A Study of the Protection of Plasmin from Antiplasmin Inhibition within an Intact Fibrin Clot during the Course of Clot Lysis*

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Previous work using soluble fibrin surrogates or very dilute fibrin indicate that inhibition of plasmin by antiplasmin is attenuated by fibrin surrogates; however, this phenomenon has not been quantified within intact fibrin clots. Therefore, a novel system was designed to measure plasmin inhibition by antiplasmin in real time within an intact clot during fibrinolysis. This was accomplished by including the plasmin substrate S2251 and a recombinant fluorescent derivative of plasminogen (S741C-fluorescein) into clots formed from purified components. Steady state plasmin levels were estimated from the rates of S2251 hydrolysis, the rates of plasminogen activation were estimated by fluorescence decrease over time, and residual antiplasmin was deduced from residual fluorescence. From these measurements, the second order rate constant could be inferred at any time during fibrinolysis. Immediately after clot formation, the rate constant for inhibition decreased 3-fold from $9.6 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ measured in a soluble buffer system to $3.2 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ in an intact fibrin clot. As the clot continued to lyse, the rate constant for inhibition continued to decrease by 38-fold at maximum. To determine whether this protection was the result of plasmin exposure of carboxyl-terminal lysine residues, clots were formed in the presence of activated thrombin-activatable fibrinolysis inhibitor (TAFIa). In the presence of TAFIa, the initial protective effect associated with clot formation occurred; however, the secondary protective effect associated with lysine residue exposure was delayed in a TAFIa concentration-dependent manner. This latter effect represents another mechanism whereby TAFIa attenuates fibrinolysis.

As a fibrin clot forms in response to a vascular injury, it regulates its own degradation by serving as cofactor for plasminogen (Pgn)$^1$ activation to plasmin (Pn) (1). Once formed, Pn directly catalyzes clot breakdown by cleaving fibrin. In the process, carboxyl-terminal lysine residues are exposed that serve to enhance Pgn activation through a positive feedback mechanism for Pn formation (2). Once formed, Pn is quickly inhibited by the potent serine protease inhibitor antiplasmin (AP), which forms a tight nearly irreversible complex with Pn. Therefore, Pn levels and consequently the rate of fibrinolysis are determined by the balance between the kinetics of Pn formation and inhibition.

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a recently identified plasmazymogen (3–5) that can be activated to the carboxypeptidase B-like enzyme TAFIa by thrombin/thrombomodulin (6), free thrombin (3, 7), or plasmin (3, 8). In the context of a fibrin clot, TAFIa removes carboxyl-terminal lysine residues from Pn-modified fibrin (9). Although no naturally occurring inhibitors of TAFIa have been identified in human plasma, TAFIa is thermally unstable and this instability is probably how TAFIa is regulated once it is activated (10, 11). Previously, a naturally occurring polymorphism at amino acid position 325 (Thr/Ile) was identified that substantially affects the thermal stability of TAFIa (12). The more stable TAFI variant with Ile-325 (TAFI-I) had a half-life of 15 min at 37 °C, whereas the less stable TAFI variant with Thr-325 (TAFI-T) had a half-life of 8 min at 37 °C. When studied using an in vitro plasma clot lysis assay, this increased stability also corresponded to a more potent clot-stabilizing effect. TAFIa-T can prolong clot lysis time by 2.7-fold at saturation, whereas TAFIa-I can prolong clot lysis by 4-fold (12). Therefore, the carboxypeptidase B-like activity of TAFIa stabilizes fibrin clots and thermal stability is a potent regulator of this antifibrinolytic potential.

Carboxyl-terminal lysine residues are important to the regulation of fibrinolysis because many key members of the fibrinolytic cascade, such as Pgn, Pn, and tissue-type plasminogen activator (tPA), contain kringle domains that mediate binding to the fibrin surface through exposed lysine residues (1, 2, 13, 14). Previously, it was shown that TAFIa removal of these carboxyl-terminal lysine residues from fibrin removes the positive feedback for Pgn activation on a Pn modified fibrin surface (9). Thus, TAFIa modulates fibrinolysis, in part at least, by regulating Pn levels through attenuation of Pgn activation.

