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 3000-fold compared to tPA alone. TAFIa attenuates fibrinolysis by removing these residues, which causes a 97% reduction in tPA catalytic efficiency. The aim of these studies 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-Pg binding site removal are 2.34 s$^{-1}$ and 142.6 nM, respectively, implying a catalytic efficiency of 16.21 μM$^{-1}$s$^{-1}$. The corresponding values of Lys-Pg binding site removal are 0.89 s$^{-1}$ and 96 nM, respectively, implying a catalytic efficiency of 9.23 μM$^{-1}$s$^{-1}$. 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 1.32 μM$^{-1}$s$^{-1}$. Interestingly, this value increases to 3.85 and 3.35 μM$^{-1}$s$^{-1}$ 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- and Lys plasminogen.

The fibrinolytic system is initiated upon release of tissue plasminogen activator (tPA) from endothelial cells at the site of vascular injury which is usually where an insoluble fibrin clot is formed (1). Initially, native or Glu-Plasminogen (Glu-Pg) weakly binds intact fibrin and is subsequently activated to plasmin (Pn) by tPA or by urokinase-type plasminogen activator (uPA). 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 (FDP) and solubilizing the clot. It also enhances fibrinolysis by converting Glu-Pg to 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).

TAFIa (activated thrombin-activatable fibrinolysis inhibitor also known as carboxypeptidase U [CPU]) 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 in order to suppress plasminogen activation and clot dissolution (7). Since thrombin clots fibrinogen and then attenuates fibrin degradation by activating TAFI, TAFI represents a link between coagulation and fibrinolysis (6). TAFIa regulates tPA dependent fibrinolysis half maximally at 1nM,
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 to the smallest fibrin degradation product (FDP; DDE, 250,000 Da) and TAFI zymogen does not show carboxypeptidase activity toward FDP (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 the current study was to determine the kinetics of removal of lysine binding sites of plasmin-modified fibrin and to compare them to the kinetics of cleavage of other substrates as measured by other investigators, the studies reported here were undertaken.

Experimental Procedures

Materials – Fibrinogen was prepared as previously described (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 β-alanine precipitation. This change in protocol allowed for a greater yield of fibrinogen. TAFI isolation and activation were essentially achieved as described (18). Since such low concentrations of TAFIa were used in experiments, TAFI was activated in the presence of 1mg/mL bovine serum albumin to prevent non-specific binding to plastics. Recombinant human Pg (S741C) and the fluorescein derivative (5IAF-Glu-Pg) were prepared as described by Horrevoets et al. (3). 5IAF-Lys-Pg was prepared by treating 5IAF-Glu-Pg with 200nM plasmin in the presence of 5mM ε-ACA for 3 hours at room temperature. Plasmin was subsequently removed using a 1mL benzamide sepharose 6B column (GE Healthcare Biosciences, Baie d’Urfé, PQ). Residual plasmin activity (<1% as determined using the chromogenic substrate, S-2251 [Diapharma, West Chester, OH]) was inhibited with 10μM D-Val-Phe-Lys-chloromethyl ketone (Calbiochem, San Diego, CA). Human Glu-Pg was isolated and Lys-Pg was prepared as previously described (18). Saccharopine dehydrogenase (SDH) was isolated as previously described (19). Solulin was a generous gift from Dr. Achim Schuttler, Paion GmbH (Aachen, Germany). QSY9 C5-maleimide and 5-iodoamidofluorescein were purchased from Invitrogen Canada Inc. (Burlington, ON). Plasmin was purchased from Haematologic Technologies Inc. (Essex Junction, VT, USA). The buffer used in all experiments was 20mM HEPES, 200mM NaCl, pH 7.4. All other reagents were of analytical quality.

