Thrombin bound to thrombomodulin activates thrombin-active fibrinolytic inhibitor (TAFI) and protein C much more efficiently than thrombin alone. Although thrombomodulin has been proposed to alter the thrombin active site, the recently determined structure of the thrombin-thrombomodulin complex does not support this proposal. In this study, the contribution of amino acids near the activation site of TAFI toward thrombomodulin dependence was determined, utilizing four variants of TAFI with specific substitutions in the P6-P3 region surrounding the Arg-92 cleavage site. Two point mutations had either the Ser-90 or Asp-87 of TAFI replaced with Ala, a third mutant had the thrombin activation site of the fibrinogen Bβ-chain substituted into positions 91–95 of TAFI, and a fourth mutant had the thrombin activation site of protein C substituted into positions 90–95 of TAFI. Each of these mutants was expressed, purified, and characterized with respect to activation kinetics and functional properties of the enzyme. Even though fibrinogen is poorly cleaved by thrombin-thrombomodulin, the fibrinogen activation site does not significantly alter the thrombomodulin dependence of TAFI activation. The TAFI variant with the protein C activation sequence is only slowly activated by thrombin-thrombomodulin, and not at all by free thrombin. Mutating Asp-87 to Ala increases the catalytic efficiency of activation 3-fold both in the presence and absence of thrombomodulin, whereas mutating Ser-90 to Ala effects only minor kinetic differences compared with wild type TAFI. The thermal stabilities and antifibrinolytic properties of the enzymes were not substantially altered by any of the mutations that allowed for efficient activation of the enzyme. We conclude that residues in the P6-P3 region of TAFI do not determine the thrombomodulin dependence of activation, which lends support to the argument that the role of thrombomodulin is to optimally orient thrombin and its substrate, rather than to allosterically alter the specificity of the thrombin active site.
the reverse is not true. Thrombin-thrombomodulin has little or no activity toward procoagulant substrates (3), whereas thrombin is at least a thousandfold less active against protein C and TAFI (8, 9). The mechanism whereby thrombomodulin alters the activity of thrombin has been widely investigated.

Thrombomodulin is a membrane-bound protein that projects into the lumen of blood vessels (2). It has a lectin-like domain, six tandem EGF domains, a Ser/Thr-rich domain, a transmembrane domain, and an intracellular domain. Although thrombin binds exclusively to the EGF 5 and 6 domains of thrombomodulin, this fragment is not sufficient to activate either protein C or TAFI (10). To effectively activate protein C, a fragment including EGF domains 4–6 plus the interdomain sequence connecting EGF 3 and EGF 4 is required (TM4–6). TAFI activation requires an additional 13 residues from the C loop of the EGF3 domain (TMc3–6). Therefore, despite the similarity in the kinetics of activation of TAFI and protein C, different structural elements of thrombomodulin are essential for each respective reaction.

Esmon and co-workers (11, 12) have used thrombin mutants and peptides corresponding to the P7–P3 region of protein C to identify unfavorable charge interactions between Glu-192 and Asp residues at the P3 and P′3 residues of the protein C activation site. They attributed part of the thrombomodulin dependence of protein C activation to the ability of thrombomodulin to minimize these charge interactions, possibly by allosterically altering the thrombin active site, including reorientation of the charged Glu-39 and Glu-192 residues. However, more recent work on the structure of the thrombin-thrombomodulin complex has shown that large allosteric changes to thrombin probably do not occur upon binding to thrombomodulin (13). Rather, thrombomodulin binds to exosite 1 of thrombin, thereby precluding binding of procoagulant substrates such as fibrinogen. Thrombomodulin also may serve as a template directing protein C or TAFI in an optimal orientation for cleavage by thrombin. In addition, extensive Ala scanning mutagenesis of thrombin found that distinct areas of thrombin structure are required for effective protein C or TAFI activation, which further supports a structural argument rather than an argument for an allosterically modified thrombin active site (14). In light of these findings, the unfavorable effect of the charged residues near the activation site of protein C may be minimized by the orientation of protein C, rather than by alterations to the thrombin active site.

