

# Kinetics of Activated Thrombin-activatable Fibrinolysis Inhibitor (TAFIa)-catalyzed Cleavage of C-terminal Lysine Residues of Fibrin Degradation Products and Removal of Plasminogen-binding Sites\*<sup>§</sup>

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Partial digestion of fibrin by plasmin exposes C-terminal lysine residues, which comprise new binding sites for both plasminogen and tissue-type plasminogen activator (tPA). This binding increases the catalytic efficiency of plasminogen activation by 3000-fold compared with tPA alone. The activated thrombin-activatable fibrinolysis inhibitor (TAFIa) attenuates fibrinolysis by removing these residues, which causes a 97% reduction in tPA catalytic efficiency. The aim of this study was to determine the kinetics of TAFIa-catalyzed lysine cleavage from fibrin degradation products and the kinetics of loss of plasminogen-binding sites. We show that the  $k_{cat}$  and  $K_m$  of Glu<sup>1</sup>-plasminogen (Glu-Pg)-binding site removal are 2.34 s<sup>-1</sup> and 142.6 nM, respectively, implying a catalytic efficiency of 16.21 μM<sup>-1</sup> s<sup>-1</sup>. The corresponding values of Lys<sup>77</sup>/Lys<sup>78</sup>-plasminogen (Lys-Pg)-binding site removal are 0.89 s<sup>-1</sup> and 96 nM implying a catalytic efficiency of 9.23 μM<sup>-1</sup> s<sup>-1</sup>. These catalytic efficiencies of plasminogen-binding site removal by TAFIa are the highest of any TAFIa-catalyzed reaction with a biological substrate reported to date and suggest that plasmin-modified fibrin is a primary physiological substrate for TAFIa. We also show that the catalytic efficiency of cleavage of all C-terminal lysine residues, whether they are involved in plasminogen binding or not, is 1.10 μM<sup>-1</sup> s<sup>-1</sup>. Interestingly, this value increases to 3.85 μM<sup>-1</sup> s<sup>-1</sup> in the presence of Glu-Pg. These changes are due to a decrease in  $K_m$ . This suggests that an interaction between TAFIa and plasminogen comprises a component of the reaction mechanism, the plausibility of which was established by showing that TAFIa binds both Glu-Pg and Lys-Pg.

The fibrinolytic system is initiated upon release of the tissue-type plasminogen activator from endothelial cells at the site of vascular injury, which is usually where an insoluble fibrin clot is formed (1). Initially, native or Glu<sup>1</sup>-plasminogen (Glu-Pg)<sup>3</sup>

weakly binds intact fibrin and is subsequently activated to plasmin by the tissue- or urokinase-type plasminogen activator. Plasmin has two major functions in fibrinolysis. It directly degrades fibrin by cleaving after specific lysine and arginine residues, thus creating soluble fibrin degradation products (FDPs) and solubilizing the clot. Plasmin also enhances fibrinolysis by converting Glu-Pg to Lys<sup>77</sup>/Lys<sup>78</sup>-plasminogen (Lys-Pg) (2). Both Glu-Pg and Lys-Pg bind intact fibrin but with very different affinities. Lys-Pg binds intact fibrin with a  $K_d$  of 0.15 μM, and Glu-Pg binds with a  $K_d$  of 30 μM (3).

The activated thrombin-activatable fibrinolysis inhibitor (TAFIa; also known as carboxypeptidase U) is central in regulating plasminogen activation and therefore fibrinolysis. TAFIa is the active form of the plasma zymogen TAFI, which is activated by thrombin, plasmin, or the thrombin-thrombomodulin complex (4–6). TAFIa is a plasma carboxypeptidase B-like enzyme that removes C-terminal lysine and arginine residues from plasmin-modified fibrin to suppress plasminogen activation and clot dissolution (7). Because thrombin clots fibrinogen and then attenuates fibrin degradation by activating TAFI, TAFI represents a link between coagulation and fibrinolysis (6). TAFIa regulates tissue-type plasminogen activator-dependent fibrinolysis half-maximally at 1 nM, which is only 1–2% of the plasma TAFI zymogen pool (6). This suggests that modest activation of TAFI can have a profound effect on inhibition of fibrinolysis. TAFIa has no known physiological inhibitor but is regulated by its own instability (8–10). It has been suggested that TAFIa inhibits fibrinolysis by a threshold mechanism (11, 12). In this mechanism, TAFIa completely inhibits fibrinolysis when its concentration is above the critical threshold; however, as TAFIa decays, it falls below the threshold, and fibrinolysis continues.

A main function of TAFIa in regulating fibrinolysis is to remove plasminogen-binding sites from plasmin-modified fibrin, thus attenuating plasminogen activation and fibrinolysis. Recently, it was demonstrated that TAFI zymogen has carboxypeptidase activity toward synthetic fibrin peptides (13), which can be attributed to the positioning of the activation peptide over the active site (9). These synthetic fibrin peptides (1400 or 2600 Da) are quite small compared with the smallest FDP

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<sup>3</sup> The abbreviations used are: Glu-Pg, Glu<sup>1</sup>-plasminogen; FDP, fibrin degradation product; Lys-Pg, Lys<sup>77</sup>/Lys<sup>78</sup>-plasminogen; TAFIa, activated thrombin-

activatable fibrinolysis inhibitor; 5IAF, 5-iodoacetamidofluorescein; ε-ACA, ε-aminocaproic acid; SDH, saccharopine dehydrogenase; QSY, QSY<sup>®</sup> 9 C<sub>5</sub>-maleimide.

