.05 ± 0.16	0.13 ± 0.02	1.17 ± 0.35
α[b]	0.34	2.73	0.34	3.04

[a] Maximal amount of plasminogen bound to the surface of fibrin covered by a volume of 50 μL. [b] α = moles of bound plasminogen (B_{max}) per mole of fibrin on the surface (365 ± 4.10^{-15}) (eq 5).

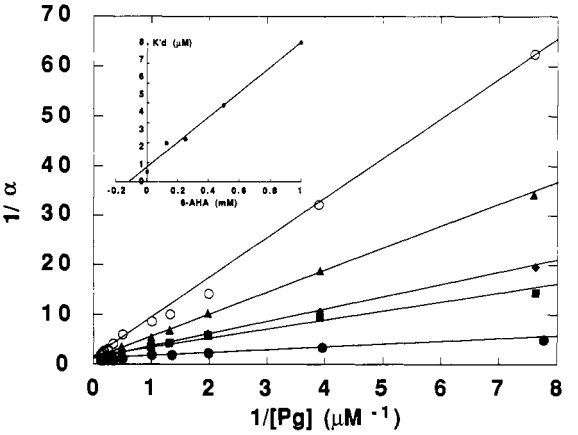

FIGURE 3: Inhibition of the binding of Glu-plasminogen to degraded fibrin by 6-AHA. Amounts of 50 μL of mass buffer containing 0–10 μM ^{125}I-Glu-plasminogen (specific activity: 2 500 000 dpm/pmol to 1000 dpm/pmol) and 0 (●), 0.125 (■), 0.25 (♦), 0.5 (▲), and 1 mM (○) 6-AHA were incubated for 18 h at 4 °C with a degraded fibrin surface. Excess proteins were discarded, the plate was washed, and the radioactivity in the wells was counted in a γ-radiation counter. The radioactivity bound was expressed in picomoles of plasminogen bound per well. In the presence of 6-AHA, the binding isotherms obey eq 6. Straight lines result from a linear fitting of the data to eq 9, which yields $1/K(=K_d)$ from the slope. The inset depicts K_d' against the concentration of 6-AHA. The K_i value was calculated from the intercept of the straight line (r = 0.994) with the abscissa axis, where $K_d' = K_d(1 + [I]/K_i)$.

with 6-AHA were performed to verify the specificity of the binding of plasminogen to intact and to degraded fibrin. As previously indicated (Figure 1B, inset), the binding of plasminogen to the fibrin surfaces was inhibited by 6-AHA in a concentration-dependent manner [IC_{50} = 0.196 mM for cleaved fibrin and 0.067 mM (not shown) for intact fibrin]. The influence of this inhibitor on the binding of plasminogen to these surfaces was further quantitated as follows. At selected concentrations, 6-AHA caused parallel shift to the right of a Bjerrum plot, demonstrating the competitive inhibition of the binding (not shown). By use of a linear transformation (eq 9) of the basic Langmuir binding isotherms obtained with plasminogen and various 6-AHA concentrations (Figure 3), an apparent K_d, K_d', was determined from the slope of the linear plot. Experiments were performed with both the intact and the degraded fibrin surfaces. To simplify, only the experiment with the degraded surface is represented in Figure 3. The values obtained are a linear function of the inhibitor concentration (eq 6) as verified from a plot of K_d' vs the 6-AHA concentration (Figure 3, inset). The K_i values, obtained from the data of three independent experiments performed in duplicate, were 130 ± 15 μM (mean ± SD) for intact fibrin and 109 ± 18 μM for plasmin-degraded fibrin.