In TAFI, the P3 and P′3 residues are the polar Ser-90 and the uncharged Ala-95, respectively (15); however, TAFI exhibits thrombomodulin dependence in its activation that is very similar to protein C (9). In the TAFI sequence, the closest charged residue is Asp-87 at P6. To explore the role of the residues of TAFI near the cleavage site with respect to the thrombomodulin dependence of activation, we made four TAFI mutants with selective alterations in the P6–P′3 region. We measured the kinetics of activation of each mutant by free thrombin and the thrombin-thrombomodulin complex and found that residues in the P6–P′3 region of TAFI do not contribute significantly to the thrombomodulin dependence of activation.

EXPERIMENTAL PROCEDURES

Materials—The synthetic carboxypeptidase substrate anisylazo-tryptophan-seryllysine (AATF) (16) was a generous gift from Dr. William L. Mock (Department of Chemistry, University of Illinois, Chicago IL). CNBr-activated Sepharose 4B was purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). Ultrafree-4 centrifugal filter devices were purchased from Calbiochem (San Diego, CA). DNA restriction and modification enzymes were purchased from Promega (Madison, WI), New England Biolabs (Mississauga, Ontario, Canada), or Stratagene (La Jolla, CA). The Sequenase version 2.0 kit for nucleotide sequence analysis was purchased from U. S. Biochemical Corp (Cleveland, OH). Dulbecco’s modified Eagle’s medium/nutrient mixture F-12, newborn calf serum, Opti-MEM, penicillin/streptomycin/Fungizone mixture, and reduced glutathione were purchased from Invitrogen. Methotrexate was obtained from Kingston General Hospital pharmacy (Wyllie-Ayestas Ltd., Montreal, QC, Canada). Baby hamster kidney cells and the pNUT mammalian expression vector were a gift from Dr. Ross MacGillivray (University of British Columbia, Vancouver, British Columbia, Canada). Thrombin was prepared from human plasma-derived prothrombin as described previously (17). Plasmin was prepared from human plasma-derived plasminogen as described previously (17). Recombinant human tPA (Actives) was generously provided by Dr. Gordon Verheijen (Genentech, Inc., South San Francisco, CA). Recombinant soluble thrombomodulin (Solulin) was obtained from Berlex Biosciences, Inc. (Richmond, CA). For immunoadsorption chromatography, a monoclonal antibody raised against purified TAFI (mAb16) (18) was coupled to CNBr-activated Sepharose 4B. Synthetic 75% phosphatidylcholine, 25% phosphatidylinositol (PCPS) vesicles were prepared as described previously (19).

Construction of TAFI Mutants—All constructs of TAFI were constructed with the sequence of the wild type described previously (15), using the TAFI soform with Ala at position 147 and Thr at position 325 (15, 20). Mutations were introduced into a plasmid coding for wild type TAFI cDNA using a QuikChange mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. A pair of primers was used to construct each of two point mutants, one with a Ser to Ala mutation at position 90 (TAFI-S90A) and one with an Asp to Ala mutation at position 87 (TAFI-D87A). Similarly, we constructed a mutant with the Protein C thrombin cleavage site (Asp-Pro-Arg-Leu-Ile-Asp) substituted into positions 90–95 of TAFI (TAFI-PCap), and another mutant with the fibrinogen Bβ thrombin cleavage site (Ala-Gly-His-Arg) substituted into positions 91–95 of TAFI (TAFI-PG-Nap). All four constructs were cloned into the pNUT expression vector as described previously (21), and the integrity of the mutations was determined by DNA sequence analysis.

