Biochemical Importance of Glycosylation in Thrombin Activatable Fibrinolysis Inhibitor

Karlien Buelens, Kerstin Hillmayer, Griet Compernolle, Paul J. Declerck, Ann Gils

Abstract—Activated Thrombin Activatable Fibrinolysis Inhibitor (TAFIa) exerts an antifibrinolytic effect by removing C-terminal lysines from partially degraded fibrin. These lysines are essential for a rapid conversion of plasminogen to plasmin by tissue type plasminogen activator. TAFI is heavily glycosylated at Asn22, Asn51, Asn63, and Asn86. Although the glycans occurring at the glycosylation sites have previously been identified, the biochemical role of these glycans is not known yet. Therefore, we have determined the biochemical importance of the glycosylation in TAFI. Four single, 6 double, 4 triple, and 1 quadruple mutant, in which asparagine was replaced by glutamine, were constructed and transfected into HEK293T cells. Based on the determination of antigen and activity levels on conditioned medium, 4 single and 1 triple mutant were purified and their biochemical properties were determined. The glycosylation knockout mutants did neither reveal an altered fragmentation pattern nor differences in TAFIa stability, but TAFI-N51Q, TAFI-N63Q, and TAFI-N22Q-N51Q-N63Q revealed a decreased TAFIa activity, an increased intrinsic catalytic activity of the zymogen, and a decreased antifibrinolytic potential compared with TAFI–wild-type, whereas TAFI-N22Q and TAFI-N86Q revealed an increased antifibrinolytic potential probably because of an increased catalytic efficiency toward the physiological substrate. From these data it can be concluded that mainly the glycosylation at Asn86 contributes to the biochemical characteristics of TAFI. Furthermore we provide evidence that the activation peptide stays in close proximity to the TAFIa moiety after activation. (Circ Res. 2008;102:295-301.)

Key Words: TAFI ■ fibrinolysis ■ glycosylation

Until recently, it was believed that blood coagulation and fibrinolysis are 2 different systems in the hemostasis and that only fibrin acts as a linker. Because of the discovery of thrombin activatable fibrinolysis inhibitor (TAFI), it became clear that there is a strong interaction between these 2 systems. On activation of TAFI by cleavage with thrombin, generated during coagulation, the active enzyme (TAFIa) is formed.1–3 TAFIa exerts an antifibrinolytic effect attributable to its carboxypeptidase activity which removes C-terminal lysines from (partially degraded) fibrin. As binding of plasminogen to these lysines strongly improves the conversion to plasmin by tPA, TAFIa slows down/decelerates the degradation of the fibrin clot.4

TAFI is synthesized in the liver and secreted as a propeptide consisting of 401 amino acids. It has a molecular weight of 56 kDa. The zymogen is activated through a proteolytic cleavage by thrombin, thrombin/thrombomodulin, and plasmin, resulting in the activation peptide (Phe1-Arg92; 20 kDa) and the catalytic domain TAFIa (Ala93-Val401; 36 kDa). TAFI harbors 4 glycosylation sites in the activation peptide (Asn22, Asn51, Asn63, and Asn86), which are always glycosylated, and 1 glycosylation site in the TAFIa moiety (Asn210), which is found in both a glycosylated and a nonglycosylated form.5 The glycans attached to Asn22 appeared to be homogeneous and their structure is a tetra-antennary complex type with bisecting N-acetylgalactosamine. On the other hand, Asn51, Asn63, and Asn86 display microheterogeneity, consisting of varying amounts of fucose and the glycans vary between hybrid and complex types of glycosylation.5

N-glycosylation is a major posttranslational modification in eukaryotic cells and takes place at the tripeptide sequence Asn-X-Ser/Thr, where X can be any amino acid with the exception of proline. The N-linked oligosaccharides, already added to the specific sequences when the polypeptide chain enters the lumen of the endoplasmic reticulum and thus before the protein is folded, may play multiple roles during the conformational maturation of glycoproteins.6 They are needed to stabilize folded domains and provide solubility enhancing polar surface groups that prevent irreversible aggregation of folding intermediates. Evidence has been provided that this posttranslational modification may also influence many of the biochemical properties of the proteins, such as stability, solubility, targeting, dynamics, and ligand binding.7 When glycosylation is inhibited, the most commonly observed effect is the generation of misfolded aggregated proteins.6,7

In TAFI, glycans are responsible for 9.2 kDa (ie, 17%) of the molecular weight.9 This high degree of glycosylation

References


hampers most likely the crystallization of TAFI and consequently the 3D structure of TAFI has not been elucidated yet.

