Characterization of the Interactions of Plasminogen and Tissue and Vampire Bat Plasminogen Activators with Fibrinogen, Fibrin, and the Complex of d-Dimer Noncovalently Linked to Fragment E*

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Vampire bat plasminogen activator (b-PA) causes less fibrinogen (Fg) consumption than tissue-type plasminogen activator (t-PA). Herein, we demonstrate that this occurs because the complex of d-dimer noncovalently linked to fragment E (DD)E, the most abundant degradation product of cross-linked fibrin, as well as Fg, stimulate plasminogen (Pg) activation by t-PA more than b-PA. To explain these findings, we characterized the interactions of t-PA, b-PA, Lys-Pg, and Glu-Pg with Fg and (DD)E using right angle light scattering spectrosopy. In addition, interactions with fibrin were determined by clotting Fg in the presence of various amounts of t-PA, b-PA, Lys-Pg, or Glu-Pg and quantifying unbound material in the supernatant after centrifugation. Glu-Pg and Lys-Pg bind fibrin with $K_d$ values of 13 and 0.13 $\mu M$, respectively. t-PA binds fibrin through two classes of sites with $K_d$ values of 0.02 and 2.6 $\mu M$, respectively. The second kringle ($K_2$) of t-PA mediates the low affinity binding that is eliminated with $\epsilon$-amino-caproic acid. In contrast, b-PA binds fibrin through a single kringle-independent site with a $K_d$ of 0.15 $\mu M$. t-PA competes with b-PA for fibrin binding, indicating that both activators share the same finger-dependent site on fibrin. Glu-Pg binds (DD)E with a $K_d$ of 5.4 $\mu M$. Lys-Pg binds to (DD)E and Fg with $K_d$ values of 0.03 and 0.23 $\mu M$, respectively. t-PA binds to (DD)E and Fg with $K_d$ values of 0.02 and 0.76 $\mu M$, respectively; interactions were eliminated with $\epsilon$-amino-caproic acid, consistent with $K_2$-dependent binding. Because it lacks a $K_2$-domain, b-PA does not bind to either (DD)E or Fg, thereby explaining why b-PA is more fibrin-specific than t-PA.

Tissue-type plasminogen activator (t-PA)$^1$ is a naturally occurring serine protease that initiates fibrinolysis by converting plasminogen (Pg) to plasmin (1). Not only is fibrin the target for plasmin attack, but fibrin also stimulates t-PA-mediated Pg activation (2, 3). To accomplish this, fibrin acts as a template to which both t-PA and Pg bind (4). The fibrin-binding properties of t-PA have been ascribed to its finger and second kringle ($K_2$) domains (5, 6), although recent studies suggest that the protease domain also influences the interaction of t-PA with fibrin (4, 7, 8). The binding of both Glu- and Lys-plasminogen (Glu-Pg and Lys-Pg, respectively) to fibrin is entirely kringle-mediated, with Lys-Pg having higher affinity for fibrin than Glu-Pg (9).

As a functional consequence of t-PA interaction with fibrin, the catalytic efficiency of t-PA-mediated Pg activation is 2–3 orders in magnitude higher in the presence of fibrin than in its absence (3, 10). In contrast to fibrin, fibrinogen (Fg) stimulates Pg activation by t-PA only 25-fold (3, 10). Based on these considerations, t-PA is designated a fibrin-specific plasminogen activator (11). Despite this designation, t-PA causes systemic plasminemia and fibrinogenolysis when given to patients (12).

In recent studies, we have demonstrated that t-PA causes systemic plasminemia, because, like intact fibrin, soluble fibrin degradation products stimulate t-PA-mediated Pg activation (13). Furthermore, we have identified the (DD)E complex as the fibrin derivative primarily responsible for this effect (14) and have shown that the stimulatory activity of (DD)E is similar to that of fibrin. (DD)E, a complex of d-dimer noncovalently bound to fragment E, is the major degradation product of cross-linked fibrin (15). As a potent stimulator of t-PA-mediated activation of Pg, (DD)E generated during thrombus dissolution has the potential to induce systemic plasminemia (12, 15).

The limited fibrin specificity of t-PA has prompted the development of plasminogen activators with greater selectivity for fibrin (16). One such agent is the plasminogen activator isolated from the saliva of vampire bats (Desmodus rotundus) (17). Full-length vampire bat salivary plasminogen activator (designated DSPA$_1$) has over 72% amino acid sequence identity to t-PA (18). The major structural difference is that vampire bat plasminogen activator (b-PA) contains only one kringle domain, whereas t-PA has two. The single kringle domain of b-PA more closely resembles the first kringle domain of t-PA in that it lacks a lysine-binding site (18, 19).