(DDE, 250,000 Da), and TAFI zymogen does not show carboxypeptidase activity toward FDPs (14). This discrepancy between fibrin peptides and FDPs as substrates for TAFI and TAFIa has prompted the development of a physiological relevant model substrate to aid in determination of the kinetics of TAFIa. The goal of this study was to determine the kinetics of the TAFIa-catalyzed release of lysine and loss of plasminogen-binding sites using well characterized, high molecular weight, soluble FDPs (15) as a physiologically relevant TAFIa substrate.

TAFIa has numerous physiologically significant substrates, one of which presumably is plasmin-modified fibrin. Studies in the TAFI knock-out mouse (reviewed by Morser *et al.* (16)) have not, however, disclosed unambiguously a role for TAFI in regulation of fibrinolysis *in vivo* in the mouse. As an indirect measure of the role of TAFIa in the regulation of fibrinolysis, as opposed to other physiological processes, the catalytic efficiency of cleavage of the various substrates associated with them would be useful. Thus, both to determine the kinetics of removal of lysine-binding sites of plasmin-modified fibrin and to compare them with the kinetics of cleavage of other substrates as measured by other investigators, the experiments reported here were undertaken.

## EXPERIMENTAL PROCEDURES

**Materials**—Fibrinogen was prepared as described previously (17) with one exception: the solution was made to 1.2% PEG 8000 instead of 2% PEG 8000 by the addition of 40% (w/v) PEG 8000 in water subsequent to  $\beta$ -alanine precipitation. This change in protocol allowed for a greater yield of fibrinogen. TAFI isolation and activation were essentially achieved as described (18). Because such low concentrations of TAFIa were used in experiments, TAFI was activated in the presence of 1 mg/ml bovine serum albumin to prevent nonspecific binding to plastics. Recombinant human plasminogen (S741C) and the fluorescein derivative (5-iodoacetamidofluorescein (5IAF)-Glu-Pg) were prepared as described by Horrevoets *et al.* (3). 5IAF-Lys-Pg was prepared by treating 5IAF-Glu-Pg with 200 nM plasmin in the presence of 5 mM  $\epsilon$ -aminocaproic acid ( $\epsilon$ -ACA) for 3 h at room temperature. Plasmin was subsequently removed using a 1-ml benzamide-Sepharose 6B column (GE Healthcare). Residual plasmin activity (<1% as determined using the chromogenic substrate S-2251 (Diapharma, West Chester, OH)) was inhibited with 10  $\mu$ M D-Val-Phe-Lys chloromethyl ketone (Calbiochem). Human Glu-Pg was isolated and Lys-Pg was prepared as described previously (18). Saccharopine dehydrogenase (SDH) was isolated as described previously (19). Solulin was a generous gift from Dr. Achim Schuttler (Paion, GmbH, Aachen, Germany). QSY® 9 C<sub>5</sub>-maleimide (QSY) and 5-iodoamidofluorescein were purchased from Invitrogen. Plasmin was purchased from Hematologic Technologies Inc. (Essex Junction, VT). The buffer used in all experiments was 20 mM HEPES and 200 mM NaCl (pH 7.4) unless otherwise noted. All other reagents were of analytical quality.

**QSY-FDP Production**—QSY-FDPs (FDPs covalently attached to the quencher QSY® 9 C<sub>5</sub>-maleimide) were prepared using methods modified from those described by Neill *et al.* (15) and Kim *et al.* (20). Briefly, 7.1 mg/ml fibrinogen was combined

with 5 nM thrombin, 2 mM CaCl<sub>2</sub>, and 40 nM plasmin (final concentrations in 7 ml) in HEPES-buffered saline (20 mM HEPES, 150 mM NaCl, pH 7.4), and coagulation and fibrinolysis were monitored by turbidity at 800 nm. Once the clot had fully lysed, thrombin and plasmin were inhibited with chloromethyl ketones (D-Phe-Pro-Arg chloromethyl ketone and D-Val-Phe-Lys chloromethyl ketone, respectively). The ionic strength was increased by the addition of solid NaCl to a final concentration of 0.5 M. The FDPs were gel-filtered using a Sephacryl 1000 column, and fractions were pooled according to the criteria described by Neill *et al.* (15). Subsequently, the FDPs were selectively reduced with 2-mercaptoethanol (50 mM final concentration) for 30 min at room temperature. 2-Mercaptoethanol was removed by dialyzing against 3  $\times$  4 liters of 20 mM HEPES and 500 mM NaCl (pH 7.4). Selectively reduced FDPs were incubated with a 30-fold excess of QSY for 1 h at room temperature. This step routinely causes some precipitation of the FDPs. Precipitate was removed by centrifugation (5000  $\times$  g), and excess QSY was removed from the sample by dialysis (3  $\times$  4 liters of 20 mM HEPES and 300 mM NaCl (pH 7.4)). QSY-FDPs were aliquoted and stored at  $-80^\circ\text{C}$  until required. QSY-FDPs are soluble, have an average molecular size of 2  $\times$  10<sup>6</sup> g/mol, and contain 7–10 QSY moieties per fragment X of fibrinogen. QSY-FDPs were characterized based on their ability to bind plasminogen and their ability to stimulate plasminogen activation. Both FDPs and QSY-FDPs yielded similar results (data not shown).