Studies using soluble fibrin derivatives have demonstrated that when Pn is bound to fibrin, it is much less susceptible to inhibition by AP (15–19). Although Pn protection from AP is potentially an important mechanism for regulating Pn levels, previous work has been hampered by the gel-like nature of native fibrin. In this study, we have designed a novel system to study protection within an intact fibrin clot in real time. To do this, identical clots were formed in the presence of the chromogenic Pn substrate S2251 and in the presence of a fluorescent Pgn derivative in which the active site serine was mutated to

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* The abbreviations used are: Pgn, plasminogen; Pn, plasmin; tPA, tissue-type plasminogen activator; AP, antiplasmin; TAFI, thrombin-activatable fibrinolysis inhibitor; TAFIa, activated TAFI; TAFI-I, TAFI variant with Thr-325; TAFI-T, TAFI variant with Ile-325; 5IAF-Pgn, fluorescein-labeled recombinant (S741C) Pgn; 5IAF-Pn, fluorescein-labeled recombinant (S741C) Pn; $r_{inh}$, rate of inhibition; $r_{F}$, rate of formation; $k_{obs}$, rate of inhibition; $k_{r}$, change in fluorescence; $dt$, change in time.
cysteine and labeled with fluorescein (5IAF-Pgn) (20, 21). One clot was monitored by fluorescence, which can be used to estimate the rate of Pn formation and the residual concentration of AP. The other clot was monitored by absorbance, which can be used to estimate the concentration of Pn at any time. By knowing the rate of Pn formation and the concentrations of Pn and AP, the second order rate constant for Pn inhibition by AP can be calculated. during fibrinolysis. In this model, it was further confirmed that modification of fibrin by Pn dramatically increased the ability of fibrin to protect Pn from inhibition. This protective effect was further characterized by forming clots in the presence of TAFIa, and it was found that TAFIa could effectively attenuate protection in a manner that depends on the concentration and thermal stability of the TAFIa isoform used. Therefore, for the first time, Pn protection from AP was measured in an intact clot and the relative roles of intact fibrin, Pn-modified fibrin, or TAFIa treated fibrin were quantified.

EXPERIMENTAL PROCEDURES

Materials—Human thrombin was prepared from plasma-derived prothrombin as described previously (22, 23). Pgn was isolated from human plasma as described previously, some of which was used to prepare human Pn (23). Baby hamster kidney cells and the pNUT expression vector were a kind gift from Dr. Ross MacGillivray (University of British Columbia, Vancouver, British Columbia, Canada). Recombinant human antithrombin was expressed in baby hamster kidney cells and isolated as described previously (3). The recombinant human Pgn (5741C) described by Horrevoets et al. (20, 21) was active site-labeled with 5-iodoacetamidofluorescein to form the fluorescent catalytically inactive Pgn derivative, 5IAF-Pgn. Solulin, a soluble derivative of thrombomodulin, was a kind gift from Dr. Olivier Kops (Paiion, GmbH, Berlin, Germany). Recombinant human TPA was a kind gift from Dr. Gordon Vehar (Genentech, Inc. South San Francisco, CA). p-Val-Phe-Lys chromothromin ketone and 5-dimethylaminophenyl-1-sulfonyl-n-D-Ala-Gly-Arg chromothromin ketone were purchased from Calbiochem.

Isolation of Fibrinogen from Human Plasma—Human fibrinogen was isolated from fresh frozen human plasma using the method described by Walker et al. (23) with the following modification. Instead of a 2% polyethylene glycol 8000 cut subsequent to the b-alanine precipitation, a final concentration of 1.2% polyethylene glycol 8000 was used. After isolation, Pgn was dialyzed into 0.02M HEPES, 0.15 M NaCl, pH 7.4, aliquoted, and stored at −70 °C until use. Recombinant TAFI Isolation and Activation—Two variants of wild-type human TAFI, the less stable TAFI-T variant (TAFI with a threonine at position 325) and the more stable TAFI-I variant (TAFI with an isoleucine at position 295), were expressed in baby hamster kidney cells using the pNUT expression vector as described previously (7, 24). Conditioned medium was collected, and the TAFI variants were isolated using affinity column chromatography (12), aliquoted, and stored at −20 °C until use. To form TAFIa, 1 µM TAFI-T or TAFI-I was incubated with thrombin (25 nm), solulin (100 nm), and CaCl2 (5 mM) for 15 min at 25 °C. The mixture was then placed on ice until use to eliminate the spontaneous thermal inactivation of the enzyme.