Discussion

Intravascular fibrinolysis is a heterogeneously catalyzed process evolving from the plasma/fibrin interface to the interior

of the clot (Krause & Deutsch, 1989). The sequence of events in such a surface-catalyzed reaction includes diffusion and adsorption of plasminogen and t-PA to the surface of fibrin, reaction of the adsorbed species leading to plasmin, and plasmin-catalyzed proteolysis of the surface of fibrin. As a consequence, fibrin degradation products are released, the reactional front is progressively displaced, and the clot is finally dissolved. Because of its importance in the progression of the fibrinolytic process we have been interested in the adsorption of reactants to the modified/degraded surface of fibrin. We have previously shown that during ongoing plasminogen activation Glu-plasminogen is continuously bound to the fibrin surface and transformed into Glu-plasmin as the fibrin is degraded (Rouy & Anglès-Cano, 1990). On the basis of a previous report (Christensen, 1985) and on the ability of 6-AHA to elute fibrin-bound plasminogen derivatives, we proposed that the binding was related to the unveiling of new carboxy-terminal lysine residues on fibrin by plasmin degradation. In the present work we have characterized the interaction of purified human Glu- and Lys-plasminogen with intact and degraded fibrin using both a radioisotopic dilution method and monospecific antibodies against human plasminogen. We have identified carboxy-terminal lysine residues on the surface of degraded fibrin as responsible for the increase in the binding of plasminogen during ongoing fibrinolysis and have determined the parameters of such an interaction.

Previous reports have already indicated that degraded fibrinogen (Suenson et al., 1984; Harpel et al., 1985) and degraded fibrin (Bok & Mangel, 1985; Tran-Thang et al., 1984) bind more plasminogen than intact fibrin. Either a one-site or a two-site binding model was postulated with a large range in the K_d values and in the number of binding sites per molecule of fibrin. This variability may be related to methodological difficulties both in the production and in the treatment of data. In the first case, the experimental problems encountered to manipulate insoluble fibrin are further complicated by physical phenomena such as diffusion-limited adsorption or nonspecific trapping of proteins produced by the dense matrix of a clot. In the present study we have avoided most of these complications by characterizing the binding of plasminogen to a well-defined surface of fibrin. We suggest that such a surface of fibrin represents a good model for a natural fibrin deposit. The procedure used for the preparation of the solid phase results in the production of a monolayer of fibrinogen (410 fmol of fibrinogen/cm^2) (Anglès-Cano & Sultan, 1984) easily transformed into a surface of fibrin by thrombin. The binding of t-PA to such a surface of fibrin is saturable and obeys the Langmuir binding isotherm equation; it dramatically enhances the activity of t-PA and has a very low dissociation constant (K_d = 1.4 nM) (Anglès-Cano, 1986). Therefore, we consider the fibrin surface obtained as described here as a suitable tool and a meaningful technique for fibrin binding studies, in agreement with the concept of fibrinolysis as a heterogeneously catalyzed process evolving at the interface of the fibrin surface and a solute phase. The modification of the surface with plasmin resulted in the release of a fibrin fragment of M_r = 39 000, corresponding most probably to the carboxy-terminal end of the α chain. As expected, the majority of fibrin fragments remained, as was the original fibrinogen, bound to the support through polyglutaraldehyde bonding. The lysines involved in the covalent binding of fibrinogen most probably are surface residues, and fibrin prepared from the immobilized fibrinogen might expose new intrachain lysyl residues to which Glu-plasminogen binds. Actually, the binding of Glu-plasminogen to a second monolayer of fibrin that was constructed over the first monolayer and was therefore oriented as in a fibrin clot showed results (amount of plasminogen bound and K_d) similar to those obtained with the first covalently bound monolayer of fibrin. This indicates that the internal lysyl residues are available on the covalently bound fibrin to the same extent as on any fibrin surface.