Expression and Purification of Recombinant TAFI Variants—Expression plasmids coding for TAFI mutant cDNAs were transfected into baby hamster kidney cells using calcium phosphate precipitation (22). Lines that stably expressed TAFI were selected by culturing cells in the presence of 400 μM methotrexate. For recombinant TAFI mutant production, stably expressing lines were cultured in triple flasks (500 cm2; Nap). All four constructs were cloned into the pNUT expression vector as described previously (21), and the integrity of the mutations was determined by DNA sequence analysis.

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Activation of TAFI and Determination of Thermal Stability—For routine activation of TAFI to TAFIa, TAFI (1 μM) was incubated with thrombin (25 μM), Solulin (100 μg/ml), and CaCl2 (5 mM) in HBS/Tween 0.01% at 37 °C. Where appropriate, the thrombin was quenched with PPAC (1 μM) before the mixture was placed on ice.

Each TAFI mutant (except for TAFI-PCap) was completely activated, as described above, and was used to determine thermal stabilities of the TAFIa mutants. The activated mutants were then transferred to a 37 °C water bath. At various times, 10–μl aliquots were removed and quenched with 190 μl of AAKF (120 μM final concentration) and PPAC (1 μM final concentration) in a microtiter plate, and the initial rates of substrate hydrolysis were measured. The amount of residual activity was calculated relative to the initial TAFIa activity, and the data were fit by nonlinear regression to the equation for exponential decay (Nonlin). The estimated first order decay constant (k) was used to determine the half-lives of each mutant.

Activation of TAFI Mutants by Thrombin and Solulin—To activate a time course of TAFI activation as described by SDS-PAGE, each TAFI mutant (1 μM) was incubated with thrombin (5 μM), Solulin...
Role of the P6-P3 Region of TAFI

Concentrations, the data were modeled with the quadratic form of the Michaelis-Menten equation, where \( Pn \) is the concentration of plasmin and TAFI is the initial concentration of each of the TAFI mutants used.

\[
v = \left(0.5 \cdot k_{cat} \cdot (Pn + TAFI) + K_{m} \right) \left(\frac{v_{max} \cdot K_{m} \cdot Pn}{(Pn + TAFI) \cdot (Pn + K_{m})}ight)
\]

To estimate \( k_{cat} \) and \( K_{m} \), the data were fitted to this equation by nonlinear regression and values are reported plus or minus the standard error of the regression (Nonlin module of SYSTAT 9).

**TAFI-deficient Plasma—**To make TAFI-deficient plasma, 150 ml of fresh frozen citrated human plasma was thawed and passed over a 1 ml mAb16-Sepharose 4B column at room temperature. The plasma was passed through the column three times, and between each pass the column was washed copiously with HBS/Tween 0.01%. To be certain the plasma was TAFI-deficient, a clot lysis assay (see below), was performed in the absence of TAFI and either in the absence or presence of Solulin (10 nM). There was no difference between the clot lysis times, and the plasma was considered to be TAFI-deficient.

**Clot Lysis Assays—**All clot lysis assays were performed in a final volume of 120 μl in microtiter plates at 37 °C. Prior to use, each microtiter plate was pre-treated as described above. TAFI-deficient plasma was diluted 1:3 in HBS/Tween 0.01% before clotting was initiated by the addition of thrombin (5 nM), CaCl\(_2\) (10 mM), PCPS (20 μM), tPA (0.3 nM), TAFI mutant zymogens (0–90 nM), and Solulin (10 nM). Reactions were covered to minimize evaporation. Clot lysis was monitored by a change in turbidity of each reaction at 400 nm in a Spectramax Plus plate reader (Molecular Devices, Sunnyvale, CA), and the time to 50% lysis was determined graphically as the midpoint between maximum turbidity of a clot and the minimum absorbance when a clot was completely lysed.