Although fibrin stimulates Pg activation by b-PA to the same extent as t-PA (10), b-PA causes less $\alpha_2$-antiplasmin and Fg consumption than t-PA in experimental animals when the two agents are used in concentrations that produce equivalent thrombolysis (20–23). This has been attributed to the fact that Fg potentiates Pg activation by t-PA more than b-PA (10, 24–26). Because our studies demonstrated that (DD)E compromises the fibrin specificity of t-PA, we examined the possibility...

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that the greater fibrin-specificity of b-PA over t-PA reflects less (DD/E)-mediated stimulation of Pг activation by b-PA relative to t-PA. Herein, we demonstrate that (DD/E) and fibrinogen stimulate plasmin formation by t-PA to a greater extent than b-PA. To explore the possibility that differences in potentiation reflect differences in binding parameters, we measured the affinities of t-PA, b-PA, Glu-Pг, and Lys-Pг to (DD/E) as well as to fibrin and Fг. Binding was quantified in the absence and presence of the lysine analogue e-amino-n-caproic acid (EACA) to identify kringle-dependent interactions.

**EXPERIMENTAL PROCEDURES**

**Materials**

Plasminogen Activators—Wild-type recombinant t-PA was kindly provided by Dr. B. Keyt (Genentech Inc., S. San Francisco, CA), and recombinant b-PA (DSPAm), was a generous gift from Dr. W. Witt (Schering AG, Berlin, Germany). t-PA and b-PA were found to be 93 and 100% single chain, respectively, when analyzed by SDS-polyacrylamide gel electrophoresis (27) on 4–15% gels (Ready-Gel; Bio-Rad, Mississauga, Canada), as determined by laser densitometry (Ultrorcan XL, LKB-Pharmacia, Baie d’Urfe, Canada). The chromogenic substrate used in Pg activation studies was the plasmin-directed substrate S-2251 (D-valyl-leucyl-lysine chloromethyl ketone, Calbiochem) at 22 °C (28). The residual activity of the active site-blocked plasminogen activators was evaluated by measuring their ability to hydrolyze the chromogenic substrate N-methylsulfonyl-n-Phe-Ala-Gly-Arg-4-nitroanilide acetate (Chromozyme t-PA; Boehringer Mannheim, Laval, Canada). t-PA activity was abolished after a 1-h incubation with dansyl-glutamyl-glutamyl-arginine chloromethyl ketone, whereas a 3-h incubation was needed to block b-PA activity. Both enzymes were then dialyzed against the pyrophosphate-containing buffer overnight at 4 °C. The protein concentrations were determined by measuring absorbance at 280 and 320 nm. Absorbance at 335 nm was used to distinguish danyl group absorbance from light scattering, as described previously (29). Based on calculations of protein concentration, 90–95% of the plasminogen activators were recovered after dialysis against pyrophosphate buffer. Active site-blocked, unlabeled derivatives of t-PA or b-PA were prepared by the same procedure, except n-prolyl-arginyl-chloromethyl ketone (PPACK, Calbiochem) was used in place of dansyl-glutamyl-glutamyl-arginine chloromethyl ketone. Under these conditions, t-PA activity was abolished after a 30-min incubation with PPACK, whereas a 2-h incubation was needed to block b-PA activity. Immediately prior to use, a 1 ml volume of the plasminogen activator was dialyzed against 2 liters of 0.02 M Tris-HCl, 0.15 M NaCl, 0.1% Tween 20, and 6 M guanidine-HCl (GHS) for 10 min at 22 °C. Plasminogen—Native Glu-Pг was isolated from freshly frozen plasma by lysine-Sepharose affinity chromatography as described previously (31) but in the absence of aprotonin. Subsequently, the column was washed extensively with 0.1 M sodium phosphate, pH 8.0, followed by 20 ml Tris-Ci, pH 8.0. Adsorbed Pg was eluted with 10 ml EACA, 20 ml Tris-Ci, pH 5.0, directly onto a DEAE-Fast Flow column (1 × 20 cm). The DEAE column was washed with 20 ml Tris-Ci, pH 8.0, to remove the EACA, and Glu-Pг was then eluted with a 0–200 ml linear NaCl gradient in TBS, pH 7.4. Glu-Pг was concentrated by ammonium sulfate precipitation with subsequent solubilization and dialysis against TBS, pH 7.4. As determined by urea/acetic acid polyacrylamide gel electrophoresis (32), isolated Glu-Pг was free of Lys-Pг and contained no plasmin chromogenic activity using S-2251. Glu-Pг concentrations were calculated by measuring absorbance at 280 nm and using a molecular weight of 90,000 and ε\text{280} = 16.1 (31). Lys-Pг was purchased from Enzyme Research Laboratories.

