Human Plasmin Enzymatic Activity Is Inhibited by Chemically Modified Dextrans*

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Dominique Ledoux‡, Dulce Papy-Garcia‡, Quentin Escartin‡, Marie-Astride Sagot§, Yihai Cao†, Denis Barritault‡‡, Josiane Courtios**, William Hornebeck‡‡, and Jean-Pierre Caruelle‡

From the ‡Laboratoire CRRET, CNRS UPRÉA 7053, Université Paris XII-Val de Marne, Avenue du Général de Gaulle, 94010 Créteil Cedex, France, §Laboratoire de Pharmacologie et d’Immunologie, Commissariat à l’Energie Atomique Saclay, 91191 Gif-sur-Yvette Cedex, France, ¶Laboratoire de Angiogenesis Research, Department of Cell and Molecular Biology, Karolinska Institute, S171 77 Stockholm, Sweden, **Laboratoire de Biotechnologie Microbienne, IUT d’Amiens, Avenue des facultés-Le Bailly, 80025 Amiens Cedex 1, France, and ‡‡Laboratoire de Biochimie, CNRS UPRÉA 6021, IFR 53 Biomolécules, Faculté de Médecine, Université de Reims, 51 rue Cognacq Jay, 51095 Reims Cedex, France

Some synthetic dextran derivatives that mimic the action of heparin/heparan sulfate were shown to promote in vivo tissue repair when added alone to wounds. These biofunctional mimetics were therefore designated as “regenerating agents” in regard to their in vivo properties. In vitro, these biopolymers were able to protect various heparin-binding growth factors against proteolytic degradation as well as to inhibit the enzymatic activity of neutrophil elastase. In the present work, different dextran derivatives were tested for their capacity to inhibit the enzymatic activity of human plasmin. We show that dextran containing carboxymethyl, sulfate as well as benzylamide groups (RG1192 compound), was the most efficient inhibitor of plasmin amidolytic activity. The inhibition of plasmin by RG1192 can be classified as tight binding hyperbolic noncompetitive. One molecule of RG1192 bound 20 molecules of plasmin with a Kd of 2.8 × 10⁻⁸ M. Analysis with an optical biosensor confirmed the high affinity of RG1192 for plasmin and revealed that this polymer equally binds plasminogen with a similar affinity (Kd = 3 × 10⁻⁸ M). Competitive experiments carried out with 6-aminohexanoic acid and kringle proteolytic fragments identified the lysine-binding site domains of plasmin as the RG1192 binding sites. In addition, RG1192 blocked the generation of plasmin from Glu-plasminogen and inhibited the plasmin-mediated proteolysis of fibronectin and laminin. Data from the present in vitro investigation thus indicated that specific dextran derivatives can contribute to the regulation of plasmin activity by impeding the plasmin generation, as a result of their binding to plasminogen and also by directly affecting the catalytic activity of the enzyme.

We have previously reported that some dextran derivatives could stimulate tissue repair when applied at the site of the injury in various in vivo models such as skin (1), bone (2), colon (3), cornea (4), and muscle (5). These biopolymers were obtained by controlled chemical substitution of dextran polymers by defined amounts of carboxymethyl, sulfate as well as hydrophobic groups such as benzylamide. As regards their in vivo properties, these biopolymers were designated regenerating agents (RGTA).1

Our initial interpretation of the ability of these biopolymers to stimulate tissue repair was to postulate that these molecules acted as functional mimetics of heparin/heparan sulfate in terms of stabilizers, protectors, and potentiators of endogenously released heparin-binding growth factors. This hypothesis was supported by in vitro experiments, which showed that these biopolymers protected some heparin-binding growth factors such as fibroblast growth factors and transforming growth factor β against proteolytic degradation and enhanced their bioavailability (3, 6). A second interpretation of the in vivo wound healing properties of these polymers, which does not exclude the first, is that they could also act on some of the proteinases involved in tissue remodeling. This led us to report in a previous study that human neutrophil elastase was inhibited by specific dextran derivatives (7). Among other known proteinases involved in tissue remodeling, plasmin plays a key role, since it acts directly by hydrolyzing components of the basement membrane such as fibrin, fibronectin, and laminin and also acts indirectly by activating other enzymes such as matrix metalloproteinases (8, 9).