Determination of the Moment to Moment Second Order Rate Constant for Pn Inhibition during Fibrinolysis—in this study, a novel system was designed for monitoring the second order rate constant for Pn inhibition by AP (kh) within an intact fibrin clot during the course of clot lysis. To do this, identical fibrin clots were formed from purified components in the wells of a clear 96-well plate and in the wells of an opaque 96-well Microfluor plate. In each case, clots were formed by adding thrombin (5 nm) and CaCl2 (5 mM) to fibrinogen (4 µM), AP (0.5 µM), Pgn (0.5 µM), 5IAF-Pgn (0.5 µM), S2251 (400 µM), and TPA (2 µM). Clots formed in the clear plate were monitored by absorbance in a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). Because 5IAF-Pgn cleavage to 5IAF-Pn by TPA resulted in a substantial loss of fluorescence (typically 45–50%), this derivative can be used to estimate the moment to moment rates of Pn formation and the residual AP concentration during fibrinolysis. Because of the large turbidity changes associated with clot formation and clot lysis, kh was estimated only within the intact clot subsequent to complete clot formation and prior to the beginning of clot lysis. All of the reactions were covered to minimize evaporation and were incubated at 25 °C.

Model—This model assumes a steady state Pn level within a fibrin clot over the time intervals. The presence of Pgn, AP, and TPA, Pn is continually being formed on the fibrin and simultaneously consumed by AP. Therefore, the rate of change in the concentration of Pn at any time during clot lysis is a result of the difference between the rate of formation (rform) and the rate of inhibition (rinh) by AP, which is described by Equation 1.

\[
\frac{d[Pn]}{dt} = r_{form} - r_{inh} \tag{1}
\]

Where the rate of change of Pn concentration within a clot over time is as shown in Equation 2.

\[
\frac{d[Pn]}{dt} = r_{form} - r_{inh} \tag{2}
\]

Because rinh depends on the concentrations of AP and Pn, the equation can be expressed as shown in Equations 3.

\[
\frac{d[Pn]}{dt} = r_{form} - k_h \times [Pn] \times [AP] \tag{3}
\]

In the steady state, the concentration of Pn within the clot is designated [Pn]ss and Equation 3 can be expressed as follows in Equation 4.

\[
0 = r_{form} - k_h \times [Pn]_{ss} \times [AP] \tag{4}
\]

Finally, rearranging Equation 4 gives k_h, the second order rate constant for Pn inhibition in terms of [Pn]ss, the rinh, and residual AP concentration as shown in Equation 5.

\[
k_h = \frac{r_{form}}{[AP]} \tag{5}
\]

Because rinh, [AP], and [Pn]ss can be measured during the course of fibrinolysis, k_h can be calculated in real time within an intact fibrin clot.

Determination of the Steady State Concentration of Pn—The fibrin clot, monitored by absorbance, exhibited changes in the absorbance measured at 405 nm because of both S2251 hydrolysis and turbidity associated with changes in clot structure as it lysed. Therefore, to determine the change in absorbance due only to S2251 hydrolysis, the absorbance of the clot was measured at both 405 and 650 nm and the change in absorbance at 650 nm was used to decouple the rate of S2251 hydrolysis from the turbidity of the clot. Turbidity measured at 405 nm was empirically found to be 2.75 times greater than turbidity measured at 650 nm. To determine the corrected rate of change at 405 nm only due to substrate hydrolysis, the rate of change measured at 650 nm was multiplied by the 2.75 correction ratio and subtracted from the rate of change measured at 405 nm.

To be able to correlate the rate of S2251 hydrolysis to the total Pn present within an unknown clot at any time, a standard curve was made by forming clots in the presence of known concentrations of Pn and in the absence of AP. To do this, a series of clots were formed in a clear microtiter plate by adding thrombin (5 nm) and CaCl2 (5 mM) to Fgn (4 µM), S2251 (400 µM), and increasing concentrations of purified human Pn (0–20 nm). The clots were monitored by absorbance at 405 and 650 nm, and the rates of S2251 hydrolysis were found by determining the slope of the change in absorbance versus change in time ratio at 405 nm over a 600-s interval subsequent to complete clot formation. The turbidity-corrected rate of change in absorbance was calculated as described and plotted against time. The slope of the relationship could then be used to derive the [Pn] from the rate of substrate hydrolysis at any time in a lysing fibrin clot.

