Generation and Characterization of a Highly Stable Form of Activated Thrombin-activable Fibrinolysis Inhibitor*

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Activated thrombin-activable fibrinolysis inhibitor (TAFIa) is a carboxypeptidase B that can down-regulate fibrinolysis. TAFIa is a labile enzyme that can be inactivated by conformational instability or proteolysis. TAFI is ~40% identical to pancreatic carboxypeptidase B (CPB). In contrast to TAFIa, pancreatic CPB is a stable protease. We hypothesized that regions or residues that are not conserved in TAFIa compared with pancreatic CPB play a role in the conformational instability of TAFIa and that replacement of these non-conserved residues with residues of pancreatic CPB would lead to a TAFIa molecule with an increased stability. Therefore, we have expressed, purified, and characterized two TAFI-CPB chimeras: TAFI-CPB-(293–333) and TAFI-CPB-(293–401). TAFI-CPB-(293–333) could be activated by thrombin-thrombomodulin, but not as efficiently as wild-type TAFI. After activation, this mutant was unstable and was hardly able to prolong clot lysis of TAFI-deficient plasma. Binding of TAFI-CPB-(293–333) to both plasminogen and fibrinogen was normal compared with wild-type TAFI. TAFI-CPB-(293–401) could be activated by thrombin-thrombomodulin, although at a lower rate compared with wild-type TAFI. The activated mutant displayed a markedly prolonged half-life of 1.5 h. Plasmin could both activate and inactivate this chimera. Interestingly, this chimera did not bind to plasminogen or fibrinogen. TAFI-CPB-(293–401) could prolong the clot lysis time in TAFI-deficient plasma, although not as efficiently as wild-type TAFI. In conclusion, by replacing a region in TAFI with the corresponding region in pancreatic CPB, we were able to generate a TAFIa form with a highly stable activity.

The coagulation system is a potent mechanism that prevents blood loss after vascular injury. It consists of a number of linked enzymatic reactions resulting in thrombin generation. Thrombin converts soluble fibrinogen into a fibrin clot. The central protein of the fibrinolytic system, plasmin, dissolves the blood clot by degrading the fibrin polymers into soluble fragments.

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Thrombin-activable fibrinolysis inhibitor (TAFI)1 (1, 2), also known as plasma procarboxypeptidase B, R, and U, provides an important link between the coagulation and fibrinolytic systems (3–5). Activated TAFI (TAFIa) down-regulates fibrinolysis, presumably by removing C-terminal lysines from fibrin that is already partially degraded by plasmin. Those lysines act as ligands for the lysine-binding sites of plasminogen and tissue-type plasminogen activator (t-PA). Removal of the lysines attenuates the fibrin cofactor function of t-PA-mediated plasminogen activation, resulting in prevention of accelerated plasmin formation and consequently down-regulation of fibrinolysis (6).

TAFI is, in analogy to other carboxypeptidases such as pancreatic procarboxypeptidase B, proteolytically activated by thrombin or plasmin by cleavage at Arg⁹, which results in release of the activation peptide from the catalytic domain (7). TAFIa is a labile enzyme, with a half-life of ~10 min at 37 °C (7–9). TAFIa is inactivated by conformational instability, after which it becomes more susceptible to proteolytic degradation by thrombin (4, 7–11). Plasmin can inactive TAFIa by proteolysis of the catalytic domain (12).

TAFI is classified as a member of the metallocarboxypeptidase subfamily. The pancreatic metallocarboxypeptidases playing a role in the digestive system are the best characterized members of this group. According to their substrate specificity, they are referred to as carboxypeptidase A, which preferentially hydrolyzes aliphatic C-terminal residues, or carboxypeptidase B (CPB), which, like TAFIa, preferentially hydrolyzes C-terminal basic amino residues. TAFI shows ~40% identity to pancreatic procarboxypeptidases; and upon activation, TAFI exerts CPB-like activity. Residues of carboxypeptidases A and B that have been implicated in catalysis (Glu⁵⁷¹ and Arg⁵⁷⁵; residue numbering of TAFIa), substrate binding (Arg⁴¹⁴, Tyr⁴⁹⁴, and Asn⁴⁹⁵), and zinc binding (His⁷⁶, Glu⁷⁰, and His⁸⁰) are conserved in the 309-amino acid catalytic domain of TAFIa (7). The presence of Asp²⁵⁷ may determine the specificity for basic amino acids. TAFI is synthesized in the liver as a pre-propeptide consisting of 423 amino acids (7). The N-terminal signal peptide is removed upon secretion (7).