As regards the pivotal role of this enzyme in tissue remodeling, we have further investigated the effect of these dextran derivatives on the enzymatic activity of plasmin. We report that as for neutrophil elastase, human plasmin activity is inhibited by specific dextran derivatives that contained the aromatic residue benzylamide. Complementary studies revealed that this type of biopolymer also bind plasminogen and modulate plasmin activity in a noncompetitive manner via regulatory sites involving the lysine-binding site (LBS) domains of plasmin.

EXPERIMENTAL PROCEDURES

Materials

The chromogenic substrates D-Val-Leu-Lys-pNA (S-2251), pyro-Glu-Gly-Arg-pNA (S-2444), and D-Ile-Pro-Arg-pNA (S-2258) as well as human tissue-type plasminogen activator (tPA) were purchased from Chromogenix (Molndal, Sweden). Ac-Arg-pNA and Suc-Gly-Gly-Phe-

1 The abbreviations used are: RGTA, regenerating agent(s); pNA, p-nitroanilide; PAGE, polyacrylamide gel electrophoresis; uPA, uroki-
Inhibition of Plasmin by Chemically Modified Dextrans

**Synthesis of Dextran Derivatives**

Water-soluble modified dextrans were prepared from T40 dextran (average M 37,000; Amersham Pharmacia Biotech) according to the method described by Mauzac et al. (12). Compound RG1100 (Fig. 1) was synthesized from dextran T40 by carboxymethylation of OH residues with monochloroacetic acid treatment in aqueous NaOH at 50 °C, pH 10, for 20 min with constant stirring. The product was then precipitated with methanol and dried under vacuum. The presence of carboxymethyl groups was confirmed by infrared spectroscopy with the appearance of a new absorption band at 1750 cm⁻¹, corresponding to the carbonyl bond of the amide. This derivatized carboxymethyl-benzylamide dextran was then isolated and its analysis by 1H NMR showed two broad multiplets at 7.3 and 7.6 ppm, assigned to the aromatic protons of unsubstituted benzylamino groups. The absence of benzylamino groups was confirmed by 13C NMR (75 MHz, D₂O) spectrum, in which the 13C signal at 142 ppm, present in the RG1100 precursor, was not detected in the RG1102 product. We then concluded that as described previously for other dextran derivatives (14), C-sulfonate groups were undetectable in the RG1102 compound and that O-sulfonation mainly occurred during treatment of derivatized carboxymethyl-benzylamide dextrans with chlorosulfonic acid. This dominant O-sulfonate formation may thus be explained by the higher content of OH groups (87%) as compared with aromatic groups (13%) present in the RG1102 precursor.

The chemical characterization of all dextran derivatives was based on the degree of substitution (d.s.) of each individual group per glucosidic unit (Table I). Each d.s. value was determined by acidimetric titration for CH₂COONa and SO₃Na content and elementary analysis for CH₂CONHCH₂C₆H₅ and SO₃Na content. All of these values were derived from Bachem (Budendorf, Switzerland). Human plasmin, human urokinase plasminogen activator (uPA), bovine pancreatic trypsin, a-chymotrypsin, bovine kidney heparan sulfate, apotinin, human Glu-plasminogen, human fibronectin, Engelbreth-Holm-Swarm mouse sarcoma laminin, polyclonal rabbit anti-fibronectin, streptavidin, and biocytin hydrazide were obtained from Sigma. Horse-radish peroxidase-conjugated goat anti-rabbit Ig were from Diagnostic Pasteur (Marne la Coquette, France). Immobilon P and ECL chemiluminescence were purchased from Millipore Corp. (Saint Quentin en Yvelines, France), and Superblock® blocking buffer was from Pierce.

**FIG. 1. Schematic structure of dextran derivatives.** Polymers were elaborated from T40 dextran by chemical substitutions as described under “Experimental procedures.” Dextran was substituted by carboxymethylation (RG1100) or O-sulfonation (RG1003); by carboxymethylation followed by O-sulfonation (RG1503); or by carboxymethylation followed by amidation with benzylamine and O-sulfonation (RG1192). The different percentages indicated in the figure were calculated from the d.s. relative to the position of each group in a glucosidic unit, as reported in Table I. For an easy representation, the substituted glucosidic units A, B, C, and D were arranged in an arbitrary combination. Their respective proportions (percentages) within each polymer were calculated according to the nature of the group linked to the C-2 position. In addition, R represents the proportion (percentage) of each substituted group in the global C-3 + C-4 positions.
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Chemical characterization of each polymer is represented through the d.s. of individual group per glucosidic unit. Each d.s. value was determined and confirmed by acidimetric titration, elementary analysis, and $^1$H NMR. A d.s. value of 3 represents the maximum of substitution, since one glucosidic unit contains three reactive OH groups on C-2, C-3, and C-4 positions. The position of each group on the C-2 versus C-3 + C-4 positions was determined by analyzing the anomeric proton signal in $^1$H NMR. In this representation, a d.s. value of 1 and 2 represents the maximum substitution of C-2 and C-3 + C-4 positions, respectively. S.D. of d.s. values were less than 5% ($\alpha = 3$).