In the assay, Pn concentrations were calculated at 300-s time intervals. The [Pn] at each point was approximated by linear regression of all the points from −300 s onwards from each point. The 600-s interval proved small enough that the regression lines were generally nearly linear. By comparing the calculated slopes to the standard curve, the corrected rates of S2251 hydrolysis can be used to determine the [Pn] in a fibrin clot at any time. Equation 6 can then be used to estimate the [Pn]ss from...
used to calculate point was approximated by linear regression of all of the points.

\( t \) was measured every 300 s. The \( dF/dt \) at each point was approximated by linear regression of all of the points.

Fluorescence showed a decrease associated with conversion of 5IAF-Pgn substrate hydrolysis by thrombin.

**Equation 7.**

\[
[Pn]_{ss} = \left( 1 - \frac{[S2251]}{K_m + [S2251]} \right) \times [Pn] \quad (\text{Eq. 6})
\]

The conditions of the assay have been chosen so that the change in substrate concentration was no more than 15% over the course of fibrinolysis, and the concentration of S2251 was assumed to be constant.

Although the assay was performed in a system of purified components, thrombin was another serine protease present that could slowly hydrolyze S2251 and skew the results slightly, especially when there was little Pn present. Therefore, for each set of experiments, a control clot was formed by adding thrombin (5 nM) and CaCl₂ (5 mm) to Fgn (4 μM) and S2251 (400 μM) in the absence of tPA to control the potential substrate hydrolysis by thrombin.

**Determination of the Rate of Pn Formation—**The clot monitored by fluorescence showed a decrease associated with conversion of 5IAF-Pgn to 5IAF-Pn. In a fibrin clot, the rate of the change in fluorescence versus change in time (dF/dt) was measured every 300 s. The dF/dt at each point was approximated by linear regression of all of the points ±300 s from each point. The 600-s interval proved small enough that the regressed points were always nearly linear. The measured dF/dt can be used to calculate \( r_{form} \), as follows where \( F_{max} \) is the initial fluorescence and \([5IAF-Pgn]_0 \) is the initial 5IAF-Pgn concentration as shown in Equation 7.

\[
r_{form} = \frac{dF}{dt} = \frac{2 \times [5IA - Pgn]_0}{F_{max}} \quad (\text{Eq. 7})
\]

Because Pgn and 5IAF-Pgn are cleaved with similar kinetics, the \( r_{form} \) can be used to estimate the rate of Pn formation and the rate of 5IAF-Pn formation.

**Determination of the Residual Concentration of AP—**The residual fluorescence can also be used to estimate the amount of AP present. Complete cleavage of the 5IAF-Pgn corresponds to complete activation of the Pgn to Pn. Once formed, the Pn is quickly inhibited by AP and most of the Pgn that has been activated will already have been inhibited. Therefore, the residual fluorescence can be measured at any time \( (F_{measured}) \) to approximate the amount of AP remaining as shown in Equation 8.

\[
[AP] = \frac{\left( F_{measured} - 0.5 \times F_{max} \right)}{0.5 \times F_{max}} \times [AP]_0 \quad (\text{Eq. 8})
\]

The Effect of TAFIa on \( k_2 \) within Intact Clots—Either TAFIa-T or TAFIa-I was activated as described and placed on ice until use. A series of fibrin clots were formed as described in the presence of increasing concentrations of TAFIa-T (0, 0.2, 0.4, 0.6, and 0.8 nM) or TAFIa-I (0, 0.1, 0.2, 0.3, and 0.4 nM). Identical clots were monitored in each case by absorbance and fluorescence, and the \( r_{form} \) and \([Pn]_{ss} \) values were measured within the intact clots at 300-s intervals as described. Using Equation 5, \( k_2 \) values were calculated during the course of clot lysis.

