Role of Zinc Ions in Activation and Inactivation of Thrombin-Activatable Fibrinolysis Inhibitor†

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ABSTRACT: Thrombin-activatable fibrinolysis inhibitor (TAFI) circulates as an inactive proenzyme of a carboxypeptidase B-like enzyme (TAFIa). It functions by removing C-terminal lysine residues from partially degraded fibrin that are important in tissue-type plasminogen activator mediated plasmin formation. TAFI was classified as a metallocarboxypeptidase, which contains a Zn^{2+}, since its amino acid sequence shows ∼40% identity with pancreatic carboxypeptidases, the Zn^{2+} pocket is conserved, and the Zn^{2+} chelator *o*-phenanthroline inhibited TAFIa activity. In this study we showed that TAFI contained Zn^{2+} in a 1:1 molar ratio. *o*-Phenanthroline inhibited TAFIa activity and increased the susceptibility of TAFI to trypsin digestion. TAFIa is spontaneously inactivated (TAFIai) by a temperature-dependent intrinsic mechanism. The lysine analogue ϵ-ACA, which stabilizes TAFIa, delayed the *o*-phenanthroline mediated inhibition of TAFIa. We investigated if inactivation of TAFIa involves the release of Zn^{2+}. However, the zinc ion was still incorporated in TAFIai, indicating that inactivation is not caused by Zn^{2+} release. After TAFIa was converted to TAFIai, it was more susceptible to proteolytic degradation by thrombin, which cleaved TAFIai at Arg^{302}. Proteolysis may make the process of inactivation by a conformational change irreversible. Although ϵ-ACA stabilizes TAFIa, it was unable to reverse inactivation of TAFIa or R302Q-rTAFIa, in which Arg^{302} was changed into a glutamine residue and could therefore not be inactivated by proteolysis, suggesting that conversion to TAFIai is irreversible.

Thrombin-activatable fibrinolysis inhibitor (*1*) [TAFI,[1] procarboxypeptidase U (*2*), procarboxypeptidase R (*3*), plasma procarboxypeptidase B (*4*); EC 3.4.17.20] is a zymogen of a carboxypeptidase B, TAFIa, which provides a link between coagulation and fibrinolysis (*5*, *6*). TAFIa efficiently attenuated fibrinolysis by removing C-terminal lysine residues from partially degraded fibrin, thereby preventing tissue-type plasminogen activator mediated accelerated plasmin formation (*7*).

TAFI shows ∼40% identity (*4*) to pancreas carboxypeptidase B, which has been studied extensively over the past decades (reviewed in ref *8*). Comparison of the sequences suggested conservation of the amino acid residues involved in the coordination of Zn^{2+}, His^{159}, Glu^{162}, and His^{288} (TAFI numbering) (*4*) in TAFI. Furthermore, TAFIa was inactivated upon addition of the Zn^{2+} chelator *o*-phenanthroline (*2*, *9*, *10*). On the basis of these observations, TAFIa has been classified as a metallocarboxypeptidase B. Metallocarboxypeptidases each carry one Zn^{2+} in their catalytic center that plays an important role in substrate hydrolysis. The Zn^{2+} is involved in the polarization of bound water by generating a hydroxyl nucleophile, which attacks the carbonyl of the peptide bond of the carboxy-terminal lysines or arginine. Furthermore, positive charges of Zn^{2+} aid to stabilize a tetrahedral intermediate. Collapse of this intermediate results in breakage of the peptide bond of the substrate. The presence of Zn^{2+} in TAFI, however, has not been shown directly.

Analogous to pancreas carboxypeptidase B (*11*, *12*), TAFI is activated to TAFIa by a single proteolytic cleavage resulting in the release of an N-terminal fragment, the activation peptide, from the 36 kDa catalytic domain (*4*). N-Terminal sequencing and mass analysis of the TAFI fragments revealed that TAFI is cleaved at Arg^{92} upon activation by thrombin, trypsin, and plasmin (*4*, *13*).

