Activated Thrombin-activatable Fibrinolytic Inhibitor Reduces the Ability of High Molecular Weight Fibrin Degradation Products to Protect Plasmin from Antiplasmin*

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Activated thrombin-activatable fibrinolysis inhibitor (TAFIa) is a carboxypeptidase B-like plasma enzyme that can slow clot lysis by removing lysine residues exposed on fibrin as it is cleaved by plasmin. Previously, it was shown that fibrin treated with TAFIa is less able to promote plasminogen activation by tissue-type plasminogen activator. In this study, the effect of TAFIa modification of a fibrin surface on the rate of plasmin inhibition by antiplasmin was studied using high molecular weight fibrin degradation products (HMw-FDPs) as a soluble model for intact plasmin-modified fibrin. To quantify the inhibition, a novel end point assay was employed where plasmin, antiplasmin, and cofactors were mixed in the presence of a chromogenic substrate and a chromogenic substrate for plasmin and antiplasmin, the rate constant for inhibition by antiplasmin was measured to determine the second order rate constant of inhibition. When HMw-FDPs were titrated in the presence of plasmin and antiplasmin, the rate constant for inhibition decreased by 16-fold at saturation (0.6 × 10⁶ M⁻¹ s⁻¹ to 0.59 × 10⁶ M⁻¹ s⁻¹). When HMw-FDPs were pretreated with TAFIa, nearly two-thirds of the protective effect was lost. When TAFIa was included in an in vitro plasma clot lysis assay, clots formed in the presence of TAFIa lysed 3–4 times more slowly than clots lysed in the absence of TAFIa (8, 14), highlighting the importance of fibrin modification to the stability of fibrin clots. Previous work has demonstrated that TAFIa modification of a partially Pla degraded clot slows the rate of Pla activation by 3-fold (1), effectively removing the positive feedback associated with carboxyl-terminal lysine exposure. Whether TAFIa treatment of fibrin also has an effect on the rate of Pla inhibition by AP has not been addressed.

Because a fibrin clot is a gel, carrying out kinetics experiments on intact fibrin was problematic. Previous work on the role of fibrin in Pla protection has utilized a variety of fibrin surrogates such as fibrin immobilized on a surface (15) or small soluble fibrin degradation products (5, 6). In this study, high molecular weight fibrin degradation products (HMw-FDPs) have been isolated to be used as a model for Pla-modified fibrin. An advantage of HMw-FDPs is that although they are soluble, they are similar to intact fibrin with respect to their ability to support Pla activation with a variety of fibrin-dependent

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The abbreviations used are: Pla, plasmin; Pla: tissue-type plasminogen activator; TAFI, thrombin activatable fibrinolytic inhibitor; TAFIa, activated TAFI; HMw-FDPs, high molecular weight fibrin degradation products; DSPAco1, D. rotundas saliva plasminogen activator; 5IIF-Pla, recombinant Pla (5741C) covalently labeled with fluorescein; PTCI, potato tuber carboxypeptidase inhibitor; Val-Pc, Pn truncated by elastase; AP, antiplasmin.
activators, including tPA and Desmodus rotundas saliva plasminogen activator α1 (DSPAα1) (16). Moreover, because they are derived from a Pn-degraded clot, the HMw-FDPs possess the carboxy-terminal lysine residues exposed during clot lysis, which are the target for TAFIa.

In this study, intact HMw-FDPs or HMw-FDPs pretreated with TAFIa were incubated in the presence of Pn and AP and the rate of inhibition was measured. To efficiently quantify the rate of Pn inhibition by AP under a variety of conditions, a modified version of a novel end point method described previously (17) was employed. In the end point assay, Pn, AP, and a chromogenic substrate (S2251) were incubated in the presence or absence of the relevant cofactors (HMw-FDPs or TAFIa pretreated HMw-FDPs) and the substrate hydrolysis by Pn was monitored by absorbance. The end point of the reaction depends on the rate of inhibition, and we can use this to measure the second order rate constant for Pn inhibition by AP.