Although some research has been devoted to study glycosylation of TAFI,\textsuperscript{5,10–12} the biochemical importance of glycosylation of TAFI has not been studied yet. The objective of this study was to elucidate the biochemical importance of glycosylation of TAFI. Glycosylation knockout mutants were constructed in which glycosylation sites in the activation peptide were deleted by replacing Asn by Gln. These mutants were then compared with wild-type TAFI (TAFI-wt, i.e., TAFI-Thr\textsuperscript{147}Ile\textsuperscript{325}) with respect to fragmentation pattern after activation, the level of TAFIa activity, intrinsic TAFI activity, TAFIa stability, TAFI activation, and TAFIa enzyme kinetics and the antifibrinolytic effect.

Materials and Methods

Materials

Recombinant TAFI-Thr\textsuperscript{147}Ile\textsuperscript{325} (TAFI-TI) and monoclonal antibodies raised against human plasma-derived TAFI were generated as described before.\textsuperscript{13} Oligonucleotides used for mutagenesis and sequencing were obtained from Sigma-Aldrich.

Human thrombin, rabbit thrombomodulin, H-D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) were obtained from Sigma, American Diagnostica, and Biomol Research Labs, respectively. Hippuryl-L-arginine was obtained from Calbiochem. Lipopectamine 2000 and Optimem 1 medium containing glutamax were purchased from Invitrogen. Citrated plasma of 21 healthy individuals purchased from Invitrogen. Citrated plasma of 21 healthy individuals was pooled for clot lysis experiments. TAFI-depleted plasma was prepared as described by Gils et al.\textsuperscript{14}

Methods

Construction, Expression, and Purification of TAFI Variants

The pcDNA5/FRT vector (Invitrogen) containing the cDNA of TAFI-Thr\textsuperscript{147}Ile\textsuperscript{325} (TAFI-TI) was used as a template for site-directed mutagenesis.\textsuperscript{13} Site-directed mutagenesis was performed with the QuickChange XL Site-directed Mutagenesis kit (Stratagene) to generate TAFI-mutants in which a N-glycosylation site was removed by replacing Asn by Gln, generating the following mutants: 4 single mutants: TAFI-N22Q, TAFI-N51Q, TAFI-N63Q, TAFI-N86Q; 6 double mutants: TAFI-N22Q-N51Q, TAFI-N22Q-N63Q, TAFI-N22Q-N86Q, TAFI-N51Q-N63Q, TAFI-N51Q-N86Q, TAFI-N63Q-N86Q; 4 triple mutants: TAFI-N51Q-N63Q-N86Q, TAFI-N22Q-N51Q-N86Q, TAFI-N22Q-N51Q-N63Q, and 1 quadruple mutant: TAFI-N22Q-N51Q-N63Q-N86Q. The mutants were transiently transfected into HEK239T cells and purified as described by Gils et al.\textsuperscript{14}

Quantification of TAFI Antigen Level and TAFIa Activity Using Conditioned Medium

TAFI antigen levels of the TAFI variants were quantified using the MA-T12D11/MA-T30E5-HRP ELISA system.\textsuperscript{15} All values were expressed relative to the conditioned medium of TAFI-wt.

TAFI activity was determined by chromogenic assay as described earlier with minor modifications.\textsuperscript{16} Briefly, conditioned medium (nondiluted; 96 hours posttransfection) was incubated with thrombin (20 nmol/L), thrombomodulin (5 nmol/L), and CaCl\textsubscript{2} (5 mmol/L) in Hepes-BSA buffer (25 mmol/L Hepes, 137 mmol/L NaCl, 3.5 mmol/L KCl, pH 7.4, containing 0.1% BSA) at 22°C for 10 minutes. Activity was stopped by addition of PPACK (30 μmol/L final concentration) and TAFIa activity was determined by measuring the hippuryl-arginine (Hip-Arg) hydrolysis as described by Hillmayer et al.\textsuperscript{17}

Evaluation of TAFI Fragments Generated on Activation With Thrombin/Thrombomodulin

TAFI fragments generated on activation of the TAFI variants with T/TM were evaluated as described before with minor modifications.\textsuperscript{18} TAFI variants (1 μmol/L) were activated by thrombin (20 nmol/L), thrombomodulin (5 mmol/L), and CaCl\textsubscript{2} (5 mmol/L) in 60 μL Tris buffer (20 mmol/L Tris, 0.1 mol/L NaCl, pH 7.4) at 37°C for 10 minutes. The reactions were stopped at the indicated time points by addition of PPACK (30 μmol/L) and sodium dodecyl sulfate (SDS, 1% final concentration). The samples were heated at 100°C during 30 seconds and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by silver staining.