Despite all the similarities between TAFI and pancreatic pro-CPB, pancreatic CPB is, in contrast to TAFIa, a stable protease (13). We hypothesized that regions or residues that are not conserved in TAFI compared with pancreatic pro-CPB play a role in the conformational instability of TAFIa and that...
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EXPERIMENTAL PROCEDURES

Materials—Rabbit lung thrombomodulin and the sheep anti-TAFI polyclonal antibody were purchased from American Diagnostica, Inc. (Greenwich, CT). Hpipurrayl-Arg and H-n-Pro-Arg chloromethyl ketone (PPACK) were from Bachem (Bubendorf, Switzerland). Porcine pancreatic CPB was from Roche Applied Science (Almere, The Netherlands), and potato carboxypeptidase inhibitor was from Calbiochem. Thrombin was a generous gift from Dr. W. Kisel (University of New Mexico, Albuquerque, NM), plasmid from Dr. A. Reijerkerk (University Medical Center, Utrecht, The Netherlands), and porcine pancreatic CPB cDNA from Drs. N. Dekker and M. Strömquist (AstraZeneca, Södertälje, Sweden). t-PA was obtained from Chromogenix (Mölndal, Sweden). Peroxidase-conjugated rabbit anti-sheep antibody, a peroxidase-conjugated rabbit anti-sheep monoclonal antibody (Nik-9H10) (0.25 mg/ml) was added to stop thrombin or plasmin activity, respectively, and hippuric acid for tritium-labeling. For the clot lysis assay, a solid-phase extraction unit (Waters Oasis, Wexford, Ireland) according to the supplier’s recommendation, and samples were analyzed by high pressure liquid chromatography as described (17). TAFIa activity is expressed in units/liter. One unit of enzyme activity is defined as the amount of enzyme required to hydrolyze 1 μmol of substrate/min at 37 °C under the conditions described (17).

Clot Lysis Assay—The clot lysis assay was performed essentially as described previously (10). Briefly, 0.7 μl of citrated human TAFI-deficient platelet-rich plasma (TIP) (19) was mixed with various concentrations of TAFIa (TAFI-CPB-(293–333), TAFI-CPB-(293–401), or porcine pancreatic CPB. The volumes were adjusted to 100 μl with HBS-buffered saline (HBS; 25 mM Heps, 137 mM NaCl, and 3.5 mM KCl, pH 7.4) containing 0.1% bovine serum albumin. For all experiments, samples (100 μl) were transferred to a microtiter plate, and turbidity was measured over time at 37 °C using a Thermomax microplate reader (Molecular Devices, Menlo Park, CA). The clot lysis time is defined as the time at which half-maximal lysis occurred.