**RESULTS**

**Construction and Expression of TAFI Mutants—**To determine whether the amino acid residues adjacent to the cleavage site influence the thrombomodulin dependence of TAFI activation, we constructed four mutants of human TAFI with alterations in the P6-P3 region. The Ser to Ala mutation at position 90 (P3) was constructed to determine whether this residue influences TAFI activation in the same way that it does for protein C activation. In TAFI the P3 residue is already an Ala, so no mutation was made at this residue. The Asp to Ala mutation at position 87 was constructed to determine whether this charged residue influences TAFI activation as charged residues near the cleavage site influence protein C activation. The other two mutants were made by replacing the P3-’P3 sequence of TAFI with the corresponding region from either the protein C or fibrinogen Bβ-chain. These mutants were used to investigate whether the thrombomodulin dependence of TAFI activation is determined by the sequence around the activation cleavage site. The alterations made in the TAFI mutants as compared with the wild type are summarized in Table I. All of the TAFI variants were purified to homogeneity by affinity chromatography, and each protein ran as a single homogenous band at approximately 60 kDa by SDS-PAGE.

**Activation of TAFI Mutants by the Thrombin-Thrombomodulin Complex—**The TAFI mutants were activated by the thrombin-thrombomodulin complex at room temperature; timed aliquots were removed and resolved by SDS-PAGE. The activation profiles of TAFI-wt, TAFI-S90A, TAFI-D87A, and TAFI-FGNap are shown in Fig. 1. Each of these mutants was used to investigate whether the thrombomodulin dependence of TAFI activation is determined by the sequence around the activation cleavage site. The altered mutants in the TAFI mutants as compared with the wild type are summarized in Table I. All of the TAFI variants were purified to homogeneity by affinity chromatography, and each protein ran as a single homogenous band at approximately 60 kDa by SDS-PAGE.
wt, TAFI-S90A, TAFI-D87A, and TAFI-FGNap, and the values of the kinetic parameters are summarized in Table II. Because TAFI-PCap was activated so inefficiently by thrombin-thrombomodulin, we estimated the $k_{cat}/K_m$ from the slope of the rate of activation of various concentrations of TAFI-PCap by a constant concentration of thrombin-thrombomodulin.

As expected from the activation gels shown in Fig. 1, TAFI-wt, TAFI-S90A, TAFI-D87A, and TAFI-FGNap were activated with similar kinetics by thrombin-thrombomodulin. Removing the charged residue at position 87 of TAFI (TAFI-D87A) increased the catalytic efficiency of activation by thrombin-thrombomodulin by approximately 3-fold. Altering the polar

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**TABLE I**

A summary of the mutations made in the P6-P'3 region of human TAFI

| Position | P6 | P5 | P4 | P3 | P2 | P1 | P'1 | P'2 | P'3 |
|----------|----|----|----|----|----|----|-----|-----|-----|
| TAFI-wt  | Asp| Thr| Val| Ser| Pro| Arg| Ala | Ser | Ala |
| TAFI-S90A| Asp| Thr| Val| Ala| Pro| Arg| Ala | Ser | Ala |
| TAFI-D87A| Ala| Thr| Val| Ser| Pro| Arg| Ala | Arg | Gly |
| TAFI-FGNap| Asp| Thr| Val| Ser| Ala| Arg| Gly | His | Arg |
| TAFI-PCap| Asp| Thr| Val| Asp| Pro| Arg| Leu | Ile | Asp |

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**FIG. 1.** Time courses of TAFI mutant activation by the thrombin-thrombomodulin complex measured by SDS-PAGE. TAFI-wt (A), TAFI-FGNap (B), TAFI-S90A (C), and TAFI-D87A (D), at a concentration of 1 μM, were activated at 22°C by thrombin (5 nM) and Solulin (50 nM). At the times indicated below each gel, aliquots were removed and quenched with PPAck (1 μM). The samples were resolved on 5–15% polyacrylamide gels and stained with Coomassie Blue.