### Table I

| Polymer  | d.s. | CM$^a$ | CMBn$^d$ | H$^t$ | Position of groups expressed in d.s. | Average M$e$
|----------|------|--------|---------|------|-----------------------------------|--------
| RG1100   | 0.49 | 2.51   | 0.29    | 0.20 | CR 2, C-3 + C-4                     | 74,000 |
| RG1503   | 0.26 | 1.92   | 0.16    | 0.10 | CR 2, C-3 + C-4                     | 47,000 |
| RG1192   | 0.31 | 1.38   | 0.19    | 0.12 | CR 2, C-3 + C-4                     | 140,000|
| RG1003   | 2.26 | 0.74   | 1.00    | 1.26 | CR 2, C-3 + C-4                     | 40,000 |

$^a$ d.s., degree of substitution of individual group in one glucosidic unit. $^b$ CM, CH$_2$COONa. $^c$ Su, SO$_3$Na. $^d$ CMBn, CH$_2$CONHCH$_2$C$_6$H$_5$. $^t$ H, Nonreacted hydroxyl groups, calculated as the residual d.s. value as compared with the total d.s. value ($\alpha = 3$). $^e$ C-3 + C-4, global substitution on C-3 + C-4 positions, calculated for each group as the difference between its total d.s. value and the one determined on the C-2 position.

confirmed by $^1$H NMR. Distribution of each group among the three reactive OH groups was also reported in Table I. Results showed that reactions of carboxymethylation and sulfonation on hydroxyl functions preferentially occurred on the C-2 position. These results are in agreement with those showing that the OH on C-2 position displayed the higher rate coefficient of dextran carboxymethylation (15). The average molecular weight of each polymer (Table I) was determined by high performance size exclusion chromatography in 0.1 mM NaNO$_3$, using KB-804 and KB-805 aqueous gel filtration columns (Shodex, Japan) applied in series. The effluent was monitored with a mini Dawn light scattering detector and a RID 10 A refractometer (Touzard & Matignon, France). The flow rate was 0.7 ml/min. All of these polymers did not present any significant anticoagulant activity (less than 5 IU/mg as compared with 173 IU/mg for heparin) (6).

**Effect of Various Polymers on the Enzymatic Activity of Proteolytic Enzymes**

Enzymatic kinetics were monitored with a Philips PU8740 spectrophotometer equipped with a thermostated cell holder. The progress curves were recorded for 0.3–5 min, depending upon the reaction velocity, and less than 5% of the substrate was hydrolyzed during the rate measurement. Plasmin, trypsin, and $\alpha$-chymotrypsin enzymatic activities were determined in 50 mM Tris/HCl buffer, pH 7.4, containing 50 mM NaCl at 37 °C and steady-state velocities were measured by following the release of p-nitroaniline at 410 nm ($e = 8800$ M$^{-1}$ cm$^{-1}$). Human tPA and uPA enzymatic activities were assayed in similar conditions at pH 7.4. We determined the kinetic constants $K_a$ and $K_c$ for all of these polymers were measured as a function of substrate concentrations, and the data were fitted to the Michaelis-Menten rate equation using the GraphPad Prism software (San Diego, CA). The $K_{max}$ and $K_e$ values for the plasmin/S-2251 system are 14 s$^{-1}$ and 0.4 mM, and those for the plasmin/S-2444 system are 9 s$^{-1}$ and 5.3 mM, respectively.