Affinities of Anti-TAFI Antibodies for TAFI and TAFI-CPB Chimeras—TAFI, TAFI-CPB-(293–333), and TAFI-CPB-(293–401) (3 μg/ml) were immobilized overnight at 4 °C on a 96-well plate (Maxisorp, Nunc International). The wells were blocked by incubation with blocking buffer (TBS containing 3% bovine serum albumin; 200 μl/well) at 37 °C for 1 h. Mouse monoclonal anti-TAFI antibody (NK-9H10 (0–10 μg/ml), 100 μl/well) was added to bind to the immobilized antigens. The plates were incubated with a peroxidase-conjugated rabbit anti-sheep anti-
body or a peroxidase-conjugated swine anti-mouse antibody, respectively, diluted 1:10000 in blocking buffer (100 μl/well) at room temperature for 1 h. After a final washing step, peroxidase activity was detected with o-phenylenediamine (0.4 mg/ml) in phosphate/citrate buffer (100 mM Na2HPO4 and 50 mM citric acid, pH 5.0) with 0.035% H2O2 (100 μl/well). Color was allowed to develop, after which the reaction was stopped by addition of 1 M sulfuric acid to each well (50 μl/well, in duplicate) of these activation mixtures revealed that the catalytic domains of inactivation could still proceed. The samples were kept at 37 °C for 2 h. Samples of TAFI, TAFI-CPB-(293–333), and TAFI-CPB-(293–401) (0.17 μM, 100 μl/well) were allowed to bind to the immobilized fibrinogen at room temperature for 1.5 h. In a separate experiment, TAFI, TAFI-CPB-(293–333), and TAFI-CPB-(293–401) (0.167 mM) were incubated with thrombin (20 nM), thrombomodulin (5 nM), and CaCl2 (5 mM) prior to binding to fibrinogen. The volume was adjusted to 180 μl with TBS. At different time points, thrombin activity was stopped by addition of 60 μl of PPACK (final concentration of 37.5 μM). Samples (100 μl/well, in duplicate) of these incubation mixtures were then allowed to bind to the immobilized fibrinogen. After washing with TBS containing 0.1% Tween 20, the wells were blocked by incubation with blocking buffer (200 μl/well) at 37 °C for 2 h. Samples of TAFI, TAFI-CPB-(293–333), and TAFI-CPB-(293–401) (0.17 μM, 100 μl/well) were allowed to bind to the immobilized fibrinogen at room temperature for 1 h. After a final washing step, peroxidase activity was detected with o-phenylenediamine (0.4 mg/ml) in phosphate/citrate buffer with 0.035% H2O2 (100 μl/well). Color was allowed to develop, after which the reaction was stopped by addition of 1 M sulfuric acid to each well (50 μl/well, and absorbance was measured at 490 nm. The data were analyzed by fitting to the one-site binding equation: $Y = B_{\text{max}}(X/K_d + X)$, where $B_{\text{max}}$ is the maximal binding, and $K_d$ is the concentration of ligand required to reach half-maximal binding.

Fibrinogen Binding—Fibrinogen (1 μg/ml in 50 mM Na2CO3/NaHCO3, pH 9.6; 100 μl/well) was immobilized overnight at 4 °C on a 96-well plate (Maxisorp). The wells were blocked by incubation with blocking buffer (200 μl/well) at 37 °C for 3 h. Samples of TAFI, TAFI-CPB-(293–333), and TAFI-CPB-(293–401) (0.17 μM, 100 μl/well) were allowed to bind to the immobilized fibrinogen at room temperature for 1.5 h. In a separate experiment, TAFI, TAFI-CPB-(293–333), and TAFI-CPB-(293–401) (0.167 mM) were incubated with thrombin (20 nM), thrombomodulin (5 nM), and CaCl2 (5 mM) prior to binding to fibrinogen. The volume was adjusted to 180 μl with TBS. At different time points, thrombin activity was stopped by addition of 60 μl of PPACK (final concentration of 37.5 μM). Samples (100 μl/well, in duplicate) of these incubation mixtures were then allowed to bind to the immobilized fibrinogen. After washing with TBS containing 0.1% Tween 20, the plates were incubated with a sheep anti-TAFI polyclonal antibody (3 CPB-(293–333), and TAFI-CPB-(293–401) to plasminogen, the same procedure as described for fibrinogen was applied in 10 mM NaAc, pH 5.0; and this chimera was 1 amino acid shorter than wild-type TAFI. TAFI-CPB-(293–401) contained another 31 extra amino acid changes in addition to the ones also present in TAFI-CPB-(293–333), again including the R302Y mutation, and included the same total number of amino acids as wild-type TAFI. In both chimeras, the polymorph residue 325 is a Ser residue. The constructs were stably expressed in baby hamster kidney cells, and the chimeric proteins were purified from culture media. The two chimeras were characterized and compared with recombinant human TAFI.