QSY-FDP production – QSY-FDPs (fibrin degradation products that are covalently attached to the quencher, QSY9 C5-maleimide) were prepared using methods modified from those described by Neill et al. (15) and Kim et al. (20). Briefly, 7.1mg/mL fibrinogen was combined with 5nM thrombin, 2mM CaCl₂, 40nM plasmin (final concentrations in 7mL) in HBS and coagulation
and fibrinolysis were monitored by turbidity at 800nm. Once the clot had fully lysed, thrombin and plasmin were inhibited with chloromethyl ketones (PPA-ck and VFK-ck, respectively). The ionic strength was increased by addition of solid NaCl to a final concentration of 0.5M. The FDPs were gel filtered using a Sephacryl 1000 column and fractions were pooled according to the criteria described by Neill et al. (19). Subsequently, the FDPs were selectively reduced with 2-mercaptoethanol (50mM final concentration) for 30 minutes at room temperature. 2-mercaptoethanol was removed by dialyzing against 3 x 4L of 20mM HEPES, 500mM NaCl pH 7.4. Selectively reduced FDPs were incubated with a 30-fold excess of QSY9 C5 maleimide for 1 hour at room temperature. This step routinely causes some precipitation of the FDPs. Precipitate was removed by centrifugation (5,000 x g) and excess QSY9 C5 maleimide was removed from the sample by dialysis (3 x 4L of 20mM HEPES, 300mM NaCl pH 7.4). QSY-FDPs were aliquotted and stored at -80°C until required. QSY-FDPs are soluble, have an average molecular weight of 2 x 10^6 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).

The binding of 5IAF-Glu- or 5IAF-Lys-Pg to QSY-FDPs – QSY-FDPs (95μL, 0 – 2μM final concentration) were added to wells of an opaque 96-well plate and monitored continually at 1-minute intervals by fluorescence in a Gemini SpectraMax XS (Molecular Devices, Sunnyvale, CA) with emission and excitation wavelengths of 480nm and 520nm, respectively, employing a cutoff filter of 515nm. After the signal stabilized, 5μL of 1μM 5IAF-Glu- or 5IAF-Lys-Pg (50nM final) was added and mixed with the QSY-FDPs. Again, after the signal stabilized, 1μL of 1M εACA was added to each well and mixed. The stable fluorescence signals obtained with only QSY-FDP (S_{QSY-FDP}), after the addition of 5IAF-Pg (S_{5IAF-Pg}) and after the addition of εACA (S_{εACA}) 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), where S_{εACA} is the fluorescence in the presence of ε-ACA and QSY-FDP, S_{QSY-FDP} is the blank in the absence of 5IAF-Pg and S_{5IAF-Pg0} is the fluorescence signal in presence of ε-ACA but in the absence of QSY-FDP. The rationale for the use of ε-ACA is that it prevents binding and therefore eliminates quenching.

\[ FF = \frac{S_{εACA} - S_{QSY-FDP}}{S_{5IAF-Pg0}} \]  

The fluorescence of 5IAF-Pg, in the presence of QSY-FDP and in the absence of ε-ACA, corrected for the internal filter effect is given by equation (2), where S_{5IAF-Pg} is the fluorescence prior to the addition of ε-ACA.

\[ S = \frac{S_{5IAF-Pg} - S_{QSY-FDP}}{FF} \]  

Similar experiments were conducted with TAFIa treated QSY-FDP. In these experiments, 20nM TAFIa (final concentration) was added to each well with QSY-FDP for 30-minutes at room temperature. After this incubation period, 5IAF-Pg and ε-ACA were added as described above.

The binding of 5IAF-Glu- 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 or 5IAF-Lys-Pg upon the addition of TAFIa. TAFI was quantitatively activated as previously described (17). When higher concentrations of TAFIa were required a stock of 5μM TAFI was activated with 50nM thrombin, 100nM Solulin and 5mM CaCl2. After quantitative TAFI activation, thrombin was inhibited using Phe-Pro-Arg-chloromethyl ketone (1 μM). A 90μL solution containing TAFIa (0 – 2.5μM final concentration) was added to wells of an opaque 96-well plate and monitored continually by fluorescence at 1-minute intervals by fluorescence.
intervals in a Gemini SpectraMax XS (Molecular Devices, Sunnyvale, CA) with emission and excitation wavelengths of 480nm and 520nm, respectively, employing a cutoff filter of 515nm in the emission beam. After the signal stabilized, 10µL of a solution containing 500nM 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 non-linear regression to the binding equation

\[ S = S_0 + \frac{\Delta S \cdot [TAFIa]}{K_d + [TAFIa]} \],

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 [TAFIa] and best-fit parameters were \( \Delta S \) and \( K_d \).