**Binding of 5IAF-Glu-Pg or 5IAF-Lys-Pg to QSY-FDPs**—QSY-FDPs (95  $\mu$ l, 0–2  $\mu$ M final concentration) were added to the wells of an opaque 96-well plate and monitored continually at 1-min intervals by fluorescence in a SpectraMax GEMINI-XS spectrofluorometer (Molecular Devices, Sunnyvale, CA) with emission and excitation wavelengths of 480 and 520 nm, respectively, employing a cutoff filter of 515 nm. After the signal stabilized, 5  $\mu$ l of 1  $\mu$ M 5IAF-Glu-Pg or 5IAF-Lys-Pg (50 nM final concentration) was added and mixed with the QSY-FDPs. Again, after the signal stabilized, 1  $\mu$ l of 1 M  $\epsilon$ -ACA was added to each well and mixed. The stable fluorescence signals obtained with only QSY-FDP ( $S_{\text{QSY-FDP}}$ ), after the addition of 5IAF-Pg ( $S_{\text{5IAF-Pg}}$ ), and after the addition of  $\epsilon$ -ACA ( $S_{\epsilon\text{-ACA}}$ ) were used to determine the  $K_d$  for the binding interaction between 5IAF-Pg and QSY-FDPs. Because the QSY-FDP absorbs significantly at the concentrations used and because of the spectral overlap between the QSY moiety and the fluorescein moiety of the labeled plasminogen, an internal filter effect is present, in addition to the quenching effect. The magnitude of the internal filter effect (FF) is calculated from the data according to Equation 1,

$$\text{FF} = \frac{S_{\epsilon\text{-ACA}} - S_{\text{QSY-FDP}}}{S_{\text{5IAF-Pg}_0}} \quad (\text{Eq. 1})$$

where  $S_{\epsilon\text{-ACA}}$  is the fluorescence in the presence of  $\epsilon$ -ACA and QSY-FDP,  $S_{\text{QSY-FDP}}$  is the blank in the absence of 5IAF-Pg, and  $S_{\text{5IAF-Pg}_0}$  is the fluorescence signal in the presence of  $\epsilon$ -ACA but in the absence of QSY-FDP. The rationale for the use of  $\epsilon$ -ACA is that it prevents binding and therefore eliminates quenching.

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The fluorescence of 5IAF-Pg in the presence of QSY-FDP and in the absence of  $\epsilon$ -ACA corrected for the internal filter effect is given by Equation 2,

$$S = \frac{S_{5\text{IAF-Pg}} - S_{\text{QSY-FDP}}}{\text{FF}} \quad (\text{Eq. 2})$$

where  $S_{5\text{IAF-Pg}}$  is the fluorescence prior to the addition of  $\epsilon$ -ACA. Similar experiments were conducted with TAFIa-treated QSY-FDPs. In these experiments, 20 nM TAFIa (final concentration) was added to each well with QSY-FDP for 30 min at room temperature. After this incubation period, 5IAF-Pg and  $\epsilon$ -ACA were added as described above.

**Binding of 5IAF-Glu-Pg or 5IAF-Lys-Pg to TAFIa**—The dissociation constants for interactions of TAFIa with Glu-Pg or Lys-Pg were determined by measuring the change in fluorescence of the fluorescein moiety of 5IAF-Glu-Pg or 5IAF-Lys-Pg upon the addition of TAFIa. TAFI was quantitatively activated as described previously (17). When higher concentrations of TAFIa were required, a stock of 5  $\mu\text{M}$  TAFI was activated with 50 nM thrombin, 100 nM Solulin, and 5 mM  $\text{CaCl}_2$ . After quantitative TAFI activation, thrombin was inhibited using Phe-Pro-Arg chloromethyl ketone (1  $\mu\text{M}$ ). A 90- $\mu\text{l}$  solution containing TAFIa (0–2.5  $\mu\text{M}$  final concentration) was added to the wells of an opaque 96-well plate and monitored continually by fluorescence at 1-min intervals in a SpectraMax GEMINI-XS spectrofluorometer with emission and excitation wavelengths of 480 and 520 nm, respectively, employing a cutoff filter of 515 nm in the emission beam. After the signal stabilized, 10  $\mu\text{l}$  of a solution containing 500 nM 5IAF-Glu or 5IAF-Lys-Pg was added to each well, and the fluorescence was measured again. The fluorescence intensity data ( $S$ ) were subjected to nonlinear regression to the binding equation  $S = S_0 + (\Delta S \cdot [\text{TAFIa}]) / (K_d + [\text{TAFIa}])$ , where  $S_0$  is the intensity in the absence of TAFIa,  $\Delta S$  is the total intensity change at saturating TAFIa, and  $K_d$  is the dissociation constant for the TAFIa-plasminogen interaction. Input variables were  $S$  and  $[\text{TAFIa}]$ , and best fit parameters were  $\Delta S$  and  $K_d$ .

**Kinetics of Plasminogen-binding Site Removal by TAFIa**—A solution (80  $\mu\text{l}$ ) containing QSY-FDP (0–1  $\mu\text{M}$  final concentration) and 50 nM 5IAF-Glu-Pg or 5IAF-Lys-Pg was added to the wells of an opaque 96-well plate and monitored continually at 1-min intervals by fluorescence in a SpectraMax GEMINI-XS spectrofluorometer with emission and excitation wavelengths of 480 and 520 nm, respectively, employing a cutoff filter of 515 nm. After the signal stabilized, TAFIa (20  $\mu\text{l}$ ) in 1 mg/ml bovine serum albumin was added to each well. The initial rate of fluorescence intensity increase for each reaction was recorded, and a model (see supplemental “Materials”) was used to convert the rate of fluorescence increase into the rate of plasminogen-binding site removal (supplemental Equation 12). The rate data were fit by nonlinear regression analysis to the Michaelis-Menten equation. The kinetic parameters thus obtained are shown in Table 1 as the means  $\pm$  S.E. ( $n = 9$ ).