Activation of TAFI by the Thrombin-Thrombomodulin Complex—TAFI, TAFI-CPB-(293–333), and TAFI-CPB-(293–401) were incubated with the thrombin-thrombomodulin complex at 37 °C. At various time points, samples were removed; the reactions were stopped by addition of PPACK; and TAFI activity was measured using hippuryl-Arg as a substrate. TAFI-CPB-(293–333) was activated by thrombin-thrombomodulin, but not as efficiently as wild-type TAFI. Under these conditions, the maximal TAFIa activity of TAFI and TAFI-CPB-(293–333) was reached by ~1 min, after which it decreased (Fig. 2A). TAFI-CPB-(293–401) was activated at a lower rate than wild-type TAFI, and TAFI-CPB-(293–401) was far more stable than wild-type TAFI.

To study the rate of activation of TAFI and the TAFI-CPB chimeras in more detail, the system had to be modified to slow down the reaction by reducing the concentration of thrombin and thrombomodulin and by incubating the reaction mixtures at 4 °C. The rates of activation of TAFI-CPB-(293–333) (8 ± 1 units/liter s−1) and TAFI-CPB-(293–401) (0.3 ± 0.1 units/liter s−1) were ~4 and ~100 times lower, respectively, than that of TAFI (32 ± 7 units liter−1 s−1) (Fig. 2B).

Stability of TAFIa, TAFI-CPB-(293–333), and TAFI-CPB-(293–401)—To investigate the stability of the various TAFIa enzymes, TAFI and TAFI-CPB-(293–333) were activated for 1 min and TAFI-CPB-(293–401) for 10 min by the thrombin-thrombomodulin complex, after which thrombin activity was or was not inhibited by addition of PPACK. In the absence of PPACK, both proteolytic and conformational inactivation could take place. In the presence of PPACK, proteolytic inactivation of TAFIa by thrombin was prevented, whereas spontaneous inactivation could still proceed. The samples were kept at 37 °C to allow spontaneous conformational inactivation. The rate of TAFIa and TAFI-CPB-(293–333) inactivation (Fig. 3A and Table I) and TAFI-CPB-(293–401) inactivation (Fig. 3B and Table I) in the presence of PPACK did not differ from the rate of inactivation in the absence of PPACK (Fig. 3A and Table I). The half-life of TAFI-CPB-(293–333) (0.9 ± 0.2 min) was reduced and that of TAFI-CPB-(293–401) (96 ± 29 min) was prolonged compared with wild-type TAFIa (2.9 ± 0.2 min).

The thrombin-thrombomodulin complex cleaves TAFI (~55 kDa) at Arg242, releasing the activation peptide (~19 kDa; not visible on Coomassie Blue-stained SDS-polyacrylamide gels) from the catalytic domain (36 kDa). The catalytic domain is degraded further by cleavage at Arg302, resulting in fragments of 25 and 11 kDa. The major thrombin cleavage site (Arg242), however, is mutated to a Tyr residue in both TAFI-CPB-(293–333) and TAFI-CPB-(293–401). SDS-PAGE analysis of samples of activation mixtures revealed that the catalytic domains (36 kDa) of TAFI-CPB-(293–333) (Fig. 4A) and TAFI-CPB-(293–401) (Fig. 4B) were indeed not proteolyzed. This indicated that TAFI-CPB-(293–333) and TAFI-CPB-(293–401) could not be proteolytically inactivated by thrombin.