**FIG. 2.** Activation of TAFI mutants by the thrombin-thrombomodulin complex. TAFI-wt (A), TAFI-FGNap (B), TAFI-S90A (C), and TAFI-D87A (D) at various concentrations were incubated with thrombin (0.5 nM) and Solulin at 1.56 nM (open squares), 3.13 nM (closed squares), 6.25 nM (open triangles), 12.5 nM (closed triangles), 25 nM (open circles), and 50 nM (closed circles) as described under “Experimental Procedures.” The rate of activation (mol of TAFIa/mol of thrombin/s) was calculated, and the data were fit globally to the model for activation described previously (9). The lines represent the best fit of the regression, and the estimated $k_{cat}$, $K_m$, and $K_d$ values are summarized in Table II. Each activation matrix was repeated at least twice, and a representative data set is shown for each mutant.
that of TAFI-wt (0.4 catalytic efficiency of activation that was approximately half cleaved when substituted into TAFI (TAFI-FGNap), with a thrombin when it is in fibrinogen, this sequence was effectively charged P3 and P4 not effectively cleaved. Perhaps, in the context of TAFI, the modulin, when the protein C cleavage sequence is in TAFI, it is remarkably efficiently in the presence of thrombomodulin, TAFI-FGNap is activated approximately half as well as TAFI-wt, it still exhibits the same thrombomodulin dependence of TAFI-FGNap activation. Although this mutant activates approximately half as well as TAFI-wt, it still exhibits the same thrombomodulin dependence in its activation. In the presence of thrombomodulin, TAFI-FGNap is activated 2000-fold more efficiently than with thrombin alone, as compared with 1360-fold for TAFI-wt.

TAFI Mutant Activation by Thrombin with Thrombomodulin Deletion Mutants—Another difference between TAFI activation and protein C activation by thrombin-thrombomodulin is the minimal elements of thrombomodulin structure required in each reaction. Protein C requires a fragment of thrombomodulin that includes EGF4-6 plus the interdomain sequence that connects EGF3 and EGF 4 (TM4–6), whereas TAFI activation requires a thrombomodulin construct that includes the C loop of the third EGF domain (TM3–6) (10). We have used these thrombomodulin constructs to activate the TAFI mutants, and compared the results to activation by full-length soluble thrombomodulin. Fig. 4 shows the effectiveness of each thrombomodulin construct to activate each mutant.

| Activator | Increase | [TAFI] μM | [Thrombin] μM | [Thrombomodulin] μM | Increase |
|-----------|----------|-----------|---------------|---------------------|----------|
| TAFI-wt | 0.000866 ± 0.000003 | 0.9 ± 0.05 | 1360 |
| TAFI-S90A | 0.00044 ± 0.000002 | 1.4 ± 0.1 | 3180 |
| TAFI-D87A | 0.0018 ± 0.000006 | 2.1 ± 0.3 | 1500 |
| TAFI-FGNap | 0.00020 ± 0.000005 | 0.4 ± 0.1 | 2000 |
| TAFI-PCap | NM | 0.0078 ± 0.0003 | NM |

Alterations near the cleavage site of TAFI do not significantly alter the thrombomodulin structural requirements for TAFI activation. In each case, TMc3–6 could effectively activate each mutant, whereas TM4–6 could not. Of the four mutants, only TAFI-D87A could be appreciably activated by thrombin-TM4–6, and even this was only a fraction (5%) of the rate of activation measured by thrombin-Solulin. Therefore, with respect to the thrombomodulin structural requirements, each mutant responds similarly to TAFI rather than protein C.

Activation of TAFI Mutants by Plasmin—Plasmin is the only other protease in human plasma that can activate TAFI (24). The activation of each mutant by plasmin is shown in Fig. 5, and the estimated $k_{cat}$ and $K_m$ values are summarized in Table III.
IV. When the mutants are compared with wild type TAFI, the largest effect is caused by removal of the charged Asp residue at position 87, which increases the efficiency of TAFI activation by 10-fold as compared with TAFI-wt. This increase is mediated by an increase in $k_{\text{cat}}$ and a decrease in $K_m$, and in this respect, this mutant responds to plasmin as it does to thrombin in the absence of thrombomodulin. TAFI-S90A exhibited an increase in affinity for plasmin, without any appreciable change in the $k_{\text{cat}}$. TAFI-FGNap is activated by plasmin with the same kinetics as TAFI-wt, whereas TAFI-PCap could not be measurably activated by plasmin.