In contrast to pancreas carboxypeptidase B, TAFIa is a labile enzyme (*4*, *9*, *13*−*17*). Upon incubation with thrombin the 36 kDa catalytic domain is proteolyzed at Arg^{302}, resulting in 25 kDa and 11 kDa fragments (*13*, *17*). However, loss of TAFIa activity precedes proteolytic degradation of TAFIa (*13*, *15*, *17*). This suggested that the cleavage site becomes more accessible to proteolysis upon inactivation. The inactivation of TAFIa is temperature-dependent and is considered to be the result of a conformational change that converts TAFIa to its inactive state TAFIai. To date, the actual event leading to the conformational change, as well as its character, and the exact role of proteolysis of TAFIa

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[1] Abbreviations: ϵ-ACA, 6-aminocaproic acid; TAFI, thrombin-activatable fibrinolysis inhibitor; TAFIa, activated TAFI; TAFIai, inactivated TAFIa; PPACK, E-D-Phe-Pro-Arg chloromethyl ketone; SBTI, soybean trypsin inhibitor.

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inactivation in vivo are unknown. In this study we investigated the importance of the coordinated zinc ion for TAFIa activity and TAFIa inactivation.

MATERIALS AND METHODS

Materials. Trypsin was purchased from Roche (Almere, The Netherlands); hippurylarginine, soybean trypsin inhibitor (SBTI), and cyanuric chloride were from Sigma. Rabbit lung thrombomodulin was from American Diagnostica (Greenwich, CT). 1,4-Dioxane was obtained from Aldrich Chemical Co. (Milwaukee, WI), and H-D-Phe-Pro-Arg-Pro chloromethyl ketone (PPACK) was from Bachem (Bubendorf, Switserland). Thrombin was a generous gift from Dr. W. Kisiel (University of New Mexico, Albequerque, NM). *o*-Phenanthroline was purchased from Fluka Chemika (Switzerland); 6-aminocaproic acid (ϵ-ACA) was from Riedel-de-Haen (Seelze, Germany). S2567 was purchased from Chromogenix (Mölndal, Sweden).

Purification of TAFI, rTAFI, and the TAFI Mutant R302Q-rTAFI. TAFI was purified, and recombinant TAFI (rTAFI) and R302Q-rTAFI were cloned, expressed, and purified as described previously (*13*). Briefly, culture media or 3 L of citrated plasma supplemented with 0.1 M ϵ-ACA was applied to a PD-10 Sephadex G-25M column (Amersham Pharmacia Biotech) connected to a CNBr-activated Sepharose column to which an antibody against TAFI (MoAb Nik-9H10, 4.5 mg/mL, 10 mL) was coupled. The columns were equilibrated in TBS (50 mM Tris, 150 mM NaCl, pH 7.4). Unbound and nonspecifically bound proteins were washed away with 0.5 M NaCl in TBS, and bound protein was eluted with 0.1 M glycine, pH 2.7. The eluted fractions were collected in 1/10 (v/v) 1 M Tris, pH 9, pooled and diluted four times in H_2O, and applied to a CNBr-activated Sepharose column to which an antibody against human serum albumin was coupled. The flow-through was applied to a protein G−Sepharose column. Both columns were equilibrated with 20 mM Tris, pH 7.4. The flow-through was applied to a MonoQ column equilibrated with the same buffer. Bound protein was eluted with a linear gradient (0−250 mM NaCl in 20 mM Tris, pH 7.4). Fractions containing TAFI were pooled and stored at −70 °C. TAFI concentrations were determined with a BCA kit (Pierce) using bovine serum albumin as a standard.

Incubation of TAFI. To investigate the importance of Zn^{2+} in TAFI activation, TAFI (270 nM) was incubated with trypsin (300 nM) in the presence of *o*-phenanthroline (10 mM) at 37 °C. Since the stock solution of *o*-phenanthroline (1 M) was dissolved in ethanol resulting in a concentration of 1% ethanol during incubations, also 1% ethanol was used in control situations. At various time points, samples were taken, and TAFIa activity was measured. To analyze the tryptic cleavage pattern, TAFI (2.7 μM) was incubated with trypsin (300 nM) in the presence of 10 mM *o*-phenanthroline at 37 °C, and samples were subjected to SDS−PAGE. To investigate the importance of Zn^{2+} in TAFIa activity, TAFI (270 nM) was activated by thrombin (20 nM), thrombomodulin (5 nM), and $CaCl_2$ (5 mM) at 37 °C. After 7.5 min the thrombin activity was inhibited by addition of PPACK (30 μM), and *o*-phenanthroline (10 mM) was added. At various time points TAFIa activity was measured. To investigate if Zn^{2+} release was reversible, TAFI (7.3 μM) was incubated with *o*-phenanthroline (10 mM) at room temperature for 5 min. Then, *o*-phenanthroline was removed by dialysis against TBS (50 mM Tris, 150 mM NaCl, pH 7.4) containing $ZnCl_2$ (10 mM) and subsequently dialyzed against TBS to remove unincorporated Zn^{2+}. Dialysis was performed at 4 °C. TAFI (270 nM) was incubated with trypsin (300 nM) at 37 °C, and samples were taken and subjected to the TAFIa activity assay. For SDS−PAGE analysis, 2.7 μM TAFI was used.