**Kinetics of Total Lysine Cleavage by TAFIa**—Lysine cleavage by TAFIa was measured using methods similar to those described by Wang *et al.* (7) and Schneider *et al.* (18). The main difference is that the time course of lysine cleavage by TAFIa

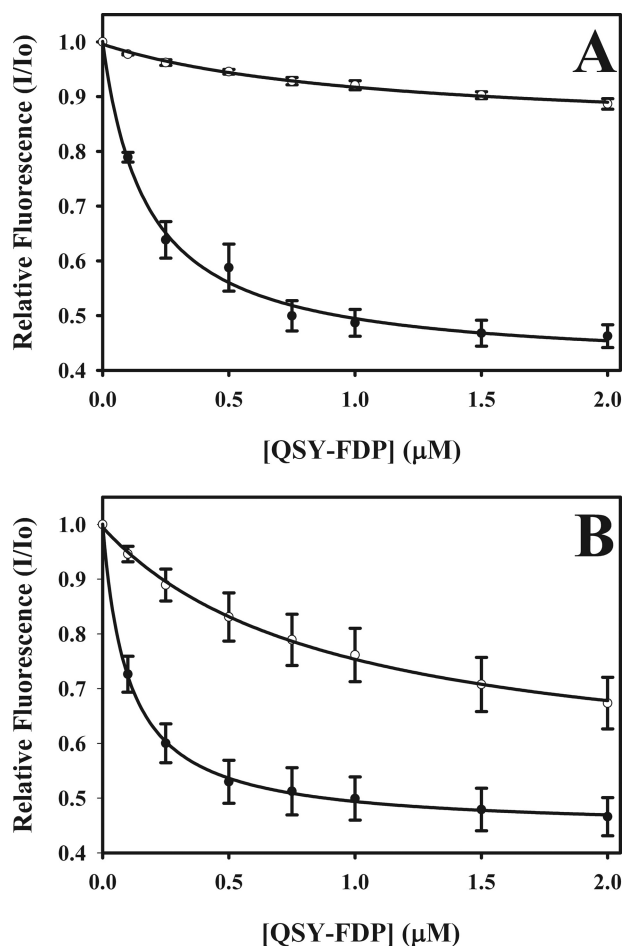


FIGURE 1. Binding of 5IAF-Glu-Pg (A) or 5IAF-Lys-Pg (B) to untreated (○) or TAFIa-treated (●) QSY-FDPs. 5IAF-Glu-Pg and 5IAF-Lys-Pg bound to QSY-FDPs with  $K_d$  values of 175 and 101 nM, respectively. Upon treatment with TAFIa, the  $K_d$  values were increased to 1.06 and 0.91  $\mu\text{M}$ , respectively. An alternative interpretation indicates that 5IAF-Glu-Pg bound TAFIa-treated QSY-FDPs with a  $K_d$  of 8.34  $\mu\text{M}$ . The data presented here are for one preparation of QSY-FDPs, but they are consistent with data from all other preparations. Data are presented as the mean  $\pm$  S.E. ( $n = 3$ ).

was measured by monitoring the rate of fluorescence change associated with the oxidation of NADH in the presence of excess SDH. A solution (50  $\mu\text{l}$ ) of unlabeled FDPs (0–10  $\mu\text{M}$  final concentration) was added to the wells of an opaque 96-well plate and monitored continually at 1-min intervals by fluorescence in a SpectraMax GEMINI-XS spectrofluorometer with emission and excitation wavelengths of 340 and 450 nm, respectively, employing a cutoff filter of 435 nm. Subsequently, the reaction was initiated by the addition of a 50- $\mu\text{l}$  solution containing 2 mM  $\alpha$ -ketoglutarate, 40  $\mu\text{M}$  NADH, 10  $\mu\text{M}$  Glu-Pg (or no plasminogen), 0.01 unit/ml SDH, and 2 nM TAFIa. Lysine cleavage by TAFIa was measured by the fluorescence change associated with NADH oxidation during SDH-mediated saccharopine formation. The initial rate for each reaction was recorded and converted to nanomolar lysine/s using a series of lysine standards. The data were fit to the Michaelis-Menten equation by nonlinear regression analysis.

## RESULTS

**Binding of 5IAF-Glu-Pg or 5IAF-Lys-Pg to QSY-FDPs**—The fluorescence intensities of 5IAF-Glu-Pg in the presence of

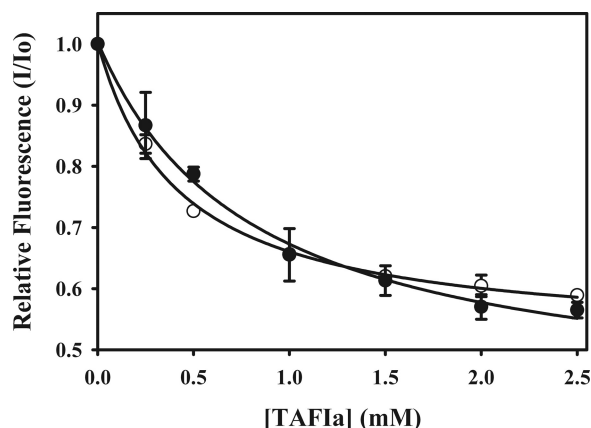


FIGURE 2. **Binding of TAFIa to 5IAF-Glu-Pg or 5IAF-Lys-Pg.** Binding of TAFIa to 5IAF-Glu-Pg (●) or 5IAF-Lys-Pg (○) was determined by fluorescence. The  $K_d$  values of the interaction with TAFIa were determined to be 900 nM for 5IAF-Glu-Pg and 425 nM for 5IAF-Lys-Pg. The maximum extents of fluorescence quenching were 62% with 5IAF-Glu-Pg and 50% with 5IAF-Lys-Pg. Data are presented as the mean  $\pm$  S.E. ( $n = 2$ ).