**Thermal Stabilities of the TAFI Mutants**—The enzyme, TAFIa, is intrinsically unstable, and TAFI mutants with altered thermal stabilities show corresponding alterations in their antifibrinolytic activity (25). Because thermal instability probably provides a mechanism for down-regulation of TAFI in vivo, we determined whether the present mutations altered their thermal stability. TAFI-wt, TAFI-S90A, TAFI-D87A, and TAFI-FGNap were each completely activated by thrombin-thrombomodulin, and the time course of activity was monitored at 37 °C, as shown in Fig. 6. Because TAFI-PCap was activated so inefficiently, the half-life of this mutant could not be reliably measured. The three mutants exhibited little change in their measured half-lives relative to TAFI-wt, indicating that alterations in this region do not have a large effect on the thermal stability of TAFIa. The residual activity was used to estimate the decay constant for each variant, and from this the half-lives were found to range between 7.4 and 7.8 min. (TAFI-wt = 7.8 ± 0.2 min, TAFI-FGNap = 7.4 ± 0.2 min, TAFI-S90A = 7.6 ± 0.2 min, and TAFI-D87A = 7.5 ± 0.3 min).

Antifibrinolytic Potential of TAFI Mutants—TAFIa attenuates clot lysis by removing lysine residues from a plasmin modified fibrin clot, which results in a 3-fold prolongation in clot lysis time in an in vitro plasma clot lysis assay (4). To determine whether these mutations alter the antifibrinolytic potential of TAFI, we have tested each mutant using an in vitro clot lysis assay, and the results are shown in Fig. 7. TAFI-wt, TAFI-S90A, TAFI-D87A, and TAFI-FGNap each behave similarly to TAFI-wt. TAFI-PCap exhibited a reduced antifibrinolytic activity with a maximum antifibrinolytic potential that was approximately 60% that of TAFI-wt. The lower antifibrinolytic potential of TAFI-PCap probably reflects its greatly reduced activation by the thrombin-thrombomodulin complex.

![Image](50x250)  
**Fig. 4. Activation of TAFI mutants by thrombin and thrombomodulin deletion mutants.** TAFI-wt, TAFI-PCap, TAFI-FGNap, TAFI-S90A, and TAFI-D87A at 1 μM were incubated for 10 min with thrombin (1 nM) in the presence of Solulin (dark gray bars), TMc3–6 (light gray bars), or TMi4–6 (black bars) at 50 nM as described under “Experimental Procedures.” The rate of activation (mol of TAFIa/mol of thrombin/s) was calculated. The lines represent the mean of two separate experiments.

![Image](50x250)  
**Fig. 5. Activation of TAFI mutants by plasmin.** TAFI-wt (closed circles), TAFI-S90A (open triangles), TAFI-D87A (closed triangles), TAFI-FGNap (open squares), and TAFI-PCap (closed squares), at various concentrations were incubated with plasmin as described under “Experimental Procedures.” The rates of activation (TAFIa/s) were calculated, and the data were fit to the quadratic form of the Michaelis-Menten equation. Each point represents the mean of at least two independent experiments, and the lines represent the best-fit line for the regression.