Zn^{2+} Release during TAFIa Inactivation. TAFI (5.5 μM) was incubated with thrombin (20 nM), thrombomodulin (5 nM), and $CaCl_2$ at 37 °C. After 10 min, thrombin activity was inhibited by hirudin (1000 units/mL), and the mixture was incubated at 37 °C for another 2 h to allow complete intrinsic inactivation of TAFIa. Subsequently, unincorporated Zn^{2+} was removed by dialysis against TBS for 2 h at 4 °C. As controls, TAFI incubated with hirudin and the activation mixture, as well as the activation mixture and hirudin, was used. The samples were analyzed for activity, protein concentration, and zinc concentration. The concentrations were corrected for the amount of protein and Zn^{2+} in the activation/hirudin mixture.

Trypsin Activity. Trypsin diluted to 60 nM in HBS (20 mM Hepes, 137 mM NaCl, 4 mM KCl, pH 7.4)/0.1% bovine serum albumin, in a volume of 30 μL, was supplemented with 10 μL of HBS/0.1% bovine serum albumin, SBTI (50 μg/mL), or *o*-phenanthroline (10 mM), and 10 μL of the chromogenic substrate S2567 (final concentration of 0.6 mM) was added. Absorbance was measured at 405 nm in a multiscan photometer (V_{max} reader; Molecular Devices, Menlo Park, CA). Experiments were performed three times in duplicate.

Reversibility of TAFIa Inactivation. rTAFI (2.7 μM) and R302Q-rTAFI (5.5 μM) were incubated with thrombin (20 nM), thrombomodulin (5 nM), and $CaCl_2$ (5 mM) at 37 °C. At different time points, TAFIa activity was measured. After 10 min, thrombin activity was quenched by the addition of PPACK (30 μM). After complete inactivation of TAFIa (60 min incubation at 37 °C) the lysine analogue ϵ-ACA was added. Samples were removed, and TAFIa activity was measured at various time points.

TAFIa Activity Assay. TAFIa activity was measured as described (*13*). Aliquots of the incubation mixtures were diluted to a final concentration of 0.2 μM rTAFI, 0.4 μM R302Q-rTAFI, or 0.16 μM plasma-derived TAFI. Dilutions were made in HBS/0.1% bovine serum albumin containing hippurylarginine (4 mM) and an inhibitor of the activating enzyme (30 μM PPACK to inhibit thrombin, 50 μg/mL SBTI to inhibit trypsin). Substrate conversion was allowed for 10 min at room temperature. Reactions were stopped by addition of 20 μL of 1 M HCl and neutralized by 20 μL of 1 M NaOH. Then 25 μL of 1 M sodium phosphate (pH 7.4) and 30 μL of 6% cyanuric chloride dissolved in 1,4-dioxane were added. Samples were vortexed and centrifuged to remove excess of cyanuric chloride and denatured proteins, and 100 μL was transferred to a 96-well microtiter plate. Absorbance was measured at 405 nm in a multiscan photometer (V_{max} reader; Molecular Devices, Menlo Park, CA). Experiments were performed three times, and data are expressed as mean ± SD.