QSY-FDPs either treated or not treated with TAFIa are shown in Fig. 1A. The data were corrected for the internal filter effect. The data obtained with 5IAF-Lys-Pg are shown in Fig. 1B. The data, interpreted as reflecting binding of 5IAF-Glu-Pg or 5IAF-Lys-Pg to the QSY-FDP, were analyzed by nonlinear regression of the data to the equation  $S = S_0 + (\Delta S \cdot [\text{FDP}]) / (K_d + [\text{FDP}])$ , where  $S$  is the fluorescence intensity (corrected for the internal filter effect),  $S_0$  is the intensity prior to the addition of QSY-FDP,  $\Delta S$  is the change in intensity at saturating QSY-FDP, and  $K_d$  is the dissociation constant for the binding interaction. With 5IAF-Glu-Pg in the absence of TAFIa, a maximum quench of 55% and a  $K_d$  of 175 nM were obtained. When QSY-FDPs were treated with TAFIa, the corresponding values were 15% and 1.06  $\mu\text{M}$ . With 5IAF-Lys-Pg in the absence of TAFIa, a maximum quench of 55% and a  $K_d$  of 101 nM were obtained. When QSY-FDPs were treated with TAFIa, the corresponding values were 45% and 91 nM. The results with TAFIa and 5IAF-Glu-Pg cannot be interpreted unambiguously because the relative change in fluorescence was small. Although the best fit of the data suggested a maximum quench of only 15% at saturation, this seems implausible because this small quench requires that 5IAF-Glu-Pg be bound to a site in the QSY-FDP sufficiently far removed from the QSY moieties to minimize energy transfer. If the data are fit assuming 55% maximum quenching, as happens in the absence of TAFIa, the fit is nearly as good, and a  $K_d$  value of 8.34  $\mu\text{M}$  is obtained (data not shown). This value is reminiscent of the 30  $\mu\text{M}$  value reported for the binding of Glu-Pg to intact fibrin (21). Regardless of the interpretation of the data, when FDPs are treated with TAFIa, the affinity of plasminogen for FDPs is reduced and likely to a greater extent with Glu-Pg compared with Lys-Pg.

**Binding of 5IAF-Glu-Pg or 5IAF-Lys-Pg to TAFIa**—To determine whether TAFIa binds to 5IAF-Glu-Pg or 5IAF-Lys-Pg, TAFIa was incubated with each 5IAF-Pg variant, and binding was assessed by a quench in fluorescence (Fig. 2). The data were analyzed as described under “Experimental Procedures.” The analysis shows that TAFIa bound Glu-Pg with a  $K_d$  of 900 nM and Lys-Pg with a  $K_d$  of 425 nM. The extent of fluorescence

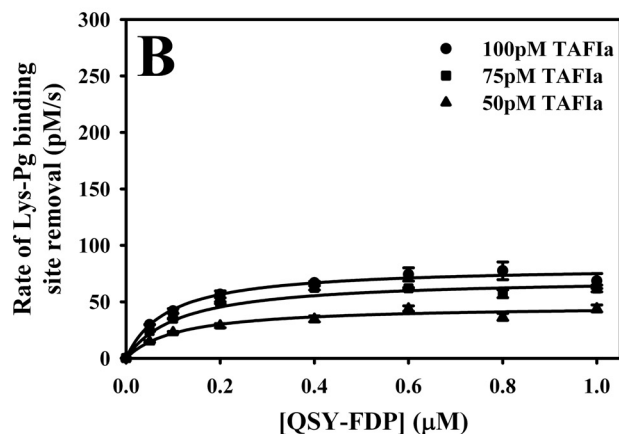
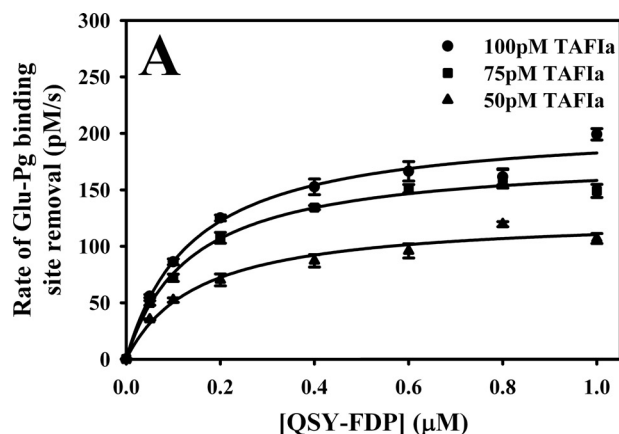