Similar to results obtained with other soluble fibrin models, HMw-FDPs effectively slow the rate of Pn inhibition by AP; however, when HMw-FDPs were preincubated with TAFIα, we found that the rate of Pn was nearly restored. Therefore, this work demonstrates that TAFIα removed the ability of HMw-FDPs to protect Pn, thereby identifying a novel mechanism where TAFIα can modulate Pn levels by increasing the rate of Pn consumption by AP.

**EXPERIMENTAL PROCEDURES**

*Materials*—Human thrombin was prepared from plasma derived pro-thrombin as described previously (18, 19). Pn was isolated from human plasma as described previously, some of which was used to prepare human Pn (19). Val-Pn, a truncated elastase-treated derivative of Pn, was formed from human Pn as described previously (19). 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 antiplasmin was expressed in baby hamster kidney cells and isolated as described previously (8). The recombinant human Pn (S741C) described by Horrovoets et al. (1, 20) was active site-labeled with 5α-fluorescein to form the fluorescent catalytically inactive Pn derivative, 5αF-Pgn. Solulin, a soluble derivative of thrombomodulin, was a kind gift from Dr. Oliver Kops (Paion, GmbH, Berlin, Germany). Recombinant human tPA was a kind gift from Dr. Gordon Vehar (Genentech, Inc. South San Francisco, CA). DSPAα1 was a kind gift from Dr. Peter Bringmann (Schering, AG, Berlin, Germany). Hirudin, n-Val-Phe-Lys chloromethyl ketone, n-Phe-Pro-Arg chloromethyl ketone, and 5-dimethylaminonaphthalene-1-sulfonyldansyl-D-Glu-Gly-Arg chloromethyl ketone were purchased from Calbiochem.

*Preparation of Human Fibrinogen*—Human fibrinogen was isolated from fresh frozen human plasma by the method described by Walker et al. (19) with the following modification. Instead of a 2% polyethylene glycol 8000 cut subsequent to the β-alanine precipitation, a final concentration of 1.2% polyethylene glycol 8000 was used. After isolation, Pfn was dialyzed into 0.02 M HEPES, 0.15 M NaCl, pH 7.4 (HBS), aliquoted, and stored at −70 °C until use.

*Preparation and Isolation of HMw-FDPs*—HMw-FDPs were derived from the lysis of purified fibrin clots and separated by size using methods similar to those described previously (19). To do this, a 6-ml mixture of purified fibrin clots and separated by size using methods similar to those described previously (19). To do this, a 6-ml was mixed vigorously for 5 min. The NaCl concentration was adjusted to 0.5 M, and then the mixture was centrifuged at 5000 × _g_ at 4 °C for 10 min. The supernatant was decanted and passed over a 30-ml Sephacryl S-1000 gel filtration column by fast protein liquid chromato-
reaction, $S_0$ is the amount of substrate remaining at any time, $S_t$ is the input concentration of S2251, and $AP_0$ is the input concentration of AP.

$$\ln \left( \frac{AP_0 - Pn}{AP_t} \right) = k_2 \cdot \ln \left( \frac{S_t}{S_0} \right)$$

(Eq. 4)

Although it is possible to measure $k_2$ at any time with this model, it is difficult to reliably know the PNAP complex concentration at any time other than at the end of the reaction where it is equal to the input PN concentration. Therefore, to solve for $k_2$, the substrate remaining at the end of the reaction ($S_0$) is measured and the residual AP concentration is inferred ($AP_0$) from the input concentrations of AP and PN ($AP_0 = AP_t - Pn$) as shown in Equation 5.

$$k_2 = \frac{\ln(AP_0/AP_t)}{\ln(S_t/S_0)} \times \frac{K_f}{K_S}$$

(Eq. 5)

As long as the starting concentration of PN (PN0) is less than the starting concentration of AP ($AP_0$), and as long as the inhibition reaction is fast relative to the rate of substrate hydrolysis by PN, the reaction will eventually go to completion before all of the substrate is consumed. In practice, $S_t$ is determined by subtracting the change in absorbance measured at the end point reaction from the change in absorbance that would be expected if all of the substrate was converted to product. Therefore, $k_2$ can be derived from Equation 5 simply by measuring the change in absorbance measured in the presence of PN and AP.