SDS−PAGE Analysis. Proteins were subjected to SDS−PAGE (*18*) analysis under reducing conditions. SDS−PAGE gels of 12.5% acrylamide were used, and proteins were

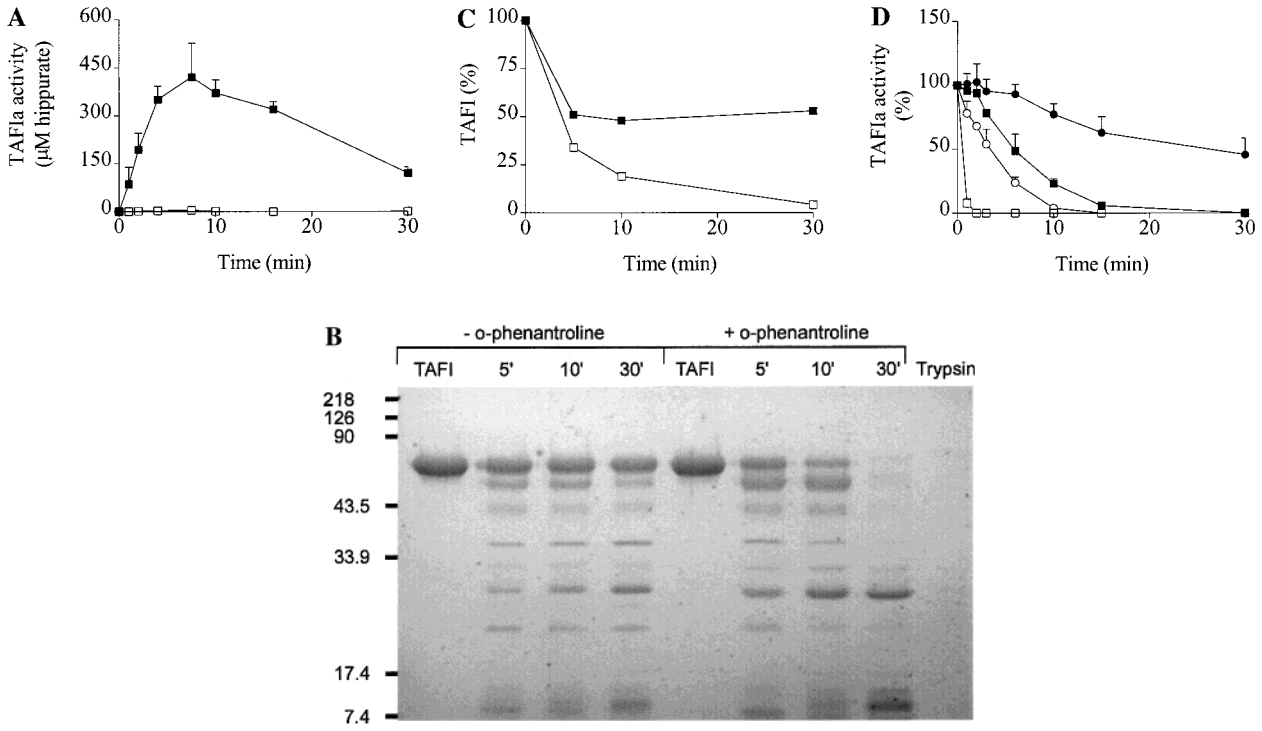

FIGURE 1: Influence of *o*-phenanthroline on TAFI. (A) TAFI (270 nM) was incubated with trypsin (300 nM) in the absence (■) or presence (□) of *o*-phenanthroline (10 mM) at 37 °C. At indicated time points trypsin activity was inhibited by SBTI (50 μg/mL), and TAFIa activity toward hippurylarginine was measured. (B) Trypsin-mediated proteolysis of TAFI was followed in time (minutes) by incubating TAFI (2.7 μM) and trypsin (300 nM) in the presence or absence of *o*-phenanthroline (10 mM) at 37 °C. Samples were analyzed by SDS−PAGE. Positions of molecular mass standards are indicated on the left. (C) The relative amount of TAFI in the absence (■) and presence (□) of *o*-phenanthroline. (D) To follow the influence of *o*-phenanthroline on TAFIa activity, TAFI (270 nM) was incubated with thrombin (20 nM), thrombomodulin (5 nM), and $CaCl_2$ (5 mM) at 37 °C. After 7.5 min, thrombin activity was inhibited by addition of PPACK (30 μM), *o*-phenanthroline (10 mM, squares) or buffer (circles) was added, and TAFIa activity was determined. The experiment was done in the presence (closed symbols) or absence (open symbols) of ϵ-ACA.

visualized by Coomassie Brilliant Blue staining. The density of the protein bands was scanned and quantified using ImageQuant 3.3 software (Molecular Dynamics).