The type of techniques and graphical representations used for the analysis of experimental data is another problem that affects the results of binding experiments. It has been repeatedly stressed (Nørby et al., 1980; Klotz, 1983; Munson, 1983; Bürgisser, 1984) that a number of experimental artifacts may contribute to misleading Scatchard representations. These are radioactivity background, nonspecific binding, improper separation of bound and free ligand, the use of a low range of ligand concentrations, and excessive scattering of data points. Even a simple one-site interaction may appear complex due to scattering. In the present study untransformed data were analyzed by nonlinear regression methods. The average binding curves represented in Figure 2 show that the plasminogen/fibrin interaction obeys the simple Langmuir equation for adsorption at interfaces, eq 4, indicating single-site binding and a single association–dissociation mechanism. The equilibrium association constant (K) and B_{max} were derived from the linearized expression, eq 9. The parameters of the binding of Glu- and Lys-plasminogen to intact fibrin (Table I) showed a difference in the affinity of the reactants (Glu-plasminogen, $K_d \sim 1$ μM; Lys-plasminogen, $K_d \sim 0.4$ μM) while their affinities with the degraded surface of fibrin were similar (Glu-plasminogen, $K_d \sim 0.6$ μM; Lys-plasminogen, $K_d \sim 0.5$ μM). These data suggest, in agreement with our previous finding, that Lys-plasminogen is an unlikely intermediary of the generation of plasmin (Rouy & Anglès-Cano, 1990). In contrast to the similarity in the affinity values, the total number of binding sites in fibrin and the fractional number of sites per molecule of fibrin (α = 0.3) increased markedly (9-fold) after plasmin degradation (α = 2.7). This indicates that as for other heterogeneously catalyzed reactions, e.g., surface-dependent reactions of coagulation (Zwaal & Hemker, 1982; Mann et al., 1990), it is the modification of the surface, i.e., the appearance of new binding sites, and not a change in affinity that is responsible for the displacement of the reaction front and finally for the lysis of the fibrin.

The K_d values we have calculated are in close agreement with those reported by Tran-Thang et al. (1986) for Glu- and Lys-plasminogen high-affinity binding sites. We were unable, however, to detect the low-affinity binding sites that have been reported previously (Bok & Mangel, 1985; Tran-Thang et al., 1986). Although this discrepancy could be related to the techniques used for the analysis of data, in our case, the simple hyperbolic expression developed by Langmuir for heterogeneous interactions and the linearized transformations used, including the Scatchard formula, were consistent with a single type of binding site in the concentration range investigated.

The nature of the binding sites appearing on the surface of fibrin upon plasmin degradation was identified by carboxy-terminal end-group digestion with carboxypeptidase B (Figure 1B). Plasminogen binding to the degraded surface of fibrin was decreased by more than 87% by treating the surface with carboxypeptidase B. Further evidence that the binding was mediated by carboxy-terminal lysine residues and not by carboxy-terminal arginines also generated by plasmin and cleaved by carboxypeptidase B was provided by the specific inhibition of the binding with the lysine analogue, 6-AHA (Figure 1B, inset). Moreover, evidence from equilibrium dialysis, affinity chromatography, and H NMR experiments

(Lerch et al., 1980; Markus et al., 1981; De Marco et al., 1982) has clearly demonstrated that the kringle-binding sites have affinity for lysine analogues, and that N^{α}-acetyl-L-arginine, a carboxy-terminal arginine analogue, does not function as a ligand (Thewes et al., 1990). These observations are in agreement with data by Christensen (1985) indicating that the carboxy-terminal lysine residues of plasmin-cleaved fibrinogen fragments are essential for their high-affinity binding to immobilized plasminogen.

The increase in the number of carboxy-terminal lysine residues upon degradation of fibrin with plasmin was predictable. Plasmin cleaves at the carboxy terminus of lysine and arginine residues in fibrin (Weinstein & Doolittle, 1972). At least 17 cleavage sites in fibrin by plasmin results in carboxy-terminal lysine fragments; four [(β-Lys^{133} and γ-Lys^{62})$_2$] have been localized in the early fragment (E) and two (α-Lys^{206} and γ-Lys^{159}) in fragment D (Henschen, 1983). The activation of plasminogen bound to such binding sites by activators having no affinity for fibrin, i.e., pro-urokinase, has been proposed as a complementary or synergistic mechanism for specific lysis of a clot (Pannel et al., 1988). The model we have characterized may constitute a useful in vitro tool to define the specificity of plasminogen activation by the different types of activators.