FIGURE 3. **Kinetics of TAFIa in removing Glu-Pg- or Lys-Pg-binding sites from QSY-FDPs.** The kinetics of 5IAF-Glu-Pg-binding site removal by TAFIa were determined by fitting the data by nonlinear regression to the Michaelis-Menten equation. A shows that the average  $k_{\text{cat}}$  and  $K_m$  values of Glu-Pg-binding site removal were 2.30  $\text{s}^{-1}$  and 142 nM, respectively, implying a catalytic efficiency of 16.21  $\mu\text{M}^{-1} \text{s}^{-1}$ . B shows that the average  $k_{\text{cat}}$  and  $K_m$  values of Lys-Pg-binding site removal were 0.89  $\text{s}^{-1}$  and 96 nM, respectively, which implies a catalytic efficiency of 9.23  $\mu\text{M}^{-1} \text{s}^{-1}$ . Data are presented as the mean  $\pm$  S.E. ( $n = 3$  for each TAFIa concentration).

decrease at saturating TAFIa was 62% with 5IAF-Glu-Pg and 50% with 5IAF-Lys-Pg. Previously, Tan and Eaton (22) measured the binding of TAFIa to immobilized Glu-Pg and Lys-Pg using surface plasmon resonance. They reported  $K_d$  values of 2000 and 380 nM for Glu-Pg and Lys-Pg, respectively. These values are similar to those found here and indicate that the binding is not appreciably affected by the existence of the fluorescein moiety in 5IAF-Glu-Pg and 5IAF-Lys-Pg.

**Kinetics of Plasminogen-binding Site Removal by TAFIa**—The calculated rate of 5IAF-Glu-Pg-binding site removal by TAFIa was determined at various QSY-FDP concentrations (0–1  $\mu\text{M}$ ) and at three concentrations of TAFIa (Fig. 3A). The data are hyperbolic in nature and were fit by nonlinear regression to the Michaelis-Menten equation. The average  $k_{\text{cat}}$  and  $K_m$  values of Glu-Pg-binding site removal for the three curves were 2.34  $\text{s}^{-1}$  and 142.6 nM, respectively, implying a catalytic efficiency of 16.21  $\mu\text{M}^{-1} \text{s}^{-1}$ . Similar experiments were conducted to determine the kinetics of 5IAF-Lys-Pg-binding site removal by TAFIa. The average  $k_{\text{cat}}$  and  $K_m$  values of Lys-Pg-binding site removal were 0.89  $\text{s}^{-1}$  and 96 nM, respectively, implying a catalytic efficiency of 9.23  $\mu\text{M}^{-1} \text{s}^{-1}$  (Fig. 3B). 5IAF-Glu-Pg- and 5IAF-Lys-Pg-binding site removal conformed well

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**TABLE 1**

Summary of the kinetics of Glu-Pg- and Lys-Pg-binding site removal by TAFIa

Data are presented as the mean  $\pm$  S.E. ( $n = 9$ ).

	$k_{\text{cat}}$ $\text{s}^{-1}$	$K_m$ $\mu\text{M}$	$k_{\text{cat}}/K_m$ $\mu\text{M}^{-1} \text{s}^{-1}$
Glu-Pg	$2.30 \pm 0.06$	$0.142 \pm 0.01$	$16.21 \pm 0.82$
Lys-Pg	$0.89 \pm 0.03$	$0.097 \pm 0.01$	$9.23 \pm 0.57$

**TABLE 2**

Comparison of TAFIa-catalyzed cleavage of several TAFIa biological substrates

OPN, osteopontin.

	$k_{\text{cat}}/K_m$ $\mu\text{M}^{-1} \text{s}^{-1}$	$k_{\text{cat}}$ $\text{s}^{-1}$	$K_m$ $\mu\text{M}$
Glu-Pg-binding site	16.21	2.30	0.142
Fibrin $\beta$ -Lys(125–133) <sup>a</sup>	0.95	13.6	14.3
Bradykinin <sup>a</sup>	0.28	19.7	70.6
C3a(69–77) <sup>a</sup>	0.23	8.4	35.9
OPN(159–168) <sup>a</sup>	0.16	2.3	142
C5a(55–74) <sup>a</sup>	0.13	29.5	219
Fibrin $\beta$ -Lys(54–62) <sup>a</sup>	0.076	2.6	34
Fibrin $\alpha$ -Arg(96–104) <sup>a</sup>	0.0042	1.5	361
RGDSTFESKSYK <sup>b</sup>	0.045	27.8	625

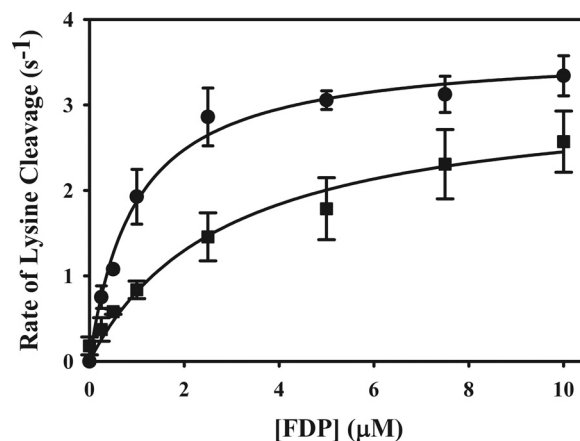
<sup>a</sup> Ref. 23.

<sup>b</sup> Ref. 13.

to the Michaelis-Menten equation, with  $R^2$  values of 0.93 and 0.92, respectively. The kinetics of TAFIa for Glu-Pg- and Lys-Pg-binding site removal are summarized in Table 1.

TAFIa has been shown by others to catalyze cleavage of lysine or arginine residues from other biological substrates such as C3a, C5a, thrombin-cleaved osteopontin, and peptides identical to those appearing in fibrin (13, 23). In Table 2, the kinetics of plasminogen-binding site removal are compared with those of several other substrates that have been reported by others. According to these data, the catalytic efficiency ( $k_{\text{cat}}/K_m$ ) for QSY-FDPs, and therefore presumably plasmin-modified fibrin, exceeds that for the other substrates by a factor of 17 or more. Although the  $k_{\text{cat}}$  values for some of the substrates exceed that for the FDPs, the very low  $K_m$  for the FDPs contributes to the high catalytic efficiency.