End point assay to monitor the $k_2$ for PN inhibition by AP—To be able to measure $k_2$, Equation 5 requires the $K_{cat}$ and $K_S$ values for PN hydrolysis of S2251. To do this, S2251 at increasing concentrations (0–800 μM final concentration) was incubated with PN (20 μM final concentration) in a final volume of 160 μL. The reactions were incubated at 25 °C and monitored at 405 nm. The initial rates of substrate hydrolysis were measured using the Spectramax Plus software (Molecular Devices, Sunnyvale, CA) and converted to $ν$ (molecular product formed per second per mol of PN). The data were then plotted as $ν$ versus starting concentration of S2251, and the $K_{cat}$ and $V_{max}$ values were estimated by non-linear regression of the hyperbolic function using SigmaPlot 6.0 (SPSS Science Inc.). Although some serine proteases can be inhibited by non-competitive inhibition, it is inferred (AP) from the input concentration of AP and PN ($AP_0 = AP_t - Pn$) as shown in Equation 5.

To study the effect of TAFI pretreatment of HMw-FDPs—To form TAFI, 1 μM TAFI was incubated with thrombin (25 nM), solulin (100 nM), and CaCl2 (5 mM) in 50 mM NaOH for 5 min at 25 °C. Reactions were then quenched by adding 450 μl of Hirudin (500 nM final concentration), and the enzyme was placed on ice until use to minimize the spontaneous thermal inactivation of the enzyme.

To measure the effect of TAFI on plasmin inhibition, two identical sets of 100-μl aliquots of HMw-FDPs (0–800 nM) were diluted in the wells of a microtiter plate. To each well of one set, 10-μl aliquots of TAFI (100 nM final concentration) were added and the reactions were quenched with 10 μl of PTCI and allowed to stand for an additional 2 min. Finally, the second set of reactions, which had PTCI but no TAFI, was mixed with 10 μl of TAFIa. All of the reactions were then diluted with 30 μl of a solution containing S2251 (2133 μM), AP (213 nm), and CaCl2 (26.7 mM), mixed thoroughly, and transferred to a Spectramax Plus plate reader. The samples were monitored at 405 nm, and the reactions were started by adding 10 μl of Pn (20 nm final concentration) to each well. After the reactions had gone to completion, the $k_2$ values were estimated from the measured absorbances as described above.

Time course of TAFIa modulation of HMw-FDPs—Into the wells of a microtiter plate, 100-μl aliquots of HMw-FDPs (800 nM) were mixed with 10-μl aliquots of TAFIa (0–5 nM final concentration). The reactions were allowed to incubate for increasing times (0–15 min) and were then quenched by adding 10 μl of PTCI (5 μM). The reactions were allowed to incubate for an additional 2 min to allow inhibition of the TAFIa by PTCI and then were diluted with 30 μl of a solution containing S2251 (2133 μM), AP (213 nm), and CaCl2 (26.7 mM). The plate was transferred to a Spectramax Plate reader, and the absorbances of the reactions were monitored simultaneously at 405 nm. Reactions were started by adding 10 μl of Pn (20 nm final concentration) to each well. And after the reactions had gone to completion, the $k_2$ values were estimated from the measured absorbances as described using Equation 5.