**Kinetics of plasminogen binding site removal by TAFIa** – A solution (80µL) containing QSY-FDP (0 – 1µM final concentration) and 50nM 5IAF-Glu- or Lys-Pg was added to wells of an opaque 96-well plate and monitored continually at 1-minute intervals by fluorescence in a Gemini SpectraMax XS (Molecular Devices, Sunnyvale, CA) with emission and excitation wavelengths of 480nm and 520nm, respectively, employing a cutoff filter of 515nm. After the signal stabilized, TAFIa (20µL) in 1mg/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 supplementary materials) was used to convert the rate of fluorescence increase into the rate of Pg binding site removal (equation 12, supplementary materials). The rate data were fit by non-linear regression analysis to the Michaelis-Menten equation. The kinetic parameters so obtained are shown in Table 1 as the mean ± standard error, 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. (17). The main difference is that the time course of lysine cleavage by TAFIa was measured by monitoring the rate of fluorescence change associated with the oxidation of NADH in the presence of excess SDH. A solution (50µL) of unlabelled FDPs (0 – 10µM final) was added to wells of an opaque 96-well plate and monitored continually at 1-minute intervals by fluorescence in a Gemini SpectraMax XS (Molecular Devices, Sunnyvale, CA) with emission and excitation wavelengths of 340nm and 450nm, respectively, employing a cutoff filter of 435nm. Subsequently, the reaction was initiated by the addition of a 50µL solution containing 2mM α-ketoglutarate (αKG), 40µM NADH, 10µM Glu-Pg (or no plasminogen), 0.01U/mL SDH and 2nM 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 nM lysine/s using a series of lysine standards. The data were fit to the Michaelis-Menten equation by non-linear regression analysis.

**Results**

The binding of 5IAF-Glu- or 5IAF-Lys-Pg to QSY-FDPs – The fluorescence intensities of 5IAF-Glu-Pg in the presence of QSY-FDP either treated or not treated with TAFIa are shown in Figure 1A. The data are corrected for the internal filter effect. The data obtained with 5IAF-Lys-Pg are shown in Figure 1B. The data, interpreted as reflecting binding of 5IAF-Glu-Pg or 5IAF-Lys-Pg to the QSY-FDP, were analyzed by non-linear regression of the data to the equation

\[ S = S_0 + \frac{\Delta S \cdot [FDP]}{K_d + [FDP]} \],

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 \( K_d \) the dissociation constant for the binding interaction. With 5IAF-Glu-Pg a maximum quench of 55% and a \( K_d \) value of 175nM were obtained in the absence of TAFIa. When QSY-FDPs were treated with TAFIa, the corresponding values were 15% maximal quench and a \( K_d \) value of 91 nM. The results with TAFIa and 5IAF-Glu-Pg cannot be interpreted unambiguously because the relative change in
fluorescence is 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 QSY-FDP sufficiently far removed from the QSY moieties to minimize energy transfer. If the data are fit assuming 55% maximal quenching, as happens in the absence of TAFIa, the fit is nearly as good and a Kd value of 8.34 μM is obtained (not shown). This value is reminiscent of the 30 μM 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 to Lys-Pg.