Functionality of TAFI, TAFI-CPB-(293–333), and TAFI-CPB-(293–401) in a Plasma System—The ability of TAFI, TAFI-CPB-(293–333), and TAFI-CPB-(293–401) to protect a fibrin clot from lysis was studied in a clot lysis assay in which t-PA-mediated clot lysis of a thrombin-induced clot was fol-
lowed spectrophotometrically over time. TdP was reconstituted with different concentrations of TAFI, TAFI-CPB-(293–333), or TAFI-CPB-(293–401) in either the absence or presence of a TAFIa-specific inhibitor, potato carboxypeptidase inhibitor (CPI). TAFI-CPB-(293–333) could hardly prolong the lysis time, whereas TAFI-CPB-(293–401) could prolong the lysis time in a concentration-dependent manner, although it was less efficient than wild-type TAFI and markedly less efficient than expected based on its half-life (Fig. 5A). Addition of CPI completely inhibited the TAFI- or TAFI-CPB-(293–401)-dependent prolongation of the lysis time. Porcine pancreatic CPB prolonged the clot lysis time in a concentration-dependent way (Fig. 5B). Because the half-life of TAFIa-CPB-(293–401) was much longer than that of wild-type TAFIa, it was expected to protect the clot much better; but, in fact, higher concentrations of TAFI-CPB-(293–401) were needed to prolong the clot lysis time to a similar extent as wild-type TAFI. To investigate if the relatively low efficiency of TAFI-CPB-(293–401) in prolonging the lysis time of TdP was due to a low rate of activation by the thrombin-thrombomodulin complex, TAFI-CPB-(293–401) was activated before it was added to the clot lysis assay. The activation mixture without TAFI-CPB-(293–401) treated in the same way had no effect on the clot lysis time. TAFIa-CPB-(293–401) was able to prolong the clot lysis time in a concentration-dependent manner slightly better than TAFI-CPB-(293–401) that had not been pre-activated; but, despite its long half-life, TAFIa-CPB-(293–401) was only marginally able to delay clot lysis (Fig. 5C). Hence, it is likely that TAFIa-CPB-(293–401) was inactivated in this plasma environment by means other than intrinsic conformational instability or that it was less functional toward fibrin.

Activation of TAFI and Inactivation of TAFIa by Plasmin—One of the possible explanations for the marginal potential of TAFI-CPB-(293–401) to attenuate fibrinolysis was that TAFI-CPB-(293–401) was inactivated by plasmin. In the past, plasmin was shown to be able to inactivate TAFIa by proteolysis. To estimate the amount of plasmin required for clot lysis, a clot lysis experiment with purified components was performed. Fibrinogen was clotted by addition of thrombin and CaCl₂, and various concentrations of plasmin were included in the mixture to allow lysis. The lysis of the clots was followed by measuring the change in turbidity for 2 h (Fig. 6A). At plasmin concentrations of 0, 10, and 25 nM, clots did not lyse completely within the 2-h timeframe. When 50 nM plasmin was used, the lysis time was ~0.5 h, which is about the lysis time of a clot of plasma in the presence of CPI or of TdP (compare Figs. 5A and 6A).
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Fig. 2 Activation and inactivation of TAFI, TAFI-CPB-(293–333), and TAFI-CPB-(293–401) during incubation with thrombin-thrombomodulin. A. TAFI (■), TAFI-CPB-(293–333) (▲), and TAFI-CPB-(293–401) (●) were incubated with thrombin-thrombomodulin at 37 °C. At various time points, aliquots were taken from the activation mixtures, and thrombin activity was inhibited by addition of PPACK. TAFI activity for the substrate hippuryl-Arg was measured (data are expressed as means ± S.D., n = 3). B. TAFI (■), TAFI-CPB-(293–333) (▲), and TAFI-CPB-(293–401) (▼) were incubated with thrombin-thrombomodulin at 4 °C. At various time points, aliquots were taken from the activation mixtures, and thrombin activity was inhibited by addition of PPACK. TAFI activity for the substrate hippuryl-Arg was measured. The lines represent the results of linear regression of the data (data are expressed as means ± S.D., n ≥ 4). Note the difference in time scale in A and B.

Fig. 3 Comparison of the stability of TAFIα, TAFIα-CPB-(293–333), and TAFIα-CPB-(293–401). TAFI (squares) and TAFI-CPB-(293–333) (triangles) were activated for 1 min (A) and TAFI-CPB-(293–401) (circles) for 10 min (B) by incubation of the respective zymogens with thrombin-thrombomodulin at 37 °C. Thrombin activity was (open symbols) or was not (closed symbols) stopped by addition of PPACK, whereas the activated species remained at 37 °C. At various times, aliquots were removed, and the TAFIα activity was measured (data are expressed as means ± S.D., n ≥ 4).