**Isolation of (DD/E)**—The soluble fibrin fragment, (DD/E), was prepared by plasmin-mediated lysis of a cross-linked fibrin clot. Briefly, a 12-ml solution of Fг (8.5 mg/ml) in 0.02 M Tris-HCi, 0.15 M NaCl, pH 7.4, was exposed to 10 mg/ml thrombin-Sepharose manufacturer (Enzyme Research Laboratories, Inc., Palo Alto, CA) equilibrated with two model 126 solvent delivery systems and a model 506 automatic injector. The presence of protein was determined with a model 167 variable wavelength absorbance detector set at 280 nm. Peak protein-containing fractions were pooled and subjected to polyacrylamide gel electrophoresis on 4–15% nondenaturing gels. (DD/E)-containing fractions were identified based on their apparent molecular weight and by immunoblot analysis using antibodies against t-dimer and fragment E (14). The (DD/E) concentrations were calculated by measuring absorbance at 280 and 320 nm using ε\text{280} = 16.0. When (DD/E) was incubated with 10 mM H-Gly-Pro-Arg-Pro-OH (Calbiochem) prior to non-denaturing polyacrylamide gel electrophoresis analysis, two lower molecular weight bands appeared, corresponding to t-dimer and fragment E, respectively.

**Methods**

**(DD/E) or Fg Stimulation of Pг Activation**—The effect of (DD/E) or Fг on t-PA- and b-PA-mediated Pг activation was determined by comparing plasmin generation in the absence of these cofactors with that in the presence of 2 mM S-2251 and 1 mM EACA or 5 mM b-PA were added to wells of a 96-well microtiter plate containing 0.4 µg Glu-Pг in the absence or presence of either (DD/E) or Fг. Plasmin generation was monitored by measuring absorbance at 405 nm at 30-s intervals for 20–30 min using a Spectramax microplate spectrophotometer (Molecular Devices, Menlo Park, CA). Point-to-point slopes were determined and converted to plasmin concentration based on the specific activity of 20 pM, with a slope of S-2251 (0.017 OD s\text{5} = 1.1), which was determined in a separate experiment. Plots of plasmin concentration versus time were used to calculate the rate of plasmin formation.

**Fluorescence and Light Scattering Measurements**—All fluorescence and light scattering intensities were measured in a LS50B luminescence spectrometer (Perkin-Elmer, Ectobicoke, Canada) using a cuvette thermostatted at 22 °C. Fluorescence measurements were performed in a 1 ml quartz microcuvette, and right angle light scattering measurements were made in a 3 ml quartz cuvette with stirring. To measure the fluorescence of individual samples, three fluorescence intensity readings, each recorded over a 3-s integration time, were averaged. Scattering intensities were continuously monitored in time drive with the interval time set at 1 or 2 s and the response time at 2 or 3 s. Intensity values were determined by averaging scattering intensities observed over a period of at least 100 s. Thus, each scattering intensity value represents the mean of 50–100 individual readings.

**Lysine Affinity of t-PA and b-PA**—To compare their affinities for lysine, fluorescently labeled t-PA and b-PA were subjected to affinity chromatography on a lysine-Sepharose column. The fluorescence intensity of a 500 µl sample of dEGR-t-PA or dEGR-b-PA was quantified with excitation light (λ\text{exc}) of 280 nm and emission wavelength set to 350 nm, respectively, a 515-mm cut-off filter, and excitation and emission slit widths both set to 5 nm. The plasminogen activator was then passed over a lysine-Sepharose column (1 × 5 cm), and, after washing, bound material was eluted with 40 mM EACA, and 500 µl fractions were collected. Fractions containing dansyl fluorescence were pooled, and total I\text{EACA} was determined. The amount of plasminogen activator that
bound was then calculated by expressing the $I_{530}$ of the eluted material as a percentage of the total $I_{530}$ loaded onto the column.

As another method of comparing the relative affinities of t-PA and b-PA for lysine, changes in tryptophan fluorescence were monitored as each plasminogen activator was titrated with the lysine analogue, EACA. Solutions of 20–40 µM of EACA were made to a 2-ml solution containing 0.3 µM PPACK-t-PA or PPACK-b-PA. Tryptophan fluorescence was monitored with $\lambda_{ex}=280$ nm, $\lambda_{em}=340$ nm, a 290-nm cut-off filter, and slit widths set to 5 nm.