A limitation of the current study was the constraints placed on the concentrations of the reagents used; specifically, the concentrations of tPA and TAFIa-T that were added. By the nature of the system, there was very little Pn present during the initial part of the experiment, and in the presence of TAFIa, there was notably less. Therefore, measuring substrate hydrolysis at this stage was difficult unless an appreciable concentration of activator was used. However, although increased concentrations of tPA made the initial rates of Pn formation easy to measure reliably, Pgn was also consumed faster, resulting in slower rates near the end of the experiment. Therefore, the tPA concentration and TAFIa-T concentrations were decided upon by titrations that determined conditions that allowed reliable measurements of all of the variables during the entire course of the experiment both in the presence and absence of TAFIa. It was for this reason that only half of the concentration range of TAFIa-I compared with TAFIa-T was used in the experiment. Higher concentrations of either TAFIa variant (greater than 1 nM TAFIa-T and greater than 0.5 nM TAFIa-I) in the presence of the 2 nM tPA resulted in initial rates of substrate hydrolysis that were very small and difficult to measure reliably. The concentrations were chosen to allow both the progress of \( k_2 \) within an intact clot to be measured and the effect of TAFIa to be measured over a concentration that is likely to be physiologically relevant (clot lysis assays show a half-maximal effect at approximately 1 nM TAFIa-T).

**RESULTS**

To observe Pn inhibition by AP, clots were formed from purified components in the presence of the Pn substrate S2251 and the fluorescent catalytically inactive plasminogen derivative, 5IAF-Pgn. By monitoring identical clots by absorbance and fluorescence in tandem \( r_{form} \), \([Pn]_{ss} \) and the [AP] could be measured within an intact clot and, from this, \( k_2 \) could be calculated in real time using Equation 5.

An example of the raw data obtained by monitoring the change in absorbance of the clot at 405 and 650 nm is shown in Fig. 1 where Fig. 1A is the absorbance profile in the absence of TAFIa and Fig. 1B is the absorbance profile in the presence of TAFIa-T. In both cases, clot formation was associated with a large increase in turbidity that can be seen at both 405 and 650 nm. In the first 600 s, as the fibrin clot formed, it was difficult to consistently measure S2251 hydrolysis against the large background change in turbidity. Similarly, as the clot lysed, there was a pronounced decrease in turbidity, which is apparent in Fig. 1A, after approximately 14,000 s. Therefore, to reliably measure \([Pn]_{ss} \) all of the inhibition studies were con-

![Figure 1](http://www.jbc.org/Downloaded from http://www.jbc.org)
fined to the time interval subsequent to clot formation and prior to clot lysis.

An example of the raw data obtained for clots monitored by fluorescence is shown in Fig. 2. As the fluorescent plasminogen derivative 5IAF-Pgn was cleaved to form the fluorescent Pn derivative 5IAF-Pn, there was a pronounced decrease in fluorescence that corresponded to the conversion. Comparing the profile of 5IAF-Pgn cleavage in the absence of TAFIa-T (Fig. 2A) to the profile obtained in the presence of TAFIa (Fig. 2B), it is clear that TAFIa alters the rate of Pgn cleavage by removing the positive feedback associated with Pn modification of the fibrin clot (9). From the fluorescence data, the $r_{\text{form}}$ and the residual $|\text{AP}|$ can be estimated at any time as described under “Experimental Procedures.”

**Protection of Pn from AP within an Intact Fibrin Clot**—Using the raw absorbance data shown in Fig. 1 and the raw fluorescence data shown in Fig. 2, [Pn]$_{\text{ss}}$, $r_{\text{form}}$, and $|\text{AP}|$ were determined during the course of clot lysis. These are shown in Fig. 3, A–C, respectively. The data from Fig. 3, A–C, were also used to calculate $k_2$ in real time using Equation 5 as described. This is shown as Fig. 3D.

In the absence of TAFIa, subsequent to clot formation, $k_2$ was found to be $3.2 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$. The $k_2$ obtained in the absence of fibrin was also measured in a soluble buffer system and was found to be $9.6 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ (data not shown), which is very similar to values measured for this interaction previously (15–17, 25, 26). Therefore, intact fibrin, prior to modification by Pn, mediates a 3-fold protective effect. As clot lysis progressed, $k_2$ continued to decrease dramatically. At maximum, $k_2$ diminished to $0.25 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$, corresponding to a 38-fold protective effect. Therefore, although intact fibrin mediates only a modest protective effect, Pn-modified fibrin is a potent protector of Pn, dramatically slowing the rate of inhibition.