### Table I

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<th>−PPACK</th>
<th>+PPACK</th>
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<td><strong>t½ (min)</strong></td>
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<tr>
<td>TAFI</td>
<td>3.3 ± 0.5</td>
<td>2.9 ± 0.2</td>
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<tr>
<td>TAFI-CPB-(293–333)</td>
<td>1.2 ± 0.04</td>
<td>0.9 ± 0.2</td>
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<tr>
<td>TAFI-CPB-(293–401)</td>
<td>115 ± 22</td>
<td>96 ± 29</td>
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6). Fifty nM plasm in is probably an underestimation of the plasm in concentration required for lysis of a plasma clot because, in the system with purified components, no α2-antiplasmin or factor XIII was included. Therefore, TAFI, TAFI-CPB-(293–333), and TAFI-CPB-(293–401) were incubated with 100 nM plasm in, and the activity was measured over time. Activation of TAFI-CPB-(293–333) by plasm in was very inefficient; hardly any activity could be measured (Fig. 6B). Although plasm in could activate TAFI, the activation of TAFI-CPB-(293–401) was far more efficient. TAFI and TAFI-CPB-(293–401) reached maximal TAFIα activity approximately at the same time (by ~5 min), after which the activity decreased again. Furthermore, the activity of TAFIα-CPB-(293–401) was lost much faster in the presence of plasm in than after addition of the plasm in inhibitor aprotinin (Fig. 6B), suggesting that plasm in is able to inactivate TAFIα-CPB-(293–401). Incubation of CPB (10 nM) with plasm in (0–2 μM) did not result in reduction of CPB activity (data not shown).

Previous research showed that plasm in cleaves TAFI at Arg⁹², resulting in release of the activation peptide from the 36-kDa fragment (7). The latter is cleaved further at several C-terminal sites, resulting in a polypeptide of 25-kDa and some smaller fragments. Some C-terminal cleavages can also occur prior to removal of the activation peptide, resulting in an ~44-kDa fragment that is no longer activable because it lacks critical amino acids involved in catalysis (12). The plasm in cleavage patterns of TAFI and TAFI-CPB-(293–333) seemed similar to that of TAFI purified from plasma (12) as assessed by SDS-PAGE analysis (Fig. 6C). The plasm in cleavage pattern of TAFI-CPB-(293–401) was, however, somewhat different (Fig. 6D). The ~44-kDa fragment and most of the smaller fragments could not be observed, whereas more of the 36-kDa fragment...
was visible. This is in agreement with the relatively high amount of activity measured when TAFI-CPB-(293–401) was incubated with plasmin (Fig. 6B). However, the exact plasmin cleavage site in TAFIa-CPB-(293–401) resulting in inactivation remains unclear.

Comparison of Fibrinogen and Plasminogen Binding—To further characterize the chimeras, the binding to fibrinogen and plasminogen was studied. Fibrinogen or plasminogen was immobilized on a 96-well plate; several dilutions of TAFI, TAFI-CPB-(293–333), and TAFI-CPB-(293–401) were applied to the plate; and bound TAFI was detected using a TAFI-specific antibody. TAFI and TAFI-CPB-(293–333) bound to the same extent to plasminogen (KD/H11005 26/H11006 25 and 25/H11006 29 nM, respectively) and fibrinogen (KD/H11005 943/H11006 383 and 239/H11006 29 nM, respectively), whereas the affinity of TAFI-CPB-(293–401) for both plasminogen and fibrinogen was severely reduced (Fig. 7, A and B). Similar results were obtained using a BIAcore biosensor system (Table II) to study the interaction between plasminogen or fibrinogen and TAFI (KD/H11005 26 and 135 nM, respectively), TAFI-CPB-(293–333) (KD = 33 and 111 nM, respectively), and TAFI-CPB-(293–401) (no binding). No binding of porcine pancreatic CPB to plasminogen or fibrinogen was observed using this system.