**Binding to Fibrin**—The binding of dEGR-t-PA or dEGR-b-PA to fibrin was determined by adding increasing concentrations of plasminogen activators to fibrin in the presence of microcentrifuge tubes (Sorvall catalog number 72.702) containing fixed amounts of Fg in TBS (29). A 10-µl aliquot of thrombin (final concentration, 10 nM) was then added to induce clotting. The final reaction volume was 200 µl. After incubation at 22 °C for 1 h, the clots were vortexed and centrifuged at 12,000 x g for 2.5 min to compact the fibrin into the 10-µl tip of the microtube. The fluorescence intensity of 150 µl of clot supernatant in 350 µl of Tris buffer was measured with $\lambda_{ex}=280$ nm, $\lambda_{em}=530$ nm, a 515-nm cut-off filter, and 15-nm slit widths. A parallel titration was done in the absence of thrombin to establish a standard curve for each ligand. The binding of Lys-Pg and Glu-Pg to fibrin was determined using the same procedure, except unbound Pg was quantified by measuring tryptophan fluorescence of the unlabelled material, and the standard curve of Pg concentration was established in the absence of fibrin. Because the affinity of Pg for fibrin is lower than that of the plasminogen activators, higher Pg concentrations were used in these experiments, thereby obviating the need to use fluorescently labeled Pg. The conditions for measuring tryptophan fluorescence include $\lambda_{ex}=280$ nm, $\lambda_{em}=340$ nm, a 290-nm cut-off filter, and slit widths set to 2.5 nm.

The effect of EACA on the binding of dEGR-t-PA, dEGR-b-PA, Glu-Pg, or Lys-Pg to fibrin was determined by repeating the same titrations in the presence of 20 mM EACA. In addition, clots formed by incubating 2 µM Fg with 10 nM thrombin in the presence of 0.8 µM dEGR-t-PA, dEGR-b-PA, Glu-Pg, or Lys-Pg were titrated with EACA (in concentrations ranging from 0 to 20 mM), and the amount of ligand displaced was determined by measuring the concentration of unbound protein in the clot supernatant as described above.

To determine whether t-PA and b-PA compete for the same fibrin binding sites, various concentrations of unlabelled, active site-blocked b-PA or t-PA, with or without 20 mM EACA, were added to a series of microcentrifuge tubes charged with 2 µM Fg and 0.8 µM dEGR-t-PA or dEGR-b-PA. Thrombin (10 nM) was added, and after incubation for 60 min at 22 °C, fibrin was pelleted by centrifugation. The amount of unbound fluorescently labeled enzyme in the supernatant was then determined by calculating the difference between the total and unbound protein concentrations. These values were divided by the Fg concentration to determine the number of moles of dEGR-t-PA, dEGR-b-PA, Lys-Pg, or Glu-Pg bound per mole of fibrin (ν). For each point in the titration, these values were then plotted against the concentration of unbound protein. Scatchard plots also were constructed, and if these appeared linear, reflecting a single class of binding sites, the binding isotherm was analyzed by nonlinear regression analysis (Table Curve, Jandel Scientific, San Rafael, CA) of the relationship,

$$I = I_0 - \frac{\alpha}{2} \left( 1 + \frac{K_d + L}{n \cdot P} \right) - \left( \frac{1}{1 + \frac{K_d + L}{n \cdot P}} - \frac{L}{n \cdot P} \right)$$

where $L$ represents the concentration of unbound protein, $n$ is the stoichiometry, and $K_d$ is the dissociation constant. All binding isotherms were linear, except for that corresponding to the binding of dEGR-t-PA to fibrin in the absence of EACA, which curved downward. These data were best fit to a two-site model by nonlinear regression analysis (Table Curve, Jandel Scientific) according to the following expression:

$$I = I_0 - \frac{\alpha}{2} \left( 1 + \frac{K_d + L}{n \cdot P} \right) - \left( \frac{1}{1 + \frac{K_d + L}{n \cdot P}} - \frac{L}{n \cdot P} \right)$$

For analysis of solution phase binding of PPACK-t-PA, PPACK-b-PA, Lys-Pg, or Glu-Pg to Fg or (DD)E, the emission intensity (I) of the incident beam after each addition of ligand was corrected for changes due to dilution and ligand scattering. Corrected values were compared with the emission intensity before the addition of ligand (I₀), and these data, together with the total ligand concentration (Lₙ), were fitted by nonlinear regression analysis (Table Curve, Jandel Scientific) to the equation,

$$I = I_0 - \frac{\alpha}{2} \left( 1 + \frac{K_d + L}{n \cdot P} \right) - \left( \frac{1}{1 + \frac{K_d + L}{n \cdot P}} - \frac{L}{n \cdot P} \right)$$

where $L$ is the concentration of ligand added, $P_z$ is the concentration of target protein, and $\alpha$ is the maximum change in emission intensity. Using $\alpha$ as a measure of 100% bound ligand, the amount of unbound ligand was determined after each addition of ligand, and Scatchard analysis was used to confirm the binding parameters derived from Equation 3.