The progress of $k_2$ in the presence of TAFIa-T is very different. Upon clot formation, $k_2$ is estimated to be $3 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$, similar to clots formed in the absence of TAFIa; however, in the presence of TAFIa-T, the dramatic increase in the protective effect associated with Pn modification of the clot was delayed. Presumably, as Pn exposed carboxyl-terminal lysine residues, TAFIa-T removed them and the protective capacity of this TAFIa treated fibrin was similar to fibrin prior to Pn modification. Therefore, within an intact fibrin clot, TAFIa not only slowed the rate of Pn formation but also made the fibrin surface less able to protect Pn from AP. As expected, this attenuation of the protective properties of Pn-modified fibrin did not persist indefinitely. Even at 25°C, TAFIa-T was thermally unstable and the enzyme had a half-life of 75 min. Eventually, the attenuation of protection was lost, and a point was reached where the concentration of active enzyme was not high enough to remove all of the lysine residues being exposed by Pn. At this point, $k_2$ began to decrease and the protection of Pn by fibrin increased to a magnitude similar to the clots lysed in the absence of TAFIa. A comparison of Fig. 3, C and D, shows that as the TAFIa activity was lost, the steady state Pn levels began to increase in the clot, most probably corresponding to the increased cofactor activity of the fibrin surface and protection of Pn from inhibition.

**The Effect of TAFIa-T and TAFIa-I on Pn Protection by Fibrin**—The thermal stability of TAFIa has been shown to modulate its antifibrinolytic effect when studied using in vitro clot lysis models (3, 12, 14). Therefore, with respect to altering the protective properties of Pn modified fibrin, the ability of TAFIa should depend on both the concentration and the thermal stability of the TAFIa variant used. To study this effect, a series of experiments were performed with increasing concentrations of the less stable TAFIa-T variant that had a half-life of 75 min at 25°C and the more stable TAFIa-I variant that had a half-life of 147 min at 25°C (12). Fig. 4 shows the dramatic effect that TAFIa had on suppressing the [Pn]$_{\text{ss}}$ within fibrin clots. It is interesting to note that although parts A and B of Fig. 4 appear similar, there is only half as much of the more stable TAFIa-I variant used to mediate nearly the same effect as the less stable TAFIa-T variant. Therefore, TAFIa suppressed Pn formation in fibrin clots in a manner that depended on the overall activity of the TAFIa added. More TAFIa or TAFIa with a prolonged half-life was more able to suppress [Pn]$_{\text{ss}}$.

Data from the same experiments were also used to compare the effects of the less stable TAFIa-T variant (Fig. 5A) and the more stable TAFIa-I variant (Fig. 5B) on the $k_2$ during clot lysis. In each case, subsequent to clot formation, the $k_2$ decreased roughly 3-fold ($2.5-3.5 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$), and in each case, the protection eventually increased 30–40-fold at maximum. However, the time scale of this decrease depended on the concentration of TAFIa added and the thermal stability of the variant used. For example, 0.2 nM TAFIa-T attenuated the increase in protection only modestly, whereas 0.2 nM TAFIa-I attenuated the protection much more efficiently. Nearly, the same effect on protection was observed in Fig. 5, compare A with B, even though we have used only half of the concentra-
DISCUSSION

In this study, an intact fibrin clot model was designed to study Pn protection from inhibition by AP. Subsequent to fibrin clot formation, $k_2$ decreased 3-fold from $9.6 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ to $3 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$. As clot lysis continued, $k_2$ eventually decreased by 38-fold overall to $0.25 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$. To demonstrate that this increase in protection was attributed to Pn exposure of carboxyl-terminal lysine residues, experiments were also performed in the presence of TAFIa. It was found that TAFIa can attenuate protection in a lysing fibrin clot, and this attenuation depends on the concentration and thermal stability of the TAFIa variant added. In the presence of TAFIa, $k_2$ decreased 3-fold, similar to clots lysed in the absence of TAFIa; however, TAFIa then attenuated the progressive decrease in the $k_2$, maintaining the protective properties of the clot at those of intact unmodified fibrin. This attenuation of protection was not absolute, and eventually, protection increased after a delay that depended on the concentration of TAFIa added. Because TAFIa was thermally unstable, activity of the enzyme will be lost over time and, eventually, there will not be enough TAFIa activity present to remove the lysine residues as they are exposed. In corroboration of this finding, a comparison of assays done with the more stable TAFIa-I variant compared with the less stable TAFIa-T variant shows that TAFIa-I has an enhanced ability to attenuate protection.