The effect of various polymers on the different enzymatic activities was determined by reacting constant concentration of enzyme with increasing concentrations of polymers for 15 min at 37 °C and measuring the residual enzymatic activity with a synthetic substrate. For plasmin, the equilibrium dissociation constants ($K_i$) were measured with 8 mM of enzyme and 0.4 mM of substrate (S-2251). The $K_i$ values and their S.E. values were calculated by nonlinear regression using the integral equation editor of the GraphPad Prism software. For the determination of inhibitor-plasmin binding stoichiometries, 750 mM plasmin was used, and the substrate was S-2444 (0.4 mM).

**Plasmin Surface Resonance Analysis**

**Biotinylation of RG1192—RG1192 polymer was labeled by reaction of its aldehydic reducing group with the hydrazide functional group of biocytin hydrazide using a modified method described by Nadkarni et al. (16). A 0.4% (w/v) solution of polymer was dissolved in 0.1 M NaOH to remove any remaining attached ligand.**

### TABLE I

**Chemical characterization of modified dextrans**

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|----------|------|--------|---------|------|-----------------------------------|--------|
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**Western Blot Analysis of Fibronectin Proteolytic Fragments**

Plasmin (1.6 µM) and RG1192 (0.05–1 µM) were incubated at 37 °C for 30 min in 50 mM Tris/HCl, pH 7.4, 50 mM NaCl, 5 mM CaCl$_2$, 0.005% P-20 were injected over the RG1192-immobilized surface, and change in resonance signal was monitored according to time. Equal volumes of each protein were also injected over a streptavidin-immobilized surface to serve as a blank for subtraction of nonspecific binding of analyte. The sensor surfaces were regenerated with a pulse of 2 mM NaCl between each injection of analyte. Equilibrium dissociation constants were determined as described in the BIAtechnology handbook (Amersham Pharmacia Biotech).

At steady state, the following is true,

$$\frac{dR}{dt} = k_c \times C \left( R_{max} - R_{eq} \right) - k_d \times R_{eq} = 0$$

which may be rearranged as follows,

$$R_{eq} = \frac{C \times R_{max}}{K_c + C}$$

where $K_c = k_c/k_d$ is the equilibrium dissociation constant, $R_{eq}$ is the response value at steady state, $R_{max}$ is the maximal capacity of the sensor chip for binding analyte, and $C$ is the molar concentration of analyte. $K_c$ was calculated by nonlinear regression analysis by fitting the $(R_{eq}, C)$ pairs to Equation 2 using the GraphPad Prism software.

Plasmin (1.6 µM) and RG1192 (0.05–1 µM) were incubated at 37 °C for 30 min in 50 mM Tris/HCl, pH 7.4, 50 mM NaCl, 5 mM CaCl$_2$, 0.001% Triton X-100 prior to the addition of 10 ng of fibronectin (0.5 mM). Following 4 h of incubation, the reaction was stopped by the addition of 1.6 µM of aprotinin, and samples were separated by SDS-PAGE (7% (w/v) acrylamide) under reducing conditions. Proteins were electrophoretically transblotted overnight at 4 °C to Immobilon-P membrane in 25 mM Tris, pH 8.3, 192 mM glycine. Membranes saturated with Superblock$^\text{B}$ blocking buffer were incubated with polyclonal rabbit anti-human fibronectin Ig at a 1:1000 dilution in phosphate-buffered saline containing 0.02% (v/v) Tween 20 and 0.3% Superblock$^\text{B}$ blocking buffer. Antibodies were detected by using horseradish peroxidase-conjugated goat anti-rabbit IgG and ECL chemiluminescence according to the manufacturer's recommendations.
Inhibition of Plasmin by Chemically Modified Dextrans

Western Blot Analysis of Laminin Proteolytic Fragments

Plasmin (0.8 μM) and RG1192 (0.05–2.5 μM) were incubated at 37 °C for 30 min in 50 mM Tris/HCl, pH 7.4, 50 mM NaCl, 0.01% Triton X-100. 50 ng of laminin (2 nM) was then added and the reaction mixture was kept for additional 60 min. The reaction was stopped with 0.8 μM aprotinin, and samples were separated by SDS-PAGE (4–15% (w/v) acrylamide gradient) under reducing conditions. Transfer and hybridization were performed in the same way as for fibronectin, except that the first incubation was performed with polyclonal rabbit anti-mouse laminin Ig at a 1:1000 dilution.