**TABLE IV**

**Kinetics of TAFI mutant activation by plasmin**

| TAFI Mutant | $k_{\text{cat}}$ | $K_m$ | $k_{\text{cat}}/K_m$ |
|------------|----------------|-------|---------------------|
| TAFI-wt    | 0.00030 ± 0.00006 | 0.029 ± 0.002 | 0.010 ± 0.001 |
| TAFI-S90A  | 0.00032 ± 0.000006 | 0.0074 ± 0.001 | 0.043 ± 0.006 |
| TAFI-D87A  | 0.00056 ± 0.0002 | 0.091 ± 0.002 | 0.092 ± 0.029 |
| TAFI-FGNap | 0.00026 ± 0.00002 | 0.021 ± 0.005 | 0.013 ± 0.002 |
| TAFI-PCap  | NM              | NM    | NM                  |

**DISCUSSION**

TAFI-S90A, TAFI-D87A, and TAFI-FGNap all behave similarly to TAFI-wt with respect to thermal stability, antifibrinolytic potential, and activation kinetics. For each mutant, activation by thrombin in the presence of thrombomodulin was approximately 3 orders of magnitude more efficient than in the presence of thrombin alone, ranging between 1350- and 3180-fold. TAFI-PCap was inefficiently activated by thrombin-thrombomodulin and was not at all activated by thrombin. Although we could not quantify the thrombomodulin dependence, even this mutant exhibited thrombomodulin dependence in its activation. Therefore, these changes to the activation sequence of TAFI do not remove the thrombomodulin dependence of TAFI activation.

Because charged residues are important to the thrombomodulin dependence of protein C activation, the role of Asp-87 in TAFI is interesting. When we mutated this residue to Ala, we found that the $k_{\text{cat}}/K_m$ was 3-fold higher than wild type, and this increase in catalytic efficiency is observed in the presence and absence of thrombomodulin. Similarly to TAFI-wt, TAFI-D87A is activated 1500-fold more efficiently in the presence of thrombomodulin. TAFI-D87A does not exhibit exactly the same thrombomodulin structural requirement as TAFI-wt, because the activation of TAFI-wt is not stimulated at all by TMi4–6, whereas TAFI-D87A is activated by TMi4–6 with approximately 5% of the efficiency of full-length thrombomodulin.
Therefore, we have identified a charged residue, Asp-87, that is probably involved in an unfavorable interaction with thrombin and that interaction is not ameliorated by formation of the thrombin-thrombomodulin complex.

As expected, mutating the Ser-90 residue of TAFI had only a small effect on the activation kinetics. Activation of TAFI-S90A by thrombin-thrombomodulin was roughly 3180-fold more efficient than by thrombin alone, whereas TAFI-wt shows a 1360-fold increase in the catalytic efficiency of activation in the presence of thrombomodulin as compared with 1360-fold for TAFI-wt. The efficiency of activation is approximately 45% of TAFI-wt, despite the change to the activation site.

FIG. 6. Time-dependent inactivation of TAFIa mutants at 37 °C. TAFI-wt (closed circles), TAFI-S90A (open triangles), TAFI-D87A (closed triangles), and TAFI-FGNap (open squares) were completely activated by thrombin-thrombomodulin as described under “Experimental Procedures.” The residual TAFIa activity was measured. The lines represent the nonlinear regression of the data to the exponential decay function. Decay constants estimated by the regression of the data were used to calculate the half-lives of the TAFIa mutants.

FIG. 7. The antifibrinolytic effect of TAFI mutants in the presence of thrombomodulin. Diluted TAFI-deficient plasma was incubated with TAFI-wt (closed circles), TAFI-S90A (open triangles), TAFI-D87A (closed triangles), TAFI-FGNap (open squares), and TAFI-PCap (closed squares) at various concentrations. Clots were formed by adding thrombin (5 nm), Solulin (10 nm), CaCl2 (5 mM), tPA (0.3 nm), and PCPS (20 µM) at 37 °C. The turbidity was monitored, and the time to 50% clot lysis was measured and plotted as a function of the initial TAFI concentration. Each point represents the mean of two independent experiments.

In fibrinogen, the fibrinogen Bβ chain is quickly cleaved by a low concentration of free thrombin, thereby forming a clot; however, the fibrinogen Bβ chain is not measurably cleaved by the thrombin-thrombomodulin complex. In the context of TAFI, this sequence has only modest effects on the kinetics of activation, which are similar to those of TAFI-wt and dissimilar to those of fibrinogen. Therefore, the structural elements that make TAFI thrombomodulin-dependent, while fibrinogen is thrombomodulin-independent, apparently are not contained in the activation sites of either protein.