To further characterize the differential binding of Glu-plasminogen to lysine side chains exposed in intact fibrin and to carboxy-terminal lysines in degraded fibrin, we determined the effect of 6-AHA, an analogue of lysine, on the binding of plasminogen to these surfaces. Analysis of data was compatible with a single binding site model as previously established (Lerch et al., 1980). It was interesting to observe that 6-AHA inhibits equally well the binding of plasminogen to intact fibrin (K_i = 130 μM) and to degraded fibrin (K_i = 109 μM). This observation is in agreement with the specificity of interaction between 6-AHA and kringle 5 recently reported (Thewes et al., 1990).

Conclusions. Altogether these data clearly indicate, in agreement with the hypothesis proposed by Christensen (1984) and with recent ligand-binding data by Petros et al. (1989), that fibrinolysis progresses via the interaction of plasminogen lysine-binding sites with the carboxy-terminal lysine residues of fibrin that are exposed by plasmin cleavage. The efficiency of the plasminogen/fibrin interaction is markedly increased by such sites. On the basis of our data, we would like to propose that it is the appearance of new binding sites and not a change in affinity that is responsible for the lysis of fibrin; i.e., the generation of carboxy-terminal lysine residues on fibrin by plasmin is a mechanism of a positive regulation that accelerates fibrinolysis by increasing the number of binding sites but does not modify the affinity of the interactions.

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Registry No. Lys, 56-87-1; plasminogen, 9001-91-6; thrombin, 9002-04-4; plasmin, 9001-90-5.

REFERENCES

Adamson, A. W. (1990) in *Physical Chemistry of Surfaces*, pp 595–606, John Wiley & Sons, Inc., New York.

Anglés-Cano, E. (1986) *Anal. Biochem. 153*, 201–210.

Anglés-Cano, E., Masson, C., Grailhe, P., Gaussem, P., & Le Bonniec, B. (1988) *Thromb. Res. 8* (Suppl.), 123–130.

Anglés-Cano, E., & Sultan, Y. (1984) *J. Immunol. Methods 69*, 115–127.

Blombäck, B., & Blombäck, M. (1956) *Ark. Kemi. 10*, 415–443.

Bok, R. A., & Mangel, W. F. (1985) *Biochemistry 24*, 3279–3286.

Bürgisser, E. (1984) *Trends Pharmacol. Sci. 5*, 142–144.

Christensen, U. (1984) *Biochem. J. 233*, 413–421.

Christensen, U. (1985) *FEBS Lett. 182*, 43–46.

De Marco, A., Hochschwender, S. M., Laursen, R. A., & Llinas, M. (1982) *J. Biol. Chem. 257*, 12716–12721.

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

Edman, P., & Henschen, A. (1975) in *Protein Sequence Determination* (Needleman, S. B., Ed.) pp 232–279, Springer, Berlin, Heidelberg, New York.

Fleury, V., & Anglés-Cano, E. (1990) *Fibrinolysis 4* (Suppl. III), 311 (Abstract).

Fraker, P. J., & Speck, J. C. J. (1978) *Biochem. Biophys. Res. Commun. 80*, 849–857.

Harpel, P. C., Chang, T. S. & Verderber, E. (1985) *J. Biol. Chem. 260*, 4432–4440.

Henschen, A. (1983) *Thromb. Res. 5* (Suppl.), 27–39.

Higgins, D., & Vehar, G. A. (1987) *Biochemistry 26*, 7786–7791.

Hoylaerts, M., Rijken, D. C., Lijnen, H. R., & Collen, D. (1982) *J. Biol. Chem. 257*, 2912–2919.

Kazal, L. A., Amsel, S., Miller, O. P., & Tocantins, L. M. (1963) *Proc. Soc. Exp. Biol. Med. 113*, 989–994.