To investigate if the capacity of TAFI to bind to fibrinogen would increase after activation/inactivation/proteolytic cleavages, TAFI, TAFI-CPB-(293–333), and TAFI-CPB-(293–401) were incubated with the thrombin-thrombomodulin complex;
thrombin activity was inhibited by addition of PPACK at different time points; and aliquots were taken and kept on ice to prevent further inactivation by conformational instability. The samples were then applied to immobilized fibrinogen, and bound TAFI was detected using a TAFI-specific antibody recognizing TAFI, TAFIa, and/or inactivated TAFIa (TAFIai) as well as the proteolytic fragments of 25 and 11 kDa. The binding of TAFI and TAFI-CPB-(293–333) increased upon longer incubation with the thrombin-thrombomodulin complex (Fig. 7C). Binding is unlikely to depend on TAFIa activity because the activity time curve did not parallel the time course of binding (compare Figs. 2 and 7C). However, proteolysis seemed to be required to increase the affinity of TAFI and/or TAFI fragments for fibrinogen. Binding of TAFI-CPB-(293–401) could not be induced by incubation with thrombin-thrombomodulin. To make sure that the absence of binding of TAFI-CPB-(293–401) to fibrinogen was not due to the immobilization process, the experiment was repeated; but this time, fibrinogen was added to the samples in solution, and binding was allowed for 30 min. Thereafter, formed complexes were captured by an immobilized antibody against fibrinogen. Binding was detected using a TAFI-specific polyclonal antibody. Also in this experimental setup, no binding of TAFI-CPB-(293–401) was observed.

To test if the differences in binding affinity of TAFI, TAFI-CPB-(293–333), and TAFI-CPB-(293–401) for plasminogen and fibrinogen could be attributed to differences in affinity for the anti-TAFI antibodies used, we determined the affinities of the two antibodies for TAFI, TAFI-CPB-(293–333), and TAFI-CPB-(293–401). The $K_D$ values of monoclonal antibody Nik-9H10 used for the plasminogen binding experiments were similar for TAFI-CPB-(293–333), TAFI-CPB-(293–401), and TAFI (0.8, 0.9, and 0.6 nM, respectively). Also, the $K_D$ values of the sheep anti-TAFI antibody used for the fibrinogen binding experiments were similar for TAFI-CPB-(293–333), TAFI-CPB-(293–401), and TAFI (7, 8, and 5 nM, respectively).
DISCUSSION

TAFIa is a labile enzyme that can attenuate fibrinolysis. Both the relevance and the exact nature of the instability are still unclear. However, Schneider et al. (15) showed that the TAFIa Ile<sup>325</sup> isoform has a longer half-life than the TAFIa Thr<sup>325</sup> isoform and that this results in an increased antifibrinolytic potential of the TAFIa Ile<sup>325</sup> isoform, suggesting that the stability of TAFIa is indeed relevant for its function.

To determine which residues in TAFIa are involved in TAFIa inactivation via intrinsic conformational instability, we replaced residues of TAFI with the corresponding residues of pancreatic CPB. The rationale behind this is that, despite conservation of many characteristics between these two homologous enzymes, pancreatic CPB activity is stable, in contrast to TAFIa activity (13). Therefore, we hypothesized that systematic replacement of TAFI residues would result in TAFI-CPB chimeras with altered stability. As a first step, we chose to

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**TABLE II**  
Characteristics of binding of TAFI, TAFI-CPB-(293–333), TAFI-CPB-(293–401), and CPB to plasminogen and fibrinogen.

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<th>Protein</th>
<th>Plasminogen</th>
<th>Fibrinogen</th>
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<tr>
<td></td>
<td>$k_a$</td>
<td>$k_d$</td>
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<tr>
<td>Plasminogen</td>
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<tr>
<td>TAFI</td>
<td>$4.7 \times 10^4$</td>
<td>$6.4 \times 10^{-3}$</td>
</tr>
<tr>
<td>TAFI-CPB-(293–333)</td>
<td>$4.8 \times 10^4$</td>
<td>$5.4 \times 10^{-3}$</td>
</tr>
<tr>
<td>TAFI-CPB-(293–401)</td>
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<td>ND</td>
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<td>CPB</td>
<td>ND</td>
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ND, no detectable binding observed.