TAFI-PCap was the only TAFI mutant that was not efficiently activated by thrombin-thrombomodulin. Intact protein C is activated with kinetics similar to TAFI, showing a large increase in the rate of activation by the thrombin-thrombomodulin complex as compared with thrombin alone. Yet, in TAFI, the protein C thrombin activation site is not efficiently cleaved by the thrombin-thrombomodulin complex, although the thrombomodulin dependence was retained. Possibly, in the context of TAFI, thrombomodulin is no longer in an appropriate orientation to minimize the unfavorable charge interactions between thrombin and the Asp residues.

TMs3–6 was able to activate all of the mutants nearly as efficiently as the full-length soluble thrombomodulin; however, TMI4–6 could not effectively activate the TAFI mutants. Therefore, despite activation site changes, the mutants have the same structural requirements for thrombomodulin as TAFI-wt. Even TAFI-PCap could not be measurably activated by TMI4–6, although this thrombomodulin deletion mutant is fully as active against protein C as full-length thrombomodulin. Therefore, none of the changes in the activation site alter thrombomodulin dependence or remove the requirement for the C loop of the third EGF domain for TAFI activation.

Plasmin is the only other identified activator of TAFI that could be physiologically relevant. Like thrombin, plasmin is a serine protease that is highly selective in its physiological targets, so we were interested to see how these mutations altered the activation kinetics by plasmin. Except for TAFI-PCap, all of the TAFI mutants activated at least as well as TAFI-wt. Most surprising was TAFI-FGNap, which was found to be activated with nearly the same kinetics as TAFI-wt. Similar to thrombin, plasmin appears to recognize the fibrinogen cleavage site in the context of TAFI, indicating that elements that make TAFI activable by plasmin probably do not reside in the cleavage site of TAFI or fibrinogen. TAFI-D87A had the most pronounced effect on the activation kinetics by plasmin, increasing the kcat and decreasing the Km. Again, similar to thrombin, the charged residue at position 87 in TAFI is probably involved in an unfavorable interaction with plasmin. TAFI-PCap was the only variant that could not be measurably activated by plasmin, perhaps because of the charged residues introduced in this variant.

The allosteric model of thrombin modulation by thrombomodulin predicts a change in the active site of thrombin that alters its specificity from one type of substrate (procoagulant targets) toward another type of substrate (protein C or TAFI). We would expect that, if the allosteric model of thrombomodulin switching is correct, the main determinants of thrombomodulin dependence would reside in the activation site. We would expect that the fibrinogen activation peptide would be thrombomodulin-independent even in the context of TAFI, cleavage site. Perhaps most surprising is how well TAFI-FGNap is activated by the thrombin-thrombomodulin complex. It shows a 2000-fold increase in the catalytic efficiency of activation in the presence of thrombomodulin as compared with 1360-fold for TAFI-wt. The efficiency of activation is approximately 45% of TAFI-wt, despite the change to the activation site.

We would expect that the fibrinogen activation peptide would be thrombomodulin-independent even in the context of TAFI.
whereas the protein C activation peptide would be thrombomodulin-dependent in both TAFI and protein C. Instead, we have found that both TAFI-FGNap and TAFI-PCap are thrombomodulin-dependent. Moreover, although TAFI-FGNap is effectively activated, TAFI-PCap is not. These findings lend support to the argument that thrombomodulin functions by orienting the active site of thrombin with the activation peptide of protein C and TAFI, rather than by creating a version of thrombin with an altered active site. More generally we have also shown that residues in the P6-P/H11032 region neither dictate the overall thrombomodulin dependence of TAFI activation nor change the thrombomodulin structural requirements for efficient TAFI activation.

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