Atomic Absorption Spectroscopy. Zinc concentrations were determined at a wavelength of 213.9 nm on a SpectraAA 220 Varian.

RESULTS

TAFI was assumed to be a Zn^{2+}-containing metallocarboxypeptidase on the basis of its homology with pancreas carboxypeptidase B (*4*), the presence of a Zn^{2+} pocket (*4*), and the observation that TAFIa activity is inhibited by the Zn^{2+} chelator *o*-phenanthroline (*2*, *4*, *9*, *10*). To establish the presence of a Zn^{2+} in TAFI, TAFI purified from human plasma was subjected to atomic absorption spectroscopy, which specifically detects Zn^{2+} (data not shown). TAFI was found to contain Zn^{2+} at a stoichiometry of 1:1, indicating that circulating TAFI contains a single Zn^{2+} per molecule.

The Zn^{2+} is essential for the hydrolysis of substrates by metallocarboxypeptidases. To investigate if Zn^{2+} is required for the generation of TAFIa activity, TAFI was incubated with trypsin in the presence or absence of the Zn^{2+} chelator *o*-phenanthroline and analyzed for TAFIa activity (Figure 1A). No activity was generated in the presence of *o*-phenanthroline. This was not caused by a direct effect on the amidolytic activity of trypsin, which was not influenced by *o*-phenanthroline (results not shown). Samples were also analyzed by SDS−PAGE (Figure 1B,C). TAFI was proteolyzed by trypsin in both the presence and absence of *o*-phenanthroline. The pattern is consistent with activation of TAFI by release of the activation peptide from the 36 kDa catalytic fragment and the generation of several other TAFI-derived fragments. Furthermore, TAFI seemed more susceptible to proteolysis in the presence of *o*-phenanthroline, suggesting that Zn^{2+} was required not only for TAFIa activity but also for maintenance of the structural integrity of the protein. To investigate if the Zn^{2+} was indeed essential for TAFIa activity, TAFI was activated with thrombin/thrombomodulin. At maximal activity, the thrombin activity was quenched by PPACK, and *o*-phenanthroline was added to complex the Zn^{2+}, after which TAFIa activity was followed in time (Figure 1D). The TAFIa activity was lost almost instantaneously in the presence of *o*-phenanthroline, indicating again that the Zn^{2+} is required for TAFIa activity. Addition of the lysine analogue ϵ-ACA, which stabilizes TAFIa activity, reduced the inhibitory effect of *o*-phenanthroline (Figure 1D). This suggested that a conformational change facilitates the release of Zn^{2+}.

To investigate if Zn^{2+} incorporation was reversible, TAFI was incubated with *o*-phenanthroline for 5 min, after which time *o*-phenanthroline was removed by dialysis against TBS or TBS containing Zn^{2+}. Free Zn^{2+} was subsequently removed by dialysis against TBS. The TAFI preparations were, after dialysis against either TBS or Zn^{2+}/TBS, incubated with trypsin, and TAFIa activity was followed in time (Figure 2A). No activity was generated in the *o*-phenanthroline-treated samples. Furthermore, SDS−PAGE analysis revealed that the susceptibility to proteolysis was increased