Klotz, I. M. (1983) *Trends Pharmacol. Sci. 4*, 253–255.

Koppert, P. W., Huijmans, C. M. G., & Nieuwenhuizen, W. (1985) *Blood 66*, 503–507.

Koppert, P. W., Koopman, J., Haverkate, F., & Nieuwenhuizen, W. (1986) *Blood 68*, 437–441.

Krause, J., & Deutsch, H. (1989) *Fibrinolysis 3*, 85–91.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lerch, P. G., Rickli, E. E., Lergier, W., & Gillessen, D. (1980) *Eur. J. Biochem. 107*, 7–13.

Liu, C. Y., Sobel, J. H., Weitz, J. I., Kaplan, K. L., & Nossel, H. L. (1986) *Thromb. Haemostasis 56*, 100–106.

Lucas, M. A., Fretto, L. J., & McKee, P. A. (1983) *J. Biol. Chem. 7*, 4249–4256.

Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P., & Krishnaswamy, S. (1990) *Blood 76*, 1–16.

Markus, G., Camiolo, S. M., Sottrup-Jense, L., & Magnusson, S. (1981) *Prog. Fibrinolysis 5*, 125–128.

Masson, C., & Anglés-Cano, E. (1988) *Biochem. J. 256*, 237–244.

Munson, P. J. (1983) *J. Recept. Res. 3*, 249–259.

Nieuwenhuizen, W., Voskuilen, M., Vermond, A., Hoegee-de Nobel, B., & Traas, D. W. (1988) *Eur. J. Biochem. 174*, 163–169.

Nørby, J. G., Ottolenghi, P., & Jensen, J. (1980) *Anal. Biochem. 102*, 318–320.

Norrman, B., Wallen, P., & Rånby, M. (1985) *Eur. J. Biochem. 149*, 193–200.

Pannel, R., Black, J., & Gurewich, V. (1988) *J. Clin. Invest. 81*, 853–859.

Petros, A., Ramesh, V., & Llinas, M. (1989) *Biochemistry 28*, 1368–1376.

Robbins, K. C., Summaria, L., & Wohl, R. C. (1981) *Methods Enzymol. 80*, 379–387.

Rouy, D., & Anglès-Cano, E. (1990) *Biochem. J. 271*, 51-57.
Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E., & Magnusson, S. (1978) *Prog. Chem. Fibrinolysis Thrombolysis 3*, 191-209.
Suenson, E., Lützen, O., & Thorsen, S. (1984) *Eur. J. Biochem. 140*, 513-522.
Suenson, E., & Thorsen, S. (1982) *Biochem. J. 197*, 619-628.
Thewes, T., Constantine, K., Byeon, I.-J., L., & Llinas, M. (1990) *J. Biol. Chem. 265*, 3906-3915.
Thorsen, S. (1975) *Biochim. Biophys. Acta 393*, 55-65.
Thorsen, S., Clemmensen, I., Sottrup-Jensen, L., & Magnusson, S. (1981) *Biochim. Biophys. Acta 668*, 377-387.
Tran-Thang, C., Kruithof, E. K. O., & Bachmann, F. (1984) *J. Clin. Invest. 74*, 2009-2016.
Tran-Thang, C., Kruithof, E. K. O., Atkinson, J., & Bachmann, F. (1986) *Eur. J. Biochem. 160*, 599-604.
Tulinsky, A., Park, C. H., Mao, B., & Llinas, M. (1988) *Proteins: Struct., Funct., Genet. 3*, 85-96.
Vali, Z., & Patthy, L. (1982) *J. Biol. Chem. 257*, 2104-2110.
Varadi, A., & Patthy, L. (1983) *Biochemistry 22*, 2440-2446.
Wallen, P., & Wiman, B. (1972) *Biochim. Biophys. Acta 257*, 122-134.
Weinstein, M. J., & Doolittle, R. F. (1972) *Thromb. Diath. Haemorrh. 28*, 289-297.
Wiman, B., & Wallen, P. (1977) *Thromb. Res. 1*, 213-222.
Wiman, B., & Collen, D. (1978) *Nature 272*, 549-550.
Zwaal, R. F. A., & Hemker, H. C. (1982) *Haemostasis 11*, 12-39.