The binding of 5IAF-Glu- or 5IAF-Lys-Pg to TAFIa – To determine whether TAFIa binds to 5IAF-Glu- or 5IAF-Lys-Pg, TAFIa was incubated with each 5IAF-Pg variant and binding was assessed by a quench in fluorescence (Figure 2). The data were analyzed as described in Experimental Procedures. The analysis shows that TAFIa binds Glu-Pg with a Kd of 900nM and Lys-Pg with a Kd of 425nM. The extents of fluorescence decrease at saturating TAFIa were 62 percent with 5IAF-Glu-Pg and 50 percent with 5IAF-Lys-Pg. Previously, Tan and Eaton measured the binding of TAFIa to immobilized Glu- and Lys-Pg using surface plasmon resonance (22). They reported Kd values of 2000nM and 380 nM for Glu- 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 – 1μM) and at three concentrations of TAFIa (Figure 3, panel A). The data are hyperbolic in nature and were fit by non-linear regression to the Michaelis-Menten equation. The average kcat and Km values of Glu-Pg binding site removal for the three curves were 2.34 s⁻¹ and 142.6nM, respectively, implying a catalytic efficiency of 16.21 μM⁻¹s⁻¹. Similar experiments were conducted to determine the kinetics of 5IAF-Lys-Pg binding site removal by TAFIa. The average kcat and Km of Lys-Pg values for binding site removal were 0.89 s⁻¹ and 96 nM, respectively, implying a catalytic efficiency of 9.23 μM⁻¹s⁻¹ (Figure 3, panel B). 5IAF-Glu- and 5IAF-Lys-Pg binding site removal conformed well to the Michaelis-Menten equation with R² values of 0.93 and 0.92, respectively. The kinetics of TAFIa on Glu- 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 site removal are compared to those of several other substrates that have been reported by others. According to these data, the catalytic efficiency (kcat/Km) for QSY-FDPs, and therefore presumably plasmin-modified fibrin, exceeds that of the other substrates by a factor of 17 or more. Although the kcat values for some of the substrates exceeds that of the FDPs, the very low Km of the FDPs contributes to the high catalytic efficiency.

Kinetics of total lysine cleavage by TAFIa – In order to determine the kinetics of cleavage of all carboxy-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 α-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 Figure 4, this method yielded a kcat of 3.15 s⁻¹ and a Km of 2.87 μM in the absence of plasminogen, which implies a catalytic efficiency of 1.10 μM⁻¹s⁻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 3.85μM⁻¹s⁻¹. This is primarily the result of changes in the Km which decreased from 2.87μM in the absence of plasminogen to 0.95μM 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 greater than 0.98. 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- and Lys-Pg with $K_d$ values in the neighborhood of 100nM. This binding is substantially weakened by TAFIa, especially with Glu-Pg. TAFIa was shown by fluorescence quenching to bind both Glu- and Lys-plasminogen. The kinetics of loss of plasminogen binding conformed to the Michaelis-Menten model. Comparison of the kinetics of loss of plasminogen binding sites to those of other potential biologic substrates of TAFIa suggest that plasmin-modified fibrin is indeed a likely physiologic 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 to 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 3 chains of fibrin have been cleaved by plasmin may be more accessible than nicked fibrin (only 1 or 2 cleaved chains) and as a result more readily cleaved by TAFIa. The higher catalytic efficiency of Glu-Pg binding site removal (16.21 s$^{-1}$µM$^{-1}$) compared to lysine cleavage (3.85 s$^{-1}$µM$^{-1}$ with 5µM Glu-Pg) is largely due to a change in the $K_m$ (0.143µM vs. 1µM, respectively), which suggests that TAFIa or TAFIa-Pg binds some lysines more tightly than others. The $k_{cat(app)}$ remains relatively unchanged when comparing the kinetics of plasminogen binding site removal to lysine cleavage (2.30 s$^{-1}$ vs. 3.66 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 alternate method using SDH does not discriminate between the different classes of C-terminal lysines and the kinetics likely approximate the average $k_{cat}$ and $K_m$ of all lysine residues cleaved by TAFIa.

Studies in the TAFI knockout 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 the present study, however, is 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 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 figure 5. The rate equation for this model is derived in Supplementary Materials (both steady state and equilibrium models).
In the absence of plasminogen $k_{\text{cat(app)}} = k_1$ and $K_{m(app)} = K_{TF}$. On the other hand, as $[\text{P}] \to \infty$, $k_{\text{cat(app)}} = k_2$ and $K_{m(app)} = K_{TFP}$.