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**Fig. 7.** Comparison of the binding of TAFI, TAFI-CPB-(293–333), and TAFI-CPB-(293–401) to plasminogen and fibrinogen. A and B, the capacity of TAFI (■), TAFI-CPB-(293–333) (▲), and TAFI-CPB-(293–401) (○) to bind to plasminogen and fibrinogen, respectively, was assessed in an enzyme-linked immunosorbent assay in which a well was coated with plasminogen or fibrinogen; TAFI was allowed to bind; and the complex was detected using an anti-TAFI antibody. The data are represented as means ± S.E. of duplicates. The lines represent the fit of the results of nonlinear regression of the data to the binding equation described under “Experimental Procedures.” C and D, TAFI, TAFI-CPB-(293–333), and TAFI-CPB-(293–401) were incubated with the thrombin-thrombomodulin complex. At the indicated time points (in minutes), aliquots were removed from the incubation mixtures; thrombin activity was quenched with PPACK; and binding to immobilized fibrinogen (following the same procedure as described for B) (C) and fibrinogen in solution (D) was allowed. To study the binding in solution, fibrinogen was added to the aliquots of the incubation mixtures supplemented with PPACK, and complex formation was allowed for 30 min. The complexes were then captured by an immobilized anti-fibrinogen polyclonal antibody and detected using an anti-TAFI polyclonal antibody.
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replace His\textsuperscript{293}–His\textsuperscript{333} (TAFI-CPB-(293–333)), a region that contains many sites known to influence TAFIa stability (Arg\textsuperscript{292}, Arg\textsuperscript{295}, Arg\textsuperscript{300}, and Thr/Ile\textsuperscript{325}) (10, 11, 15) and that differs substantially from pancreatic CPB. The majority of the amino acids replaced in TAFI-CPB-(293–333), residues 306–326, are predicted by the three-dimensional TAFI model proposed by Barbosa Pereira et al. (21) to form an α-helix across the surface of the protein. The second chimera (TAFI-CPB-(293–401)) included the same stretch of amino acids from CPB as TAFI-CPB-(293–333), but the region was extended to the C terminus.

The mutations severely altered the characteristics of the enzymes compared with wild-type TAFI. The rate of activation of TAFIa-CPB-(293–333) was reduced 4-fold, and that of TAFI-CPB-(293–401) by 100-fold. This suggests that the C-terminal part of TAFI is involved in the activation process, proteolysis at Arg\textsuperscript{292}. This may be explained by a disturbed interaction with the thrombin-thrombomodulin complex or by a different accessibility of the proteolytic cleavage site due to a changed orientation of the activation peptide of the chimeric proteins.

The half-life by spontaneous decay of TAFIa-CPB-(293–333) was reduced to 0.9 ± 0.2 min compared with wild-type TAFIa, with a half-life of 2.9 ± 0.2 min, whereas the half-life of TAFI-CPB-(293–401) was strikingly prolonged to 96 ± 29 min. This showed that the C terminus of TAFIa was involved in the determination of the (in)stability of TAFI. Moreover, it showed that our approach is suitable for identifying the molecular determinants that regulate TAFIa (in)stability.

In the concentration range tested, TAFI-CPB-(293–333) could not prolong the clot lysis time of TdP. Its short half-life is probably one of the reasons for this. It has been shown that the antifibrinolytic potential of TAFIa depends on the stability and that a low antifibrinolytic potential of a particular TAFI variant cannot be overcome by increasing the concentration (22). Furthermore, TAFI-CPB-(293–333) bound to plasminogen and fibrinogen with affinities similar to those of TAFI. Thus, it can prolong the lysis time of TdP. It is more likely that there are other reasons yet to be identified, which may include a reduced rate of activation and inactivation, why TAFIa-CPB-(293–401) is, despite its stability, not a good attenuator of clot lysis.

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