TAFIa Activity Measurements on Purified Material

Two different batches of each TAFI variant were used to determine TAFIa activity by a chromogenic assay as described earlier with minor modifications.\textsuperscript{16} Purified TAFI variants (0.25 μmol/L) were incubated with thrombin (5 nmol/L) and thrombomodulin (20 nmol/L) and CaCl\textsubscript{2} (1.25 mmol/L) in Hepes-BSA buffer (25 mmol/L Hepes, 137 mmol/L NaCl, 3.5 mmol/L KCl, pH 7.4, containing 0.1% BSA) at 25°C for 5 to 10 minutes (quantitative conversion into TAFIa). Activation was stopped by addition of PPACK (30 μmol/L final concentration). Subsequently, Hip-Arg was added to a final concentration of 4 mmol/L and substrate conversion was allowed to proceed for 10 minutes at 25°C. Reactions were stopped by addition of HCl (1 mol/L), neutralized with NaOH (1 mol/L), and buffered with NaHPO\textsubscript{4} (1 mol/L pH 7.4). Subsequently, 6% cyanuric chloride, dissolved in 1,4-dioxane, was added and the mixtures were vortexed and centrifuged. The supernatant was transferred to a 96-well microtiter plate and the absorbance was measured at 405 nm using an EL ×808 Absorbance Microplate Reader (Bio-tek Instruments Inc.).

We expressed the specific activity of TAFIa under the described conditions in U mg\textsuperscript{-1} TAFI. A standard spanning 62.5 μmol/L to 2 mmol/L hippurate was used to quantify the amount of hippurate.\textsuperscript{17}

Analysis of Intrinsic Catalytic Activity of the Zymogen

The intrinsic catalytic activity of the zymogen was determined of all TAFI variants by a chromogenic assay as described above with some small modifications. TAFI variants (0.27 μmol/L) were incubated with the synthetic substrate Hip-Arg (12.5 μmol/L). Substrate conversion was allowed to proceed for 1 hour at 37°C. Reactions were stopped, the absorbance measured, and the TAFI activity calculated as described above.

TAFI Activation by Thrombin/Thrombomodulin

TAFI activation by T/TM was performed as described by Ceresa et al with some small modifications.\textsuperscript{18} The amount of TAFI-variant generated after each activation time was obtained using specific standard calibration curves for each TAFI-variant (absorbance/min per nmol/L TAFI-variant). TAFI variants (0.06–1 μmol/L) were activated for different time intervals at 25°C (ranging between 0 to 2 minutes) in the presence of CaCl\textsubscript{2} (5 mmol/L), thrombin (0.5 mmol/L), and thrombomodulin (1 nmol/L) in Hepes-BSA buffer. Under these conditions, less than 20% of total TAFI was converted to TAFIa and rates of activation were linear over time of measurements. Activation was stopped by addition of PPACK (30 μmol/L). Samples were incubated for 20 minutes at 25°C with Hip-Arg. The apparent k\textsubscript{cat} and K\textsubscript{m} values for activation of each TAFI variant were determined by nonlinear regression of the data to the Michaelis-Menten equation using GraphPad Prism 4.01.

TAFI Hydrolysis of Hippuryl-L-Arginine

Characterization of TAFIa hydrolysis of Hip-Arg was determined as described by Ceresa et al with some small modifications.\textsuperscript{19} TAFI variants (1 μmol/L) were activated for 5 to 10 minutes at 25°C. Under these conditions TAFI variants are quantitatively converted to TAFIa without conversion to TAFIa (confirmed with SDS-PAGE analysis in parallel with measurement of TAFIa activity). The k\textsubscript{cat}...
and $K_v$ values for Hip-Arg hydrolysis were determined following the procedures described by Ceresa et al.18

**Determination of TAFIa Stability**

Samples were activated with thrombin/thrombomodulin (T/TM) at 22°C for 10 minutes, as described in TAFIa activity measurements, generating a high and reproducible amount of TAFIa. The reaction was stopped with PPACK and the samples were subsequently incubated at 37°C for heat inactivation. After 0, 5, 10, 15, and 20 minutes, aliquots were put on ice until substrate conversion with Hip-Arg was performed. Activities were expressed as percentage of maximum activity and half-lives were calculated by 1-phase exponential decay regression with the GraphPad Prism software.

**Clot Lysis Assay**

The clot lysis assay was performed based on procedures described by Leurs et al with some modifications.19 TAFI-depleted citrated pooled human plasma was supplemented with TAFI variants at a final concentration of 0 to 90 nmol/L, t-PA (40 ng/mL in Hepes 20 mmol/L, 0.1% Tween 20, pH 7.4) and thrombolamin (1 nmol/L) were added, and clot formation was initiated by addition of CaCl2 (12.5 mmol/L final concentration). The final plasma concentration was 50%. All reagents (except t-PA) were diluted in Heps buffer (Hepes 20 mmol/L, pH 7.4) and the reaction was performed at 37°C. Clot formation/dissolution was monitored measuring turbidity at