Photoaffinity Labeling of Human Serum Vitamin D Binding Protein and Chemical Cleavages of the Labeled Protein: Identification of an 11.5-kDa Peptide Containing the Putative 25-Hydroxyvitamin D_3 Binding Site†

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ABSTRACT: In this paper, we describe photoaffinity labeling and related studies of human serum vitamin D binding protein (hDBP) with 25-hydroxyvitamin D_3 3β-3′-[*N*-(4-azido-2-nitrophenyl)amino]propyl ether (25-ANE) and its radiolabeled counterpart, i.e., 25-hydroxyvitamin D_3 3β-3′-[*N*-(4-azido-2-nitro-[3,5-^{3}H]phenyl)amino]propyl ether (^{3}H-25-ANE) (Ray et al., 1986, 1991). We have carried out studies to demonstrate that (1) 25-ANE competes with 25-OH-D_3 for the binding site of the latter in hDBP and (2) ^{3}H-25-ANE is capable of covalently labeling the hDBP molecule when exposed to UV light. Treatment of a sample of purified hDBP, labeled with ^{3}H-25-ANE, with BNPS-skatole produced two Coomassie Blue stained peptide fragments, and the majority of the radioactivity was associated with the smaller of the two peptide fragments (16.5 kDa). On the other hand, cleavage of the labeled protein with cyanogen bromide produced a peptide (11.5 kDa) containing most of the covalently attached radioactivity. Considering the primary amino acid structure of hDBP, this peptide fragment (11.5 kDa) represents the N-terminus through residue 108 of the intact protein. Thus, our results tentatively identify this segment of the protein containing the binding pocket for 25-OH-D_3.

Vitamin D binding protein (DBP), identical with the group-specific component (Gc), is an abundant serum protein, which contains a single polypeptide chain and 0-4% neuraminidase-sensitive sialic acids (Cooke & Haddad, 1989). It is well established that the primary function of DBP is to act as a plasma carrier for vitamin D and its metabolites, a property manifested by its high-affinity binding to metabolites of vitamin D, of which one of its highest affinities is toward 25-hydroxyvitamin D_3 (25-OH-D_3), the major circulatory form of vitamin D_3 (Francheschi et al., 1981; Daiger et al., 1975; Haddad & Walgate, 1976). Recently high-affinity binding of DBP by a variety of other substrates, which include monomers of actin (Van Baelen et al., 1980; Haddad, 1982), polyunsaturated fatty acids (Williams et al., 1988; D. Z. Xiang and R. Bouillon, unpublished results), and membrane immunoglobulins of lymphocytes (Petrini et al., 1983, 1985), has been described. The full-length primary amino acid structure of hDBP has been determined recently, which shows remarkable homology with albumin and α-fetoprotein (Schoentgen et al., 1986; Cooke & David, 1985; Yang et al., 1985a).

The photoaffinity labeling technique has been used extensively to probe proteins and other biomolecules for their organelle distribution, polymorphic behavior, ligand interaction, and ligand-binding-site structure (Bayley & Knowles, 1977; Sweet & Murdock, 1987). For the past several years we have been interested in obtaining information about the ligand-binding site in DBP. To this effect, we reported the synthesis of a photoaffinity analogue of 25-OH-D_3 and successful labeling of rat serum DBP with this analogue (Ray et al., 1986). However, instability of this analogue in basic reaction conditions prompted us to develop a second-generation photoaffinity analogue of 25-OH-D_3. Recently, we reported the synthesis of 25-hydroxyvitamin D_3 3β-3′-[*N*-(4-azido-2-nitro-[3,5-^{3}H]phenyl)amino]propyl ether (^{3}H-25-ANE), a

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