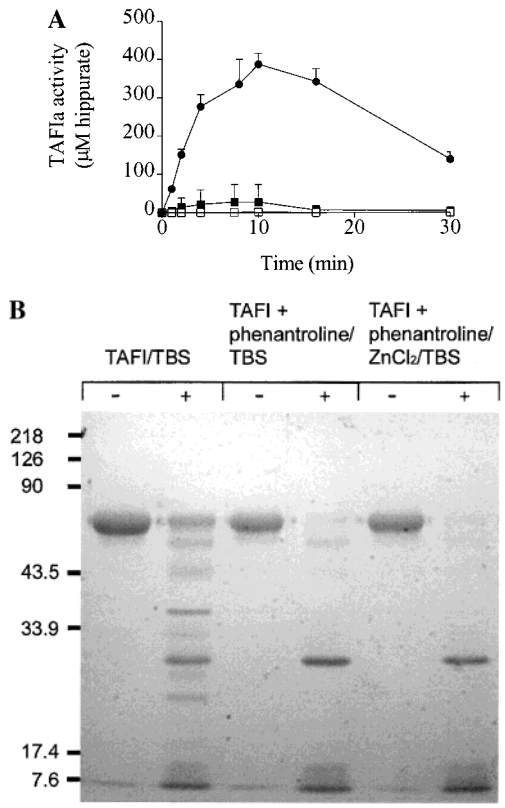

FIGURE 2: Reversibility of Zn^{2+} incorporation in TAFI. (A) TAFI (7.2 μM) was incubated with *o*-phenanthroline (10 mM) for 5 min, after which time it was extensively dialyzed against TBS (■) or $ZnCl_2$ (10 mM)/TBS and subsequently against TBS to remove free Zn^{2+} (□). TAFI incubated with buffer instead of *o*-phenanthroline and dialyzed against TBS (●) was taken in parallel as control. The activatability of the different TAFI preparation was analyzed by incubating TAFI (270 nM) with trypsin (300 nM). At the indicated times, samples were removed, trypsin activity was inhibited by addition of SBTI, and TAFIa activity toward hippurylarginine was measured. (B) Samples of the different preparations (2.7 μM TAFI) were analyzed by SDS−PAGE [−, not incubated with trypsin; +, incubated with trypsin (300 nM) at 37 °C for 5 min].

in the samples treated with *o*-phenanthroline (Figure 2B). We therefore concluded from Figure 2A,B that Zn^{2+} incorporation in TAFI was not reversible after removal by *o*-phenanthroline.

TAFIa activity is unstable at 37 °C (*4*, *9*, *13*−*17*). The enzyme is considered to convert spontaneously into an inactive form, TAFIai, by a process referred to as conformational instability (*13*, *17*). The presence of ϵ-ACA prevents this conformational change (*13*, *17*, *19*). Since TAFIa is inactivated upon addition of a Zn^{2+} chelator, a process that can be slowed by addition of ϵ-ACA, we investigated if inactivation results from release of the Zn^{2+} from TAFIa. TAFI was activated at 37 °C by the thrombin/thrombomodulin complex, and the TAFIa activity was monitored. At maximal activity, the thrombin activity was quenched by hirudin to prevent further cleavage, and complete inactivation of TAFIa was allowed by incubating the sample for another 2 h at 37 °C. To remove possibly released Zn^{2+}, the sample was dialyzed against TBS for 2 h at 4 °C. TAFI incubated with the activation mixture to which hirudin was added prior to incubation, as well as the activation mixture with hirudin, was used as control. Semiquantification of the protein bands on SDS−PAGE (Figure 3) showed that 40% of TAFI had