### RESULTS

**Influence of (DD)E or Fg on t-PA- and b-PA-mediated Activation of Pg**—To compare the effect of (DD)E and Fg on t-PA and b-PA-mediated Pg activation, 0.4 µM Glu-Pg was incubated with 1 nM t-PA or 5 nM b-PA in the absence or presence of various concentrations of (DD)E or Fg for 10 min at 37 °C, and the rate of plasmin formation was monitored (Fig. 1). In the presence of (DD)E, the rate of t-PA-mediated plasmin formation is increased a maximum of 244-fold (from $2.5 \times 10^{-4}$ s⁻¹ to $6.1 \times 10^{-4}$ s⁻¹). Fg increases the rate of t-PA-mediated plasmin formation 25-fold (from $2.5 \times 10^{-4}$ s⁻¹ to $6.2 \times 10^{-4}$ s⁻¹). In contrast, b-PA-mediated plasmin formation is increased only 20-fold with (DD)E (from $1.3 \times 10^{-4}$ s⁻¹ to $2.6 \times 10^{-4}$ s⁻¹) and 8-fold with Fg (from $1.3 \times 10^{-4}$ s⁻¹ to $1.0 \times 10^{-4}$ s⁻¹). Thus, (DD)E and, to a lesser extent, Fg are more potent stimulators of Pg activation by t-PA than by b-PA.

**Affinities of t-PA and b-PA for EACA**—To begin to explore why (DD)E and Fg are less potent stimulators of Pg activation by b-PA than t-PA, we first compared the lysine-binding properties of the plasminogen activators because the affinity of t-PA for lysine determines, at least in part, its affinity for fibrin (33). To compare their relative affinities for lysine, aliquots containing 0.32 mg/ml dEGR-t-PA or 0.2 mg/ml dEGR-b-PA were subjected to affinity chromatography on a lysine-Sepharose column. Plasminogen activator that bound to the lysine-Sepharose was eluted with 40 mM EACA. Whereas 90% of the t-PA bound to lysine-Sepharose, only 3% of the b-PA bound. The affinities of t-PA and b-PA for the lysine analogue, EACA, were compared by quantifying changes in tryptophan fluorescence when ligand was titrated with EACA. Titrations of active site-blocked t-PA with EACA results in a concentration-dependent and saturable increase in its tryptophan fluorescence (Fig. 2). Based on analysis of these data, EACA binds to t-PA with a $K_d = 214$ µM and $n = 0.91$ EACAs/t-PA. In contrast, there is no change in tryptophan fluorescence when active site-blocked b-PA is titrated with EACA (Fig. 2). This finding is
consistent with our observation that unlike t-PA, b-PA does not bind lysine-Sepharose.

**Interactions of t-PA, b-PA, Glu-Pg, and Lys-Pg with Fibrin**—Since fibrin has been reported to stimulate Pg activation by t-PA and b-PA to a similar extent (10), we quantified the binding of the plasminogen activators and Pg to fibrin. The Scatchard plot for the binding of dEGR-t-PA is nonlinear (Fig. 3A), indicating heterogeneous binding sites or negative cooperativity (34). A plot of the double reciprocal (1/B versus B) yields a straight line, whereas a plot of 1/F versus 1/F yields a sigmoidal curve, where B and F represent the amount of bound and free t-PA, respectively (data not shown). These findings are indicative of binding site heterogeneity (34). Accordingly, the data were fit to a two-site model (Equation 2) by nonlinear regression analysis, and the resulting binding parameters are $K_{d1} = 0.053 \mu M$ ($n_1 = 0.25$ t-PA/fibrin) and $K_{d2} = 2.6 \mu M$ ($n_2 = 1.4$ t-PA/fibrin). When fibrin is titrated with dEGR-t-PA in the presence of 20 mM EACA (Fig. 3B), Scatchard analysis yields a straight line, indicating a single class of binding sites ($K_{d} = 0.47 \mu M$ ($n = 0.25$ t-PA/fibrin)) that more closely resembles the high affinity interaction of t-PA with fibrin seen in the absence of EACA. Like other investigators (29), we interpret this as further evidence that t-PA binds to fibrin through two classes of sites: a high affinity, finger-independent site and a low affinity, kringle-dependent site. In contrast, b-PA binds to fibrin through a single class of high affinity, kringle-independent sites.