Our data showed that plasminogen has little effect on $k_{\text{cat}}$, which implies that, to a good first approximation, $k_1 = k_2$. Plasminogen, however, decreased the $K_{m(app)}$ by about 3-fold, which suggests that $K_{TFP}$ is 3-fold smaller than $K_{TF}$. In other words, the TAFIa-Pg complex binds FDP 3-fold more tightly than TAFIa does. This, in turn, implies 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 FDP. These results are consistent with the notion that plasminogen (Glu- or Lys-) binds TAFIa at a different site than it binds FDPs. If the tight interaction between plasminogen and FDPs (Figure 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 plasminogen-TAFIa complex and FDPs is higher than the binding energy of TAFIa binding FDPs. This suggests that plasminogen dependent binding linkage, similar to the linkage described by Wyman et al. (24), occurs to lower the $K_m$ of plasminogen binding site removal by TAFIa.

Acknowledgements

We would like to thank Tom Abbott for his assistance in preparing the manuscript. This work was supported by grants from The National Institute of Health, USA [HL 46703, Project 4 (MEN)] and The Heart and Stroke Foundation of Ontario (T5575). JHF was supported by a Doctoral Fellowship from the Heart and Stroke foundation of Canada.

Figure 5. 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 P or F 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 $K_{TF}$, $K_{TP}$, $K_{TFP}$ and $K_{TFP}$. According to this model TAFIa (T) can bind FDP (F) or it can bind Pg (P) to form the corresponding binary complexes. The TF complex can turn over to form cleaved FDP (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 in the Supplementary Materials, the rate equation for this model is given by the Michaelis-Menten equation (17), with $k_{\text{cat(app)}}$ and $K_{m(app)}$ terms that depend in characteristic ways on the plasminogen concentration, given by equations (18) and (19).

\[
\frac{v}{[T_0]} = \frac{k_{\text{cat(app)}} [F]}{K_{m(app)} + [F]} \tag{17}
\]

\[
k_{\text{cat(app)}} = \frac{k_1 K_{TFP} + k_2 [P]}{K_{TFP} + [P]} \tag{18}
\]

\[
K_{m(app)} = \frac{K_{TF} K_{TFP} + K_{TFP} [P]}{K_{TFP} + [P]} \tag{19}
\]
References

Figure and Table Legends

**Figure 1.** Binding of 5IAF-Glu-Pg (panel A) or 5IAF-Lys-Pg (panel B) to untreated (○) or TAFIa-treated (●) QSY-FDPs. 5IAF-Glu- and 5IAF-Lys-Pg bind to QSY-FDPs with K_d values of 175nM and 101nM, respectively. Upon treatment with TAFIa, the K_d values are increased to 1.06µM and 0.91 µM, respectively. An alternative interpretation indicates that 5IAF-Glu-Pg binds TAFIa treated QSY-FDPs with a K_d value of 8.34 µM. 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 ± SE (n=3).

**Figure 2.** Binding of TAFIa to 5IAF-Glu- or 5IAF-Lys-Pg. Binding of TAFIa to 5IAF-Glu- (●) or 5IAF-Lys-Pg (○) was determined by fluorescence. The K_d of the interaction with TAFIa was determined to be 900nM for 5IAF-Glu-Pg and 425nM for 5IAF-Lys-Pg. The maximum extents of fluorescence quench were 62 percent with 5IAF-Glu-Pg and 50 percent with 5IAF-Lys-Pg. Data are presented as the mean ± SE (n=2).

**Figure 3.** The kinetics of TAFIa in removing Glu- 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 non-linear regression to the Michaelis-Menten equation. Panel A shows that the average k_cat and K_m values of Glu-Pg binding site removal were 2.30 s^{-1} and 142 nM, respectively, implying a catalytic efficiency of 16.21 µM^{-1}s^{-1}. Panel B shows that the average k_cat and K_m values of Lys-Pg binding site removal were 0.89 s^{-1} and 96 nM, respectively, which implies a catalytic efficiency of 9.23 µM^{-1}s^{-1}. Data are presented as the mean ± SE (n=3 for each TAFIa concentration).

**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_cat did not vary to any great extent whether plasminogen was added or not; however, the K_m was decreased from 2.87µM to 0.95 in the presence of Glu-Pg. This resulted in an increase in the catalytic efficiency from 1.10 µM^{-1}s^{-1} in the absence of plasminogen to 3.85 µM^{-1}s^{-1} in the presence of Glu-Pg, respectively. Data are presented as the mean ± SE (n=4).