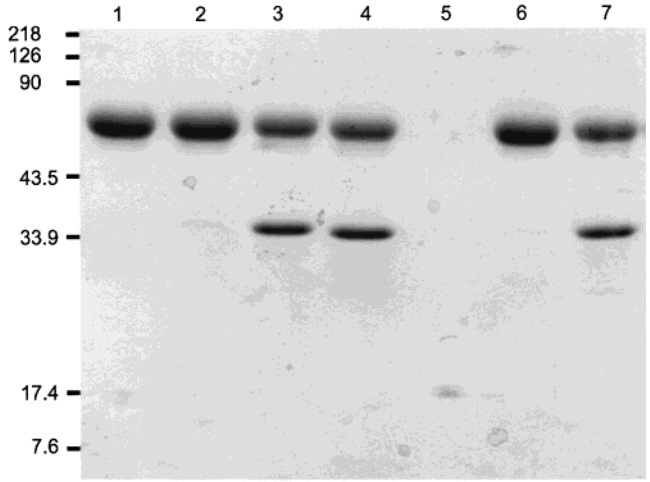

FIGURE 3: Presence of Zn^{2+} in TAFI and TAFIai. TAFI (5.5 μM) was incubated with an activation mixture containing thrombin (20 nM), thrombomodulin (5 nM), and $CaCl_2$ (5 mM) at 37 °C. After 10 min, thrombin activity was inhibited by the addition of hirudin (1000 units/mL). Samples of the mixture were withdrawn, the TAFIa activity was measured, and the fragment generation was visualized by SDS−PAGE. The mixture was incubated for another 2 h at 37 °C to allow complete inactivation of TAFIa as determined by the activity assay. The TAFI incubated with the activation mixture and hirudin, as well as the activation mixture with hirudin, was taken as control. The three reaction mixtures were dialyzed against TBS at 4 °C for 2 h to remove possibly released Zn^{2+}, and the Zn^{2+} concentration was measured using atomic absorption spectroscopy. Samples were analyzed by SDS−PAGE to establish the relative amount of TAFIai formed. Lanes: 1, TAFI; 2, TAFI + hirudine + IIa/TM; 3, TAFIa + IIa/TM, 10 min at 37 °C, + hirudine; 4, TAFIa + IIa/TM, 10 min at 37 °C, + hirudine, 2 h at 37 °C; 5, IIa/TM + hirudine; 6, same as lane 2 but after dialysis; 7, same as lane 3 but after dialysis. Positions of molecular mass standards are indicated on the left.

been converted to TAFIa within the first 10 min incubation period and that the amount of the 36 kDa fragment that had been proteolyzed further was negligible. After an additional 2 h of incubation at 37 °C all the TAFIa had been converted to TAFIai, since no activity was detected, and no further proteolysis was observed. The preparations were dialyzed to remove unincorporated Zn^{2+}, after which the protein concentration was determined in each sample. Dialysis had no influence on the ratio of TAFI to TAFIai as checked by SDS−PAGE (Figure 3, lanes 6 and 7). We therefore expected that if Zn^{2+} would be released upon TAFIa inactivation, the Zn^{2+} concentration would be reduced by ~40%. However, the Zn^{2+} concentration in TAFIai (4.8 μM protein and 5.7 μM Zn^{2+}) was not decreased compared to TAFI (5.8 μM protein and 4.0 μM Zn^{2+}). The conformational change involved in TAFIa inactivation is apparently not caused by Zn^{2+} release from TAFIa.

After TAFIa is converted to its inactive state, it becomes more susceptible to proteolytic degradation by thrombin, which cleaves TAFIai at Arg^{302} (*13*, *17*). Proteolysis may make the process of inactivation by a conformational change irreversible. It is unclear, however, if TAFIa inactivation is reversible. To address this question, recombinant wild-type TAFI, rTAFI, and a rTAFI mutant in which Arg^{302} was mutated into a glutamine, R302Q-rTAFI, were incubated with thrombin/thrombomodulin for 7.5 min at 37 °C to allow activation of TAFI. At maximal TAFIa activity, thrombin activity was stopped by addition of PPACK to prevent any

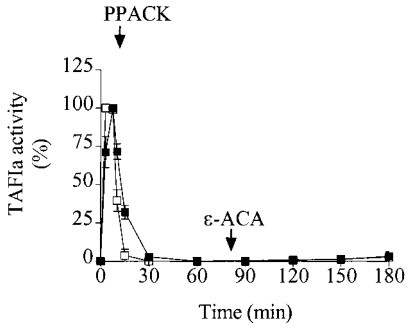

FIGURE 4: Reversibility of TAFIa inactivation. rTAFI (2.7 μM, ■) and R302Q-rTAFI (5.5 μM, □) were incubated with thrombin (20 nM), thrombomodulin (5 nM), and $CaCl_2$ (5 mM) at 37 °C. After 7.5 min, thrombin activity was inhibited with PPACK (30 μM), and incubation was continued to allow inactivation of TAFIa by conformational instability. After 60 min of incubation, ϵ-ACA (5 mM) was added. At the indicated time points, aliquots were removed, and the activity toward hippurylarginine was measured.

further proteolysis. Incubation was continued at 37 °C to allow complete inactivation of TAFIa, after which ϵ-ACA was added. At several time points TAFIa activity was measured (Figure 4). Since ϵ-ACA stabilizes TAFIa activity (*13*, *15*, *16*), we argued that if TAFIa inactivation was reversible, incubation with ϵ-ACA would lead to the recurrence of TAFIa activity. However, no activity was observed after ϵ-ACA was added, suggesting that the conformational change leading to TAFIa inactivation is irreversible.