The ability of t-PA and b-PA to compete for the same fibrin binding sites was assessed by titrating fibrin clots containing fixed amounts of either dEGR-t-PA or dEGR-b-PA with increasing concentrations of PPACK-t-PA or PPACK-b-PA, respectively. As illustrated in Fig. 4, t-PA competes for virtually all of the b-PA binding sites on fibrin. In contrast, b-PA is only able to compete for about 50% of the t-PA binding to fibrin. However, the combination of excess b-PA and EACA competes for almost all of the t-PA binding sites on fibrin. These data support the concept that t-PA and b-PA share a high affinity, finger-independent site and a low affinity, kringle-dependent site. The Scatchard plots for the binding of Glu-Pg and Lys-Pg to fibrin are linear (data not shown), indicating that both Glu-Pg and Lys-Pg interact with fibrin through a single class of binding sites. Glu-Pg binds to fibrin with a $K_{d} = 13 \mu M$ and $n = 0.72$ Glu-Pg/fibrin, whereas Lys-Pg binds to fibrin with a $K_{d} = 0.13 \mu M$ and $n = 0.71$ Lys-Pg/fibrin. No binding of either Glu-Pg or Lys-Pg to fibrin was detected when the experiments were repeated in the presence of 20 mM EACA, indicating that their interaction with fibrin is entirely kringle-dependent.

**Interactions of t-PA, b-PA Glu-Pg, and Lys-Pg with Fg**—The relative scatter plots for the interactions of t-PA and b-PA with Fg are shown in Fig. 5. Under the conditions outlined under “Methods” ($\lambda_{ex}, \lambda_{em} = 400$ nm, slit widths = 12 nm), the
t-PA and b-PA Binding to Fibrinogen, Fibrin, and (DD)E

scattering intensity of 0.1 μM PPACK-t-PA is 1.0 \( \langle I_0 \rangle \). At saturating levels of Fg, the maximum relative scattering intensity \( \langle III_s \rangle \) is 42, a value in good agreement with a calculated maximum relative scattering intensity of 39 if the stoichiometry is 1:1 (35). The solid line represents the fit of the data to Equation 3 by nonlinear regression analysis. Based on this analysis, t-PA binds to Fg with a \( K_d = 0.76 \) μM and \( n = 0.59 \) t-PA/Fg. When t-PA is titrated with Fg in the presence of 20 mM EACA, there is no increase in the scattering intensity relative to Fg alone. Thus, the binding of t-PA to Fg is entirely kringle-dependent. The scattering intensity of 0.1 μM PPACK-b-PA is 0.8, and the relative scattering intensity does not change when Fg is added. Therefore, in contrast to the findings with t-PA, b-PA does not interact with Fg.

The interactions of Glu-Pg and Lys-Pg with Fg are shown in Fig. 6. Relative scattering intensity increases when Lys-Pg is titrated with Fg. \( I_o \) for 0.3 μM Lys-Pg (\( \lambda_{wv} = 440 \) nm, width = 12 nm) is 2.7. If one Lys-Pg molecule binds to each Fg molecule, the theoretical \( III_s \) at saturating Fg concentrations is 24. Titrations of Lys-Pg with Fg reach a maximum \( III_s \) value of 19, a value compatible with 1:1 stoichiometry. Analysis of the binding data by nonlinear regression analysis indicates that Lys-Pg interacts with Fg with a \( K_d = 0.23 \) μM and \( n = 0.64 \) Lys-Pg/Fg. The interaction of Lys-Pg with Fg is kringle-dependent, because it is completely abrogated by EACA (data not shown). In contrast to Lys-Pg, there is almost no increase in the scattering intensity over base line when Glu-Pg is titrated with Fg.
Fig. 6. The binding of Glu-Pg or Lys-Pg to Fg. 0.3 \( \mu \text{M} \) Lys-Pg (○) or Glu-Pg (□) was titrated with Fg at the concentrations indicated, and light scattering was monitored at 440 nm (I). Since Glu-Pg was titrated with high concentrations of Fg, excitation and emission slit widths were both narrowed to 8 nm; in contrast, interactions with Lys-Pg were monitored with slit widths of 12 nm. Under these conditions, \( I_o \) values for Glu- and Lys-Pg were 1.6 and 2.7, respectively. Increases in the scattering intensities when Lys-Pg is titrated with Fg indicate saturable binding of Lys-Pg to Fg with a \( K_d \) of 0.23 \( \mu \text{M} \) and \( n = 0.64 \) Lys-Pg/Fg. The solid line represents the best fit to Equation 3. In contrast, when compared with the scattering caused by Glu-Pg alone, Fg does not increase the relative scattering intensity, indicating that Glu-Pg does not bind to Fg.