Electrophoretic Analysis of Plasminogen Activation

The generation of plasmin from Glu-plasminogen in the presence of RG1192 was analyzed in 50 mM Tris/HCl, pH 7.4, 50 mM NaCl, 0.01% Triton X-100 as follows. 5 μg of Glu-plasminogen (0.8 μM) was preincubated for 30 min at 37 °C with increasing concentrations of RG1192 (0.05–1 μM) before the addition of uPA (20 nM). After 2 h of incubation, samples were separated by SDS-PAGE (10% (w/v) acrylamide) under reducing conditions. Gels were then fixed and stained with Coomassie Brilliant Blue R-250.

RESULTS

Effect of Dextran Derivatives on the Amidolytic Activity of Plasmin, α-Chymotrypsin, Trypsin, uPA, and tPA—Fig. 2 shows the influence of increasing concentrations of various dextran derivatives obtained by sequential chemical substitution of dextran polymers on the amidolytic activity of plasmin. Dextran substituted with carboxymethyl functions (RG1100) did not affect the activity of plasmin even for concentrations greater than 1 μM. Following O-sulfonation, the resulting derivatized polymer (RG1503) showed an inhibitory potential toward human plasmin (IC50 = 20 nM) with 70% of residual enzyme activity. This potential was, however, better than the one obtained with a dextran sulfate (RG1003), indicating a beneficial contribution of the carboxymethyl groups with respect to the antiprotease activity of this polymer. Interestingly, when both carboxymethyl, sulfate and benzylamide groups were coupled to dextran glucosidic units (RG1192), a potent inhibitory activity of plasmin was observed. The IC50 value was 2 nM with 20% of residual enzyme activity. In contrast, heparin and heparan sulfate did not affect the activity of plasmin.

Among the various dextran derivatives tested, RG1192 was the most efficient inhibitor of plasmin activity. The antiprotease potency of this polymer was therefore investigated toward other serine proteinases such as trypsin, α-chymotrypsin, and the two human plasminogen activators, uPA and tPA (Fig. 3).

![Fig. 2. Effect of different polymers on the enzymatic activity of human plasmin. Increasing concentration of RG1100 (⧫), RG1503 (○), RG1102 (●), RG1003 (▲), heparin (■), or heparan sulfate (◆) were added to plasmin (8 nM) at pH 7.4 and 37 °C, and the residual enzymatic activity was measured with S-2251 (0.4 mM). The experimental points obtained for RG1103 were fitted to Equation 3 by nonlinear regression, and the line represents the best fit curve. Ordinates, V/V0, initial rate in the presence of inhibitor/initial rate in its absence.](Image 56x527 to 290x729)

![Fig. 3. Influence of RG1192 on the activity of several serine proteinases. For experimental details see “Experimental Procedures.” V, trypsin (30 nM); the substrate was Ac-Arg-Pro-NA (1 mM); ●, α-chymotrypsin (60 nM); the substrate was Suc-Gly-Gly-Phe-pNA (1.5 mM); ▲, uPA (12 nM); the substrate was S-2444 (1 mM); ○, tPA (10 nM); the substrate was S-2288 (2 mM). Plasmin as reference (8 nM); the substrate was S-2251 (0.4 mM). V/V0, see legend to Fig. 2.](Image 314x525 to 548x729)

![Ordinates](Image 68x437)

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![Western Blot Analysis of Laminin Proteolytic Fragments](Image 201x201)

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In the same concentration range used for plasmin, trypsin and uPA were insensitive to RG1192. In the same concentration range used for plasmin, trypsin and uPA were insensitive to RG1192. α-Chymotrypsin activity was slightly affected by the presence of RG1192, whereas 70% of the tPA activity was inhibited at a saturating concentration of inhibitor. However, the inhibitory potential of RG1192 on tPA was lower than that of obtained with plasmin: IC50 equal to 34 nM as compared with 2 nM for plasmin.

**Study of the Mechanism of Plasmin Inhibition by RG1503 and RG1192**—The mechanism of the inhibition pattern of a single-substrate reaction can be schematically represented as follows.

\[ nE + nS \rightleftharpoons K_s n(ES) \rightarrow nE + nP \]

\[ K_i \]

\[ K_0 \]

\[ E, I + nS \rightleftharpoons aK_r (ES)_I \rightarrow E, I + nP \]

**Scheme I**

Where \( n \) is the number of free or substrate-bound plasmin molecules per molecule of inhibitor, \( K_i \) is the equilibrium dissociation constant of the enzyme-inhibitor complex, \( a \) is a dimensionless number that affects the binding of substrate to the plasmin-inhibitor complex or that of inhibitor to enzyme-substrate complex, and \( b \) is a dimensionless factor that affects the catalytic constant \( k_{cat} \). Hyperbolic noncompetitive inhibition is characterized by \( a = 1 \) and \( 0 < b < 1 \).