DISCUSSION

On the basis of the homology of TAFI with other known carboxypeptidases such as pancreas carboxypeptidases A and B (*4*), TAFI was classified as a metallocarboxypeptidase. Comparison of the amino acid sequence of TAFI to that of pancreas carboxypeptidase B indicated that the Zn^{2+} is probably coordinated to His^{159}, Glu^{162}, and His^{288} in TAFI (TAFI numbering) (*4*). Although TAFIa activity was inhibited upon addition of *o*-phenanthroline (*2*, *9*, *10*), the presence of a Zn^{2+} in its active center had not been proven directly. In this study, we showed that TAFI purified from human plasma contained one Zn^{2+} per molecule. Furthermore, no TAFIa activity could be generated by incubation of TAFI with trypsin in the presence of *o*-phenanthroline, and TAFIa activity was instantaneously lost upon addition of *o*-phenanthroline to TAFIa, whereas there was no direct effect of *o*-phenanthroline on the trypsin activity. This indicated that the Zn^{2+} is essential for the expression of TAFIa activity. Once Zn^{2+} was removed from TAFI by *o*-phenanthroline, the Zn^{2+}-depleted TAFI was unable to incorporate Zn^{2+}. However, we cannot rule out that the structural change, indicated by increased susceptibility to trypsin-mediated proteolysis observed after pretreatment of TAFI with *o*-phenanthroline, prevented incorporation of Zn^{2+}, whereas in the absence of *o*-phenanthroline binding might be reversible and a number of divalent cations might be able to replace the Zn^{2+}. Tan et al. (*19*), for example, showed that Co^{2+} stimulated the activity toward an ester substrate and inhibited the activity toward a peptide substrate. The opposite effect was observed with Cd^{2+}. Similar results were reported by others (*9*, *10*); however, in all of these experiments Co^{2+} and Cd^{2+} were additives and Zn^{2+} was not removed prior to the experiment.

As mentioned above, TAFI was more susceptible to proteolysis upon *o*-phenanthroline incubation, indicating a change within the TAFI three-dimensional structure. TAFIa is an unstable enzyme, which can be stabilized by ϵ-ACA by keeping TAFIa in its active conformation. Addition of ϵ-ACA to TAFIa slowed inhibition of TAFIa activity by *o*-phenanthroline, suggesting that ϵ-ACA delayed the release of the Zn^{2+}. It might do so by hindering the structural changes observed upon *o*-phenanthroline treatment, which might either be a prerequisite or a consequence of Zn^{2+} release. Alternatively ϵ-ACA might hinder *o*-phenanthroline directly. We investigated if the conformational change that is associated with TAFIa inactivation resulted from Zn^{2+} release from TAFIa or, in other words, if the Zn^{2+} is still incorporated in TAFIai. Atomic absorption spectroscopy showed that this is indeed the case, indicating that Zn^{2+} release is not the cause of the conformational change leading to TAFIa inactivation.

To investigate the role of proteolysis and conformational instability of TAFIa in TAFIa inactivation, we activated TAFI with thrombin/thrombomodulin, stopped further proteolysis by thrombin by addition of PPACK, and allowed full inactivation of TAFIa, after which ϵ-ACA was added. Although ϵ-ACA stabilizes TAFIa, it was unable to restore TAFIa activity. The same results were obtained with R302Q-rTAFI, in which the major thrombin cleavage site, Arg^{302} of TAFIa, was changed into a glutamine residue and could therefore not be inactivated by proteolysis. This suggested that conversion of TAFIa into TAFIai is irreversible and that proteolysis is not required for this process. Proteolysis, however, may serve other functions, which might be hard to appreciate in a purified system. In the presence of C-terminal lysines, for example, present on fibrin degradation products, TAFIa may be stabilized, in analogy to the situation observed with ϵ-ACA. In this situation TAFIa inactivation might depend on proteolysis rather than conformational instability. One report stated a role for inactivated TAFIa preparations in inhibition of plasmin activity and clot lysis (*20*). Furthermore, TAFIai may still compete for TAFI(a) binding sites, thereby limiting the protective effect of TAFIa on clot lysis. Proteolysis of TAFIai may result in impaired binding, which might neutralize this limiting effect on TAFIa's function. Finally, proteolysis may play a role in completely different processes such as clearance from the circulation or cell signaling. Further research on the separately purified TAFI fragments may generate clues on their specific functions.

In conclusion, Zn^{2+} is important for TAFIa activity but does not seem to play a role in the inactivation of TAFIa by intrinsic conformational instability.

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