Interaction of t-PA, b-PA, Glu-Pg, and Lys-Pg with (DD)E–
The interactions of t-PA and b-PA with (DD)E are illustrated in Fig. 7. Titrations with (DD)E were performed under the same conditions as titrations with Fg titrations, and \( I_o \) values for the plasminogen activators were identical to those previously determined (1.0 for t-PA and 0.8 for b-PA). When t-PA is titrated with (DD)E, the maximum \( I/I_o \) observed is 22; a value identical to theoretical \( I/I_o \) for a 1:1 t-PA/(DD)E interaction. Based on analysis of the binding data, t-PA binds to (DD)E with a \( K_d \) of 0.023 \( \mu \text{M} \) and \( n = 0.8 \) t-PA/(DD)E. No increase in scattering intensity was detected when t-PA was titrated with (DD)E in the presence of 20 mM EACA, indicating that the interaction of t-PA with (DD)E is entirely kringile-dependent. In contrast to the findings with t-PA, no increase in scattering occurred when b-PA was titrated with (DD)E, indicating that b-PA does not interact with (DD)E.

The interactions of Glu-Pg and Lys-Pg with (DD)E are illustrated in Fig. 8, A and B, respectively. With \( \lambda_{\text{ex}} \) and \( \lambda_{\text{em}} \) = 440 nm and slit widths set to 12 nm, 0.1 \( \mu \text{M} \) (DD)E has a scattering intensity of 7.4. Titration of (DD)E with Glu-Pg results in a maximum \( I/I_o \) of 2.0; a value similar to a predicted \( I/I_o \) of 1.9 for a 1:1 Glu-Pg to (DD)E interaction. Analysis of the binding curve indicates that Glu-Pg binds to (DD)E with a \( K_d \) = 5.4 \( \mu \text{M} \) and \( n = 1.2 \) Glu-Pg/(DD)E. Lys-Pg titration of (DD)E results in a maximum \( I/I_o \) of 1.8, a value identical to that predicted by 1:1 stoichiometry. Nonlinear regression analysis of the data indicates saturable binding of Lys-Pg to (DD)E with a \( K_d \) = 0.03 \( \mu \text{M} \) and \( n = 1.1 \) Lys-Pg/(DD)E. The interactions of both Glu-Pg and Lys-Pg with (DD)E are completely inhibited by 20 mM EACA, indicating that their binding is kringle-dependent (data not shown).

DISCUSSION

Previously, we demonstrated that t-PA causes systemic plasminemia and subsequent fibrinogenolysis because (DD)E generated during the thrombolytic process stimulates t-PA-mediated Pg activation (13, 14). We and others (20–23) have shown that t-PA produces more Fg consumption than b-PA in experimental animals. Fig. 1 provides a plausible explanation for the greater fibrin specificity of b-PA over t-PA. Thus, (DD)E and Fg are less potent stimulators of Pg activation by b-PA than t-PA. To explore the possibility that this reflects differences in the affinities of the plasminogen activators for (DD)E and Fg, we
compared the binding interactions of t-PA and b-PA with (DD)E and Fg. Since efficient Fg activation requires the formation of a ternary enzyme-cofactor-substrate complex (4), the affinity of both native Glu-Pg and plasmin-derived Lys-Pg for (DD)E and Fg also were quantified. For comparative purposes, we also measured the affinities of the activators and substrates for fibrin.

The binding parameters for the interactions of the plasminogen activators (t-PA and b-PA) and substrates (Glu-Pg and Lys-Pg) with the cofactors (Fg, fibrin, and (DD)E) are listed in Table I, and the structural domains responsible for these interactions are summarized in Table II. Interactions of t-PA and b-PA with (DD)E and Fg elucidate the principal differences between the two activators. t-PA binds to both Fg and (DD)E via its K2 domain. In contrast, b-PA does not bind Fg or (DD)E because it lacks a functional lysine-binding site. Thus, the presence of a lysine-binding kringle, in addition to its finger domain to fibrin binding sites.