Under the conditions used in the assay, the maximum protection was found to be 38-fold, which is in close agreement with previous work that has found that soluble fibrin surrogates can protect Pn by 10–45-fold (15–19). A model using immobilized fibrin was also used to study protection; however, with this model, the protection was found to be 398-fold (27), which is 10 times more potent than the effect measured with the intact clot model. Until now, studies on the protection of Pn from AP have been essentially end point studies on a single fibrin state. An advantage of this work is that the end result of the dynamic balance of Pn formation and inhibition and how this changes during the course of fibrin modification and breakdown can be observed. By measuring the $k_2$ in real time, the dramatic role that carboxyl-terminal lysine residue exposure has on protecting Pn becomes apparent. Under the conditions used in this assay, the modified fibrin surface is 10 times better at protecting Pn than the unmodified surface. Moreover, by including TAFIa, the effect of lysine residue exposure can be countered.

Previous work has demonstrated that AP has a critical carboxyl-terminal lysine residue that can be removed by carboxypeptidase B and is therefore a potential target for TAFIa. The original work on this phenomenon used peptides corre-
sponding to the carboxyl terminus of the AP (28); however, more recent work with recombinant AP variants that have the carboxyl-terminal lysine mutated still retains much of the ability of the AP to inhibit Pn (17). If TAFIa removal of lysine from the carboxyl terminus of AP were affecting the inhibitory potential of AP in this system, increased concentrations of TAFIa would correspondingly decrease $k_2$; however, the opposite effect was observed within the intact clots studied here. TAFIa maintained the system in a decreased protective state, and the greater the concentration of TAFIa added, the longer this decreased protection can be maintained. In the context of this study, either TAFIa was not included at high enough concentrations to appreciably modify the large AP pool or, alternatively, TAFIa may have had little or no activity toward AP. In either case, this potential side reaction was not observed. Therefore, this work provides some functional corroboration to the idea that TAFIa has little effect on the inhibitory capacity of AP, which makes sense when the antifibrinolytic role of TAFIa is considered.

For the first time, the protection of Pn from AP within an

**Fig. 4.** The effect of the less stable TAFIa-T and the more stable TAFIa-I variants on the steady state concentration of Pn during clot lysis. A, clots were formed in the presence of 0 nM (closed circles), 0.2 nM (open squares), 0.4 nM (closed squares), 0.6 nM (open triangles), and 0.8 nM (closed triangles) of the less stable TAFIa-T variant. The [Pn]$_{ss}$ was estimated as described during the course of clot lysis. B, similarly, clots were formed in the presence of the 0 nM (closed circles), 0.1 nM (open squares), 0.2 nM (closed squares), 0.3 nM (open triangles), and 0.4 nM (closed triangles) of the more stable TAFIa-I variant. The [Pn]$_{ss}$ was estimated as described during the course of clot lysis. TAFIa has a concentration dependent effect on the [Pn]$_{ss}$ within the clots, and the more stable variant is nearly twice as potent as the less stable variant.

**Fig. 5.** The concentration and stability of TAFIa modulates the ability of a lysing clot to protect Pn from AP. A, clots were formed in the presence of 0 nM (closed circles), 0.2 nM (open squares), 0.4 nM (closed squares), 0.6 nM (open triangles), and 0.8 nM (closed triangles) of the less stable TAFIa-T variant. B, similarly, clots were formed in the presence of the 0 nM (closed circles), 0.1 nM (open squares), 0.2 nM (closed squares), 0.3 nM (open triangles), and 0.4 nM (closed triangles) of the more stable TAFIa-I variant. The $k_2$ at each concentration of TAFIa is plotted against time. TAFIa modulation of $k_2$ depends on the concentration and thermal stability of the TAFIa variant added to the system.

**Fig. 6.** Concentration dependence of the TAFIa-mediated alteration of $k_{2a}$. The ability of TAFIa-T (closed circles) or TAFIa-I (open circles) to attenuate the decrease in $k_2$ was estimated from the plots shown in Fig. 5. The time to half-maximal protection was estimated graphically and plotted against TAFIa concentration for each variant.
intact fibrin clot has been characterized and how this dramatically alters steady state Pn levels within a clot has been demonstrated. This work shows that while intact fibrin is able to modestly protect Pn, modification of fibrin by Pn serves as a potent protective surface, further emphasizing the importance of this feedback mechanism to fibrinolysis. In addition, these studies have demonstrated that increased protection associated with the exposure of the lysine residues is reversible and a novel means by which TAFIa could modulate clot stability has been identified.

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