RESULTS

End point model used to study Pn inhibition by AP—HMw-FDPs were prepared by partially lysing a fibrin clot, separating the soluble fraction by centrifugation, and purifying the high molecular weight fragments using gel filtration chromatography. Fractions containing high molecular weight fragments were pooled and concentrated, and the size distribution was measured using multi-angle laser light scattering. The average molecular mass of the HMw-FDPs was 2 × 10^6 daltons (data not shown). Isolated HMw-FDPs were used as a model of modified fibrin.

Previously, a novel end point assay was developed to efficiently study inhibition kinetics (17). In this study, a modified version of this model has been derived that allows direct measurement of $k_2$. The rationale for this model is outlined under “Experimental Procedures.” If the model is valid, it predicts that the estimated $k_2$ value should remain constant even when the input concentrations of Pn and AP are varied. Therefore, in Fig. 1A, the AP concentration was varied nearly 2-fold while keeping the Pn concentration constant and, as expected, the $k_2$ values remained constant over this range. Similarly, in Fig. 1B, the Pn concentration was varied 3-fold while the AP concentration was kept constant and only a slight decrease in the $k_2$ values over the concentrations used was measured. These experiments demonstrate that, as predicted by the model, the $k_2$ value is insensitive to changes in the relative concentrations of Pn and AP.

$H_{Mw}$-FDPs protect Pn from inhibition by AP—Previous work using various soluble models of fibrin has demonstrated that fibrin surrogates or lysine analogues can protect Pn from inhibition by AP (2–6). The data in Fig. 2 demonstrate that the HMw-FDPs are similarly able to protect Pn from inhibition by AP. In the absence of HMw-FDPs, the $k_2$ for Pn inhibition by AP is 9.72 ± 0.6 × 10^5 s^-1, which is similar to values measured previously for this interaction (0.7–2 × 10^6 s^-1)

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(2–6, 22, 23). With increasing concentrations of HMw-FDPs, the $k_2$ decreased by 7-fold at 600 nM HMw-FDPs ($1.41 \pm 0.09 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$) and by 16-fold at saturation ($0.59 \pm 0.24 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$). In contrast, a similar experiment with Val-Pn, a truncated derivative that is missing kringle domains 1–4, measured the $k_2$ value to be between 0.54 and $0.56 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$. The measured $k_2$ value using the end point assay is very similar to the previously reported value for this interaction ($0.65 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$) (3). HMw-FDPs did not measurably affect the rate of inhibition of Val-Pn. It is interesting that the $k_2$ measured for Val-Pn is approximately the same as the $k_2$ estimated for Pn completely saturated with HMw-FDPs, highlighting the importance of the kringle interactions in modulating protection.

TAFIa Removes the Ability of HMw-FDPs to Protect Pn from AP—The affinity of Pn for fibrin and AP depends on the lysine-dependent binding properties of Pn. Therefore, the HMw-FDPs were treated with TAFIa to determine whether removal of the carboxyl-terminal lysine residues would increase the rate of Pn inhibition by AP. To make TAFIa-pretreated HMw-FDPs, HMw-FDPs were incubated at various concentrations with 10 nM TAFIa for 20 min and then with PTCI to inhibit the TAFIa. The untreated HMw-FDPs were not incubated with TAFIa; however, at the end of a 20-min incubation, TAFIa was added after adding 5 μM PTCI. Including preinhibited TAFIa in the untreated HMw-FDPs assures that the loss of protection is not due to PTCI or inhibited TAFIa. In all of the experiments, TAFIa was inhibited with PTCI before AP or Pn was added to the reaction, thereby minimizing the possibility that TAFIa is modifying AP.

Fig. 3 presents the raw absorbance data obtained when the end point assay was performed with unmodified HMw-FDPs at increasing concentrations (Fig. 3A) or TAFIa-pretreated HMw-FDPs (Fig. 3B). With untreated HMw-FDPs at increasing concentrations, there was a corresponding increase in the end points and consequently a corresponding decrease in the $k_2$. In contrast, with HMw-FDPs pretreated with TAFIa, there was a dramatic decrease in the magnitude of the measured end points, which corresponds to a diminished ability of the HMw-FDPs to protect Pn from AP.