**Kinetics of Total Lysine Cleavage by TAFIa**—To determine the kinetics of cleavage of all C-terminal lysine residues from FDPs and not just those involved in plasminogen binding, we used an enzyme (SDH) that catalyzes the formation of saccharopine from lysine and  $\alpha$ -ketoglutarate via an NADH-linked reaction (7, 17). SDH was added at a high enough concentration to make the TAFIa reaction rate-limiting. Thus, the kinetics of lysine cleavage by TAFIa were monitored by following the fluorescence decrease associated with NADH oxidation. As shown in Fig. 4, this method yielded a  $k_{\text{cat}}$  of  $3.15 \text{ s}^{-1}$  and a  $K_m$  of  $2.87 \mu\text{M}$  in the absence of plasminogen, implying a catalytic efficiency of  $1.10 \mu\text{M}^{-1} \text{ s}^{-1}$  when the data were fit to the Michaelis-Menten equation. Interestingly, upon the addition of Glu-Pg, the catalytic efficiency was increased by 3.5-fold to  $3.85 \mu\text{M}^{-1} \text{ s}^{-1}$ . This is primarily the result of changes in the  $K_m$ , which decreased from  $2.87 \mu\text{M}$  in the absence of plasminogen to  $0.95 \mu\text{M}$  in the presence of Glu-Pg. This suggests that plasminogen augments the binding of TAFIa to FDPs, and this has a positive effect on the catalytic efficiency. The data fit well to the Michaelis-Menten equation in that all  $R^2$  values were  $>0.98$ .



**FIGURE 4. Effect of Glu-Pg on lysine cleavage by TAFIa.** The kinetics of lysine cleavage by TAFIa were analyzed in the presence of Glu-Pg (●) or in the absence of plasminogen (■). The  $k_{\text{cat}}$  did not vary to any great extent whether plasminogen was added or not; however, the  $K_m$  was decreased from 2.87 to  $0.95 \mu\text{M}$  in the presence of Glu-Pg. This resulted in an increase in the catalytic efficiency from  $1.10 \mu\text{M}^{-1} \text{ s}^{-1}$  in the absence of plasminogen to  $3.85 \mu\text{M}^{-1} \text{ s}^{-1}$  in the presence of Glu-Pg, respectively. Data are presented as the mean  $\pm$  S.E. ( $n = 4$ ).

**TABLE 3**

Summary of the kinetics of lysine cleavage by TAFIa

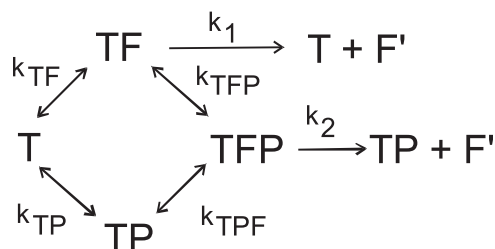
	$k_{\text{cat}}$ $\text{s}^{-1}$	$K_m$ $\mu\text{M}$	$k_{\text{cat}}/K_m$ $\mu\text{M}^{-1} \text{ s}^{-1}$
+ Glu-Pg	3.66	0.95	3.85
No Pg	3.15	2.87	1.10

The kinetic parameters associated with lysine cleavage by TAFIa are summarized in Table 3.

## DISCUSSION

This work shows that QSY-FDPs, and presumably plasmin-modified fibrin for which the QSY-FDPs are a surrogate, bind both Glu-Pg and Lys-Pg with  $K_d$  values in the neighborhood of  $100 \text{ nM}$ . This binding is substantially weakened by TAFIa, especially with Glu-Pg. TAFIa was shown by fluorescence quenching to bind both Glu-Pg and Lys-Pg. The kinetics of loss of plasminogen binding conformed to the Michaelis-Menten model. Comparison of the kinetics of loss of plasminogen-binding sites with those of other potential biological substrates of TAFIa suggests that plasmin-modified fibrin is indeed a likely physiological substrate. The catalytic efficiency of cleaving all lysine residues from FDPs was curiously less than that of cleaving lysines involved in plasminogen binding. Interestingly, the catalytic efficiency of cleaving all lysine residues was increased by including plasminogen in the reaction. This suggests that lysine residues associated with plasminogen binding are cleaved by TAFIa with relatively high efficiency compared with others and that plasminogen promotes the interaction of TAFIa with FDPs.

The presumed different classes of lysine residues may differ in their ability to bind plasminogen and/or TAFIa, which would greatly influence the kinetics of TAFIa. For example, exposed C-terminal lysines at a site where all three chains of fibrin have been cleaved by plasmin may be more accessible than nicked fibrin (only one or two cleaved chains) and, as a result, more readily cleaved by TAFIa. The higher catalytic efficiency of Glu-Pg-binding site removal ( $16.21 \mu\text{M}^{-1} \text{ s}^{-1}$ ) compared with lysine



SCHEME 1. **Equilibrium binding model of lysine cleavage by TAFIa.** TAFIa (T) binds either FDPs (F) or plasminogen (P) to form the corresponding binary complexes, TF or TP. These then bind either plasminogen or FDPs to form the ternary TFP complex. The binary complex yields TAFIa plus cleaved FDPs (F') with rate constant  $k_1$ , and the ternary complex TFP yields F' and TP with rate constant  $k_2$ . The constants  $k_1$  and  $k_2$  are  $k_{\text{cat}}$  values. The binding interactions are characterized by equilibrium constants  $K_{\text{TF}}$ ,  $K_{\text{TP}}$ ,  $K_{\text{TFP}}$ , and  $K_{\text{TFPF}}$ .