While classical inhibition does not depend upon the total enzyme concentration, tight binding inhibition does (17), since the concentration of the bound inhibitor \( [E_I] \) is no longer negligible with respect to that of total inhibitor concentration \( [I]_0 \). We therefore analyzed whether RG1192 and RG11503 behaved as tight binding inhibitors by measuring the inhibition of plas-
min activity using different enzyme concentrations. The inhibition profiles obtained with RG1192 (Fig. 4) indicated tight binding inhibition, since plasmin inhibition decreased with the total enzyme concentration \([E]_0\); IC\(_{50}\) = 2 and 8 nM for \([E]_0 = 8\) and 150 nM, respectively. At high enzyme concentration that ensured pseudo-reversible binding of plasmin to inhibitor, the tight binding inhibitor titrated the enzyme (17). Indeed, at \([E]_0 = 750 \text{nM (Fig. 4, see inset)}\), RG1192 titrated the enzyme with an equivalence point corresponding to the binding of about 20 molecules of plasmin per molecule of RG1192. Similar experiments demonstrated tight binding inhibition of plasmin by RG1503 and a 1:6 inhibitor/plasmin binding stoichiometry (data not shown).

Since these two polymers behave as tight binding inhibitors, the analysis of steady-state velocities data and \(K_i\) determinations by means of the Dixon (18) and/or Cornish-Bowden (19) representation is unsuitable. We therefore used the complex steady-state rate equation (Equation 3) derived by Szedlaczek et al. (20), which takes into account both the tightly binding behavior of the inhibitors and the residual enzymatic activity of the plasmin/inhibitor complexes,

\[
\frac{v}{v_0} = \frac{v_i - v_o}{2v_0} \left( \left( A + B \left[ \frac{[I]_0}{[E]_0} \right] \right) + 4A \right)^{1/2} + \frac{v_i + v_o}{v_0} - A + B \left[ \frac{[I]_0}{[E]_0} \right] \ (\text{Eq. 3})
\]

with

\[
A = \frac{1 + [S]/K_m}{\alpha + [S]/K_m} 	imes aK_i
\]

and

\[
v_e = k_{cat} \frac{[E][S]}{[S]_{\text{cat}} + aK_m}
\]  

where \(v/v_0\) = enzyme velocity in the presence of inhibitor/enzyme velocity in the absence of inhibitor, \(v_e\) = limit of \(v_i\) for saturating concentrations of inhibitor, and \([I]_0\), \([S]_0\), and \([E]_0\) are the total concentration of inhibitor, substrate and plasmin, respectively. To calculate \(\alpha\) and \(\beta\), we measured and compared the kinetic parameters of the enzymatic reaction in the absence (\(k_{cat}, K_m\)) and in the presence (\(b k_{cat}, a K_m\)) of a saturating concentration of inhibitors. The \(K_i\) values were calculated by nonlinear regression analysis by fitting the \((v/v_0, [I]_0)\) pairs to Equation 3, in which \(k_{cat}, [S]_0/K_m, [E]_0\), \(\alpha, \beta, \) and \(n\) were set as fixed parameters. The inhibition curves obtained with RG1503 (Fig. 2) and RG1192 (Fig. 4) have been calculated using 8 nM of plasmin, 0.4 mM of S-2251, and the best estimates of \(K_i\). A good fit was obtained between the oretical curves and experimental data (\(R > 0.99\)).

Table II summarizes the parameters describing the inhibition of plasmin by RG1503 and RG1192. These two compounds behaved as tight binding hyperbolic noncompetitive inhibitors (\(a = 1, \beta = 0\)) with \(K_i\) values in the 30 nM range for RG1192, whereas that for RG1503 is 3-fold higher. All measurements reported here were performed in 50 mM Tris-HCl buffer containing 50 mM NaCl. Increasing the ionic strength by the addition of NaCl resulted in a progressive reduction of the inhibitor potency of all dextran derivatives tested. At physiological ionic strength, only RG1192 retained an inhibitory capacity toward plasmin with 30% of residual enzymatic activity (data not shown). In these conditions, the new binding characteristics of RG1192-plasmin complex were \(\alpha = 1, \beta = 0.3\), and \(K_i = 3.1 \pm 0.2 \times 10^{-5} \text{M}\).