The $k_2$ values obtained from the raw data in Fig. 3, A and B, are plotted in Fig. 3C. Pretreatment of the HMw-FDPs with TAFIa removes much of the protective properties of the HMw-FDPs. For the TAFIa-treated HMw-FDPs, the second order rate constant decreased from $7.4 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ in the presence of HMw-FDPs to $4.9 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ in the presence of 600 nM FDPs. In contrast, for a similar experiment performed with untreated HMw-FDPs, the $k_2$ decreased from $8.7 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ in the absence of FDPs down to $1.87 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ in the presence of 600 nM FDPs. In this system, HMw-FDPs were able to protect Pn by 4.7-fold, whereas HMw-FDPs pretreated with TAFIa were able to protect Pn by only 1.5-fold. Therefore, TAFIa modification of HMw-FDPs caused a 3-fold loss in their ability to protect Pn.

Loss of Protection Depends on TAFIa Concentration and Incubation Time—To further characterize the role of TAFIa in this reaction, a series of end point assays were performed where 730 nM HMw-FDPs were pretreated with TAFIa at increasing concentrations (0–5 nM) for increasingly long incubation times (0–20 min). The results of this experiment are shown in Fig. 4, and as expected, the extent to which TAFIa removes the protective properties of the HMw-FDPs depends on the concentration of TAFIa added and the length of incubation. In this experiment, the $k_2$ increased from $1.94 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ for HMw-FDPs that were not allowed to incubate with TAFIa up to $6.22 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ for HMw-FDPs that had been incubated with 5 nM TAFIa for 20 min. This set of end
point assays demonstrates that the maximum 3-fold loss of protection depends on the TAFIa concentration and incubation time.

DISCUSSION

The role of fibrin in slowing the rate of Pn inhibition by AP has been extensively studied with lysine analogues (2-4, 23) and soluble (5, 6) or immobilized (15) fibrin derivatives. In every case, occupation of the kringle domain of Pn slowed inhibition by 10-398-fold depending on the system used to study the reaction. As a fibrin clot was broken down by Pn and carboxyl-terminal lysine residues were exposed, the modified fibrin surface constituted a better protective surface for Pn. It follows then that the removal of newly exposed carboxyl-terminal lysine residues will decrease the affinity of Pn binding through its kringle domains, and consequently, the Pn pool will be less protected from inhibition by AP. This is especially interesting because the recently identified antifibrinolytic carboxypeptidase, TAFIa, effectively removed carboxyl-terminal lysine residues and in this way slowed fibrin clot lysis (13). In this study, HMw-FDPs were used as a model for Pn-modified fibrin to study elements of the protection of Pn inhibition by AP. HMw-FDPs slowed Pn inhibition by 16-fold from 9.6/10^6 M in the absence of HMw-FDPs to 0.59/10^6 M at saturation, which is very similar to observations made using other soluble fibrin models. When the HMw-FDPs were pretreated with TAFIa, their ability to protect Pn from AP was diminished by 3-fold. This loss of protection depends on the concentration of TAFIa and the length of the incubation. Therefore, this work identifies a novel mechanism whereby TAFIa can modulate steady state Pn levels, and consequently, fibrinolysis by diminishing the ability of Pn degraded fibrin to protect Pn from AP.

For the first time, Pn inhibition by AP was measured using a novel chromogenic substrate end point assay. Previous work has measured a k2 of 0.7-24 × 10^6 M^-1 s^-1 for Pn inhibition by plasma-derived AP (2-6, 22, 23) and a k2 of 10.8 × 10^6 M^-1 s^-1 for Pn inhibition by recombinant AP (4). The k2 measured in this study was 9.6 × 10^6 M^-1 s^-1, which is in close agreement with previously measured values, supporting the validity of the end point model for studying Pn inhibition.