It is evident from Table I that kringle-dependent affinities of t-PA and Pg vary depending on the fibrin(ogen) derivative. Kringle-dependent interactions with Fg and fibrin are weak, whereas (DD)E binding is much stronger. The affinity of the site on (DD)E that binds to fibrin is 112-fold higher than its counterpart on fibrin. Consequently, t-PA binds to (DD)E via its K2 domain with an affinity similar to that of its K2 domain on fibrin. The reduced affinity attributed to finger-mediated binding may reflect the conformational changes in t-PA that occur when its K2 domain is occupied by EACA, a concept supported by our observation that EACA induces changes in the tryptophan fluorescence of t-PA (Fig. 2), and the report that the fluorescence of eosin-t-PA binding to fibrin in a concentration-dependent fashion. In contrast, b-PA partially inhibits t-PA binding by competing only with fibrin, as evidenced by competition studies (not shown). Plasmin-mediated exposure of new carboxyl-terminal lysine residues may explain why the affinities of Glu-Pg and Lys-Pg for (DD)E are higher than those for fibrin. In support of this concept, fibrin exposed to limited plasmin digestion has been reported to exhibit higher affinity for both forms of Pg (39).

Three lines of evidence indicate that (DD)E and Fg serve as templates onto which the enzyme and substrate assemble. First, near unity stoichiometries for the interactions of t-PA, Glu-, and Lys-Pg with (DD)E and Fg were obtained by nonlinear regression analysis of the binding data. Second, as an independent assessment of stoichiometry, increases in right angle light scattering intensities were compared with those predicted by 1:1 interactions, based on the observation that right angle scattering intensity is related to the square of the molecular mass (35). In all cases, the observed increase was similar to that predicted for simple binary interactions. Third, t-PA and Lys-Pg bind to distinct sites on (DD)E and Fg because high concentrations of Lys-Pg have no effect on t-PA binding to these derivatives (not shown), a finding similar to that observed with intact fibrin (29). Taken together, these data suggest that the cofactor serves as a template onto which one enzyme and one substrate molecule assemble. This hypothesis is supported by the recent observation that t-PA-mediated

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**Table I**

| Ligand | Kd (μM) | n | Kd (μM) | n | Kd (μM) | n |
|--------|---------|---|---------|---|---------|---|
| t-PA   | 0.053 ± 0.019 | 0.25 ± 0.08 | 0.023 ± 0.008 | 0.80 ± 0.16 | 0.76 ± 0.04 | 0.59 ± 0.28 |
| b-PA   | 0.15 ± 0.04  | 1.0 ± 0.2  | NB*      | NB      | NB      | NB |
| Glu-Pg | 13 ± 4     | 0.72 ± 0.21 | 5.4 ± 1.1 | 1.2 ± 0.2 | NB      | NB |
| Lys-Pg | 0.13 ± 0.03 | 0.71 ± 0.21 | 0.030 ± 0.005 | 1.1 ± 0.2 | 0.23 ± 0.05 | 0.64 ± 0.16 |

* NB, no binding detected.

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**Table II**

| Domains responsible for the binding of t-PA, b-PA, and Pg to Fg, fibrin, and (DD)E |
|-----------------------------|---------|---------|---------|---------|---------|---------|
| Ligand                      | t-PA    | b-PA    | Pg      |
| Fibrin                      | Finger  | Kringle | NB*     | Kringle |
| (DD)E                       | Finger + kringle | Kringle | Kringle |
| Finger                      | NB      | Kringle |

* NB, no binding detected.
stimulation of Pg activation by fibrin requires binding of both t-PA and Pg to fibrin (4).

Our results suggest that the affinity of the plasminogen activator for fibrin(ogen) derivatives determines the stimulatory activity of the cofactor. Thus, we have shown that high affinity plasminogen activator-cofactor interactions (b-PA/fibrin, t-PA/fibrin, and t-PA/(DD)E) result in marked stimulation of Pg activation, whereas weaker interactions (t-PA/Fg, b-PA/Fg, and b-PA/(DD)E) elicit modest to poor stimulation. A correlation between a cofactor’s affinity for t-PA and its ability to stimulate Pg activation is supported by kinetic models of plasminogen activation (2) and why Fg is a weaker stimulator of Pg activation than fibrin. Fibrinolysis of Pg corresponds with their ability to degrade plasma clots (41, 42).

Our studies give considerable insight into the biochemical differences between t-PA and b-PA and provide direction for further study. Although t-PA-mediated Pg activation is stimulated in the presence of fibrin, t-PA has only modest fibrin specificity, because it binds to (DD)E and fibrin with equally high affinity and displays moderate affinity for Fg. These data explain why (DD)E is almost as potent as fibrin at stimulating Pg activation and why Fg is a weaker stimulator of Pg activation. In contrast, b-PA is more fibrin-specific than t-PA (20–22), because it only has affinity for fibrin. Since it is the K2 domain of t-PA that limits its fibrin specificity by mediating t-PA-mediated Pg activation (2) and why Fg is a weaker stimulator of Pg activation, b-PA/(DD)E and Fg, our studies also suggest that the K2 domain would render t-PA as fibrin-specific as b-PA.

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