**Analysis of Plasmin Binding to RG1192 by Surface Plasmon Resonance**—Surface plasmon resonance performed on a BIAcoreTM system was used to confirm the interaction between plasmin and RG1192. The biotinylated polymer was immobilized to a streptavidin-bound carboxymethyl dextran surface, and this biosensor chip was used to determine the equilibrium dissociation constant of the plasmin-RG1192 complex. Increasing concentrations of plasmin were injected over the sensor surface, and surface plasmon resonance response values were reached at steady states, representing the equilibrium binding of plasmin to immobilized RG1192 (Fig. 5A). The equilibrium dissociation constant \(K_d\) was calculated by a nonlinear least square fit of the data to Equation 2, as described under “Experimental Procedures” (Fig. 5B). \(K_d\) was found to be 6.0 \(\pm 0.3 \times 10^{-8} \text{M}\), which is closed to the enzymatically determined constant \((2.9 \times 10^{-8} \text{M})\).

**Identification of the Kringle Domains of Plasmin as the RG1192 Binding Sites**—The plasmin molecule contains its A-heavy chain five homologous kringle structures in which LBS are located. Kringles 1, 4, and 5 possess LBS that mediate interactions of plasminogen with fibrin (21), laminin, and fibronectin (22) as well as \(\alpha\)-antiplasmin (23) and possess significant affinity for \(\alpha\)-amino acids such as 6-aminohexanoic acid (6-AHA) (24, 25). Furthermore, it has been previously shown that fibrinogen (26), penicillin (27), and oleic acid (28) modulate plasmin activity by interacting through the LBS modules. We therefore investigated whether the ability of

| Polymers | \(\alpha\) | \(\beta\) | \(K_i\) | \(n\) |
|----------|--------|--------|--------|-----|
| RG1503   | 1      | 0.62   | \(1.10 \pm 0.06 \times 10^{-7}\) | 6   |
| RG1192   | 1      | 0.2    | \(2.8 \pm 0.2 \times 10^{-8}\) | 20  |
RG1192 to suppress plasmin activity depends on its interaction with LBS in plasmin. To explore such a possibility, plasmin activity was first measured in the presence of increasing concentrations of 6-AHA and a constant RG1192 concentration known to produce 50% inhibition. As shown in Fig. 6A, increasing concentrations of 6-AHA relieve the inhibition of plasmin activity, and the inhibitory effect of RG1192 was completely abolished at 5 mM 6-AHA. We then analyzed the effect of plasmin-derived kringle fragments on the plasmin inhibition by RG1192. As described above, plasmin activity was measured in the presence of a constant concentration of RG1192, yielding 50% of enzyme inhibition and an increasing excess of kringle 1–3, kringle 4, or recombinant kringle 5. As shown in Fig. 6B, a 300-fold molar excess of isolated kringle 1–3 as well as kringle 5 restored up to 100% of plasmin activity despite the presence of RG1192. In contrast, only a slight restoration of plasmin activity was observed in the presence of kringle 4. All of these results indicate that RG1192 mediates its inhibitory effect through binding to the LBS-containing kringles but also suggest that these kringles display different affinities toward RG1192.

Interaction of Plasminogen with RG1192—The experiments mentioned above indicate that RG1192 interacts with the kringle domains of plasmin. Since these characteristic structures are equally present within plasminogen, the inactive zymogen of plasmin, we analyzed the capacity of the RG1192 polymer to interact with plasminogen. To investigate such a possibility, we first analyzed the effect of RG1192 on the proteolytic cascade of plasminogen activation. uPA was chosen as the plasminogen activator, since the polymer did not affect its enzymatic activity (Fig. 3). Direct analysis by SDS-PAGE of Glu-plasminogen activation by uPA was documented by the disappearance of the Glu-plasminogen band (95 kDa) and the emergence of the plasmin light B-chain band (25 kDa) and the NH₂-terminal activation peptide (9 kDa) (Fig. 7, lane 2). The inclusion of increasing concentrations of RG1192 in the activation process impeded the conversion of Glu-plasminogen to plasmin in a concentration-dependent manner (Fig. 7, lanes 3–7). Surprisingly, in the presence of RG1192, a new 85-kDa band was observed instead of the native 95-kDa Glu-plasminogen band, and RG1192 did not reduce the level of the 9-kDa activation peptide. The 85-kDa polypeptide may represent Lys-plasminogen, the shortened form of the inactive protein, generated by trace amounts of active plasmin. These results therefore indicated that the presence of RG1192 in the reaction mixture rendered the cleavage of plasminogen.
masses of the polypeptides expressed in kDa are indicated on the left. The molecular masses of the polypeptides expressed in kDa are indicated on the left.