The end point model depends on the inhibitor being present in excess of the enzyme so that the reaction will go to completion and substrate hydrolysis will stop. However, a potential
confounding interaction could occur if Pn is inactivating AP without remaining consumed in the PnAP complex. To address this possibility, a series of end point assays have been performed where the measured $k_2$ values were determined in the presence of 40 nM AP and 10–30 nM Pn (Fig. 1B). In each reaction, a well defined end point was reached and the estimated $k_2$ values remained constant over the concentration range. If the AP pool was appreciably cleaved by Pn, the $k_2$ values would vary with changes in the relative concentrations of Pn and AP. However, this experiment demonstrates that nearly stoichiometric concentrations of Pn and AP do not appreciably alter the $k_2$ values, indicating that this potential side reaction is not occurring, at least not to a measurable extent.

HMw-FDPs were used as a model for Pn-modified fibrin because of their ability to support Pgn activation by both tPA and DSPA (16), the latter being an activator with no kringle domains, and therefore requiring structural elements on intact fibrin that were not present on smaller soluble fibrin derivatives. However, besides being a good model for intact fibrin, HMw-FDPs were also potentially interesting as probable degradation products released as the mechanism of fibrin clot breakdown was studied with a perfused clot model, the degradation products released as the fibrin clot was degraded. TAFIa is used as the activator, even though urokinase does not exhibit increased stability in the presence of TAFIa because of their ability to support Pgn activation by both tPA and DSPA (16). From this results obtained in this study and from previous work (13, 16), it is probable that the fibrinolytic potential of released fibrin degradation products in vitro would depend on the degree to which they retain carboxyl-terminal lysine residues. Unmodified HMw-FDPs would probably be good activators of Pgn and good protectors of Pn, whereas TAFIa-modified FDPs would probably have a diminished potential to activate Pgn and a diminished ability to protect any Pn that was formed.

Intact AP had a carboxyl-terminal lysine residue that was a potential target for TAFIa. Initial research on the role of this residue used small peptides corresponding to the carboxyl terminus of AP, and it was found that removal of this residue hampered the binding to Pn (24). It was proposed that removal of this residue on intact AP would affect its inhibitory properties; therefore, it is possible that TAFIa modification of AP would have unexpected profibrinolytic activity. More recent work, however, has used intact mutants of AP where the carboxyl-terminal lysine was mutated to either threonine or glutamic acid and it was found that these derivatives had similar inhibitory potential to the wild type antiplasmin (4). In light of this, it seems probable that if TAFIa does target the carboxyl terminus of AP, it will only have a modest effect on the inhibitory potential of the protein. Nevertheless, to forego the possibility of TAFIa-modifying AP thereby altering its inhibitory potential, in each experiment TAFIa was inhibited by a large excess of the potent TAFIa inhibitor, PTCI, prior to adding AP to the reactions.

Initial work on the role of TAFIa in fibrinolysis demonstrated that within an intact Pn-modified fibrin clot, TAFIa mediated its activity by removing carboxyl-terminal lysine residues from the fibrin (13). A loss of the carboxyl-terminal lysine residues reduced Pgn binding and, in turn, slowed the rate of activation with tPA by 3-fold. It is interesting then that TAFIa is also quite capable of stabilizing fibrin clots even if urokinase is used as the activator, even though urokinase does not depend on the surface of fibrin for Pn formation (25). If TAFIa was mediating its effect solely through the modulation of Pgn binding to the fibrin surface, it is difficult to imagine how this would alter the effect of urokinase; however, if part of the role of TAFIa is to reduce the protective properties of the fibrin surface and thereby increase the rate of Pn inhibition, clots lysed by urokinase would exhibit increased stability in the presence of TAFIa. With the identification of this novel mechanism whereby TAFIa can regulate Pn levels, these observations are reasonable and expected.

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