cleavage ( $3.85 \mu\text{M}^{-1} \text{s}^{-1}$  with  $5 \mu\text{M}$  Glu-Pg) is largely due to a change in the  $K_m$  ( $0.143$  versus  $1 \mu\text{M}$ , respectively), which suggests that TAFIa or TAFIa-Pg binds some lysines more tightly than others. The  $k_{\text{cat(app)}}$  remains relatively unchanged when comparing the kinetics of plasminogen-binding site removal with lysine cleavage ( $2.30$  versus  $3.66 \text{s}^{-1}$  for Glu-Pg, respectively). The fluorescence method for measuring plasminogen-binding site removal by TAFIa, by definition, measures the kinetics of cleavage of lysine residues involved in plasminogen binding. The alternative method using SDH does not discriminate between the different classes of C-terminal lysines, and the kinetics likely approximate the average  $k_{\text{cat}}$  and  $K_m$  of all lysine residues cleaved by TAFIa.

Studies in the TAFI knock-out mouse have not disclosed an obvious and overt hyperfibrinolytic phenotype that might be expected based on studies done *in vitro* (15). This suggests that down-regulation of fibrinolysis by TAFIa might not be physiologically relevant. Rather, TAFIa might be involved in other processes such as inflammation and wound healing. The catalytic efficiency of removal of plasminogen-binding sites from plasmin-modified fibrin measured in this study is, however, the highest by far of all the biological substrates measured to date, including those thought to be involved in inflammation. Thus, in a complex milieu *in vivo* with numerous TAFIa substrates, the preferred substrate would be plasmin-modified fibrin. This argues quite strongly for a physiologically relevant role for TAFIa in the down-regulation of fibrinolysis.

The enhancement of lysine cleavage by plasminogen was a surprising finding. It was presumed that plasminogen would inhibit of C-terminal lysine cleavage by TAFIa by sterically hindering access of the lysine residues to TAFIa; however, Glu-Pg actually increased the catalytic efficiency of TAFIa in cleaving lysines from FDPs. The fact that both TAFIa and plasminogen bind to each other and to fibrin can rationalize this phenomenon. This is done through the model depicted in Scheme 1. The rate equation for this model is derived under [supplemental "Materials"](#) (both steady-state and equilibrium models). According to this model, TAFIa (T) can bind FDPs (F), or it can bind plasminogen (P) to form the corresponding binary complexes. The TF complex can turn over to form cleaved FDPs (F') with  $k_{\text{cat}} = k_1$ . The TP complex can bind F to form the ternary TFP complex, which can turn over to F' with  $k_{\text{cat}} = k_2$ . As shown under [supplemental "Materials,"](#) the rate equation

for this model is given by the Michaelis-Menten equation (Equation 3), with  $k_{\text{cat(app)}}$  and  $K_{m(\text{app})}$  terms that depend in characteristic ways on the plasminogen concentration, given by Equations 4 and 5.

$$\frac{v}{[T_0]} = \frac{k_{\text{cat(app)}}[F]}{K_{m(\text{app})} + [F]} \quad (\text{Eq. 3})$$

$$k_{\text{cat(app)}} = \frac{k_1 K_{\text{TFP}} + k_2 [\text{Pg}]}{K_{\text{TFP}} + [\text{Pg}]} \quad (\text{Eq. 4})$$

$$K_{m(\text{app})} = \frac{K_{\text{TF}} K_{\text{TFP}} + K_{\text{TFP}} [\text{Pg}]}{K_{\text{TFP}} + [\text{Pg}]} \quad (\text{Eq. 5})$$

In the absence of plasminogen,  $k_{\text{cat(app)}} = k_1$ , and  $K_{m(\text{app})} = K_{\text{TF}}$ . On the other hand, as  $[\text{Pg}] \rightarrow \infty$ ,  $k_{\text{cat(app)}} = k_2$ , and  $K_{m(\text{app})} = K_{\text{TFP}}$ . Our data show that plasminogen has little effect on  $k_{\text{cat}}$ , which implies that, to a good first approximation,  $k_1 = k_2$ . However, plasminogen decreased the  $K_{m(\text{app})}$  by  $\sim 3$ -fold, which suggests that  $K_{\text{TFP}}$  is 3-fold smaller than  $K_{\text{TF}}$ . In other words, the TAFIa-plasminogen complex binds FDPs 3-fold more tightly than TAFIa does. This implies, in turn, that plasminogen promotes the interaction of TAFIa with its substrate, FDP. In this way, plasminogen increases the catalytic efficiency of cleavage of lysine residues from FDPs. These results are consistent with the notion that plasminogen (Glu-Pg or Lys-Pg) binds TAFIa at a different site than it binds FDPs. If the tight interaction between plasminogen and FDPs (Fig. 1) remains intact in the presence of TAFIa, plasminogen may act as a template that can be used to position TAFIa within close proximity to its substrate, *i.e.* lysine residues on FDPs. The binding energy between the TAFIa-plasminogen complex and FDPs is higher than the binding energy between TAFIa and FDPs. This suggests that plasminogen-dependent binding linkage, similar to the linkage described by Wyman (24), occurs to lower the  $K_m$  of plasminogen-binding site removal by TAFIa.

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