Effects of RG1192 on the Plasmin-mediated Fibronectin and Laminin Proteolysis—Enzymatically active plasmin is able to hydrolyze extracellular matrix proteins such as fibronectin and laminin (8, 9). Use was made of this property to analyze the effect of RG1192 on a more representative substrate of the biological activity of plasmin. Limited proteolysis of fibronectin by plasmin yielded several polypeptide fragments with apparent molecular masses ranging from 150 to 50 kDa (Fig. 8A, lane 2). The addition of increasing concentrations of RG1192 to the incubation mixtures completely abolished the proteolytic cleavage of fibronectin in a dose-dependent manner (Fig. 8A, lanes 3–7). When the same experiment was done with laminin as the natural substrate, RG1192 was also able to prevent its plasmin-mediated proteolysis (Fig. 8B). However, no complete inhibition of this natural substrate against proteolysis was attained even for RG1192 concentrations up to 2.5 \( \mu \text{M} \) (Fig. 8, lane 6).

**DISCUSSION**

It was previously shown that some dextran derivatives, which imitate the action of heparin/heparan sulfate, were able in vitro to protect various heparin-binding growth factors against proteolytic degradation (6) as well as to inhibit the enzymatic activity of neutrophil elastase (7). In this study, we report that these biopolymers are also able to inhibit the enzymatic activity of plasmin, another known proteinase involved in extracellular matrix and tissue remodeling. The extent of plasmin inhibition varied with the nature of the substituted chemical groups; the most efficient inhibitors were substituted with carboxymethyl, sulfate as well as benzylamine groups (RG1192), those without benzylamine were less efficient (RG1503), and those containing only carboxymethyl were ineffective (RG1160). Moreover, the natural glycosaminoglycans heparin and heparan sulfate had no effect upon the enzymatic activity of plasmin. Our data as well as those previously reported concerning heparin (29) as well as chondroitin sulfate and dermatan sulfate (30) indicated that sulfated glycosaminoglycans are devoid of significant antiplasmin activity.

To understand the mechanism by which RG1192 affected the catalytic activity of plasmin, competitive experiments were carried out with 6-AHA and kringle proteolytic fragments. The results reveal that a kringle-dependent interaction occurs between RG1192 and plasmin. RG1192 thus binds plasmin at regulatory sites that were clearly distinct from the catalytic site of the enzyme, hence confirming the noncompetitive character of the inhibition, which was originally determined enzymatically. Remarkably, tPA, whose activity was affected by RG1192, but to a lesser extent than plasmin, contained two homologous kringle domains that exhibited lysine and 6-AHA binding properties (31). Hence, it would be interesting to investigate whether other kringle-containing proteins possessing this characteristic property should be potential ligands of RG1192.

Inhibition of plasmin by RG1503 and RG1192 was of the reversible, tight binding hyperbolic noncompetitive type. Such a mechanism was already described concerning inhibition of elastase and cathepsin G by heparin (32). The plasmin inhibition data could be satisfactorily fitted to Equation 3, which assumes that the inhibition potency depends on the binding stoichiometry \( n \), the equilibrium dissociation constant \( K_a \), and the dimensionless numbers \( \alpha \) and \( \beta \). The mechanism of plasmin inhibition by the two polymers was substrate-independent \( (\alpha = 1) \) and proceeded by a decrease of \( K_{ci} \) \( (\beta < 1) \). This implies that at the concentration used, the substrate did not significantly affect the affinity of the inhibitor for the enzyme \( (K_a = K_{ci}^{-1}) \) and that its rate of hydrolysis was lowered by the presence of the polymer. The experimentally determined values of \( \beta \) (Table II) were equivalent to the residual enzymatic
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