**Table 1.** A summary of the kinetics of Glu- and Lys-Pg binding site removal by TAFIa. Data are presented as the mean ± SE (n=9).

**Table 2.** Comparison of TAFIa-catalyzed cleavage of several TAFIa biological substrates

**Table 3.** A summary of the kinetics of lysine cleavage by TAFIa
Figure 1.
Figure 2.
Figure 3.

A

Rate of Glu-Pg binding site removal (pM/s)

[QSY-FDP] (μM)

B

Rate of Lys-Pg binding site removal (pM/s)

[QSY-FDP] (μM)
Figure 4.
Table 1.

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}/K_m$ (µM$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu-Pg</td>
<td>2.30 ± 0.06</td>
<td>0.142 ± 0.01</td>
<td>16.21 ± 0.82</td>
</tr>
<tr>
<td>Lys-Pg</td>
<td>0.89 ± 0.03</td>
<td>0.097 ± 0.01</td>
<td>9.23 ± 0.57</td>
</tr>
</tbody>
</table>

Table 2.

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}/K_m$ (µM$^{-1}$s$^{-1}$)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu-Pg Binding Site</td>
<td>16.21</td>
<td>2.30</td>
<td>0.142</td>
</tr>
<tr>
<td>FBβ-Lys (125-133)$^1$</td>
<td>0.95</td>
<td>13.6</td>
<td>14.3</td>
</tr>
<tr>
<td>Bradykinin$^1$</td>
<td>0.28</td>
<td>19.7</td>
<td>70.6</td>
</tr>
<tr>
<td>C3a (69-77)$^1$</td>
<td>0.23</td>
<td>8.4</td>
<td>35.9</td>
</tr>
<tr>
<td>OPN (159-168)$^1$</td>
<td>0.16</td>
<td>2.3</td>
<td>142</td>
</tr>
<tr>
<td>C5a (55-74)$^1$</td>
<td>0.13</td>
<td>29.5</td>
<td>219</td>
</tr>
<tr>
<td>FBβ-Lys (54-62)$^1$</td>
<td>0.076</td>
<td>2.6</td>
<td>34</td>
</tr>
<tr>
<td>FBα-Arg (96-104)$^1$</td>
<td>0.0042</td>
<td>1.5</td>
<td>361</td>
</tr>
<tr>
<td>RGDSTFESKSYK$^2$</td>
<td>0.045</td>
<td>27.8</td>
<td>625</td>
</tr>
</tbody>
</table>


Table 3.

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}/K_m$ (µM$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ Glu-Pg</td>
<td>3.66</td>
<td>0.95</td>
<td>3.85</td>
</tr>
<tr>
<td>No Pg</td>
<td>3.15</td>
<td>2.87</td>
<td>1.10</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue-type plasminogen activator</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu-Pg</td>
<td>Native or Glu(^1)-plasminogen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys-Pg</td>
<td>Plasmin cleaved or Lys(^{77/78})-plasminogen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pn</td>
<td>Plasmin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase-type plasminogen activator</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FDP</td>
<td>Fibrin degradation products</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAFI(a)</td>
<td>(Activated) thrombin activatable fibrinolysis inhibitor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDH</td>
<td>Saccharopine dehydrogenase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5IAF</td>
<td>5-iodoacetamidofluorescein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QSY</td>
<td>QSY9 C5-maleimide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VFK-ck</td>
<td>D-Val-Phe-Lys chloromethyl ketone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPA-ck</td>
<td>D-Phe-Pro-Arg chloromethyl ketone</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The kinetics of TAFIa-catalyzed cleavage of carboxy terminal lysine residues of fibrin degradation products and removal of plasminogen binding sites
Jonathan H. Foley, Paul F. Cook and Michael E. Nesheim

J. Biol. Chem. published online April 5, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M110.215061

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2011/04/05/M110.215061.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2011/04/05/jbc.M110.215061.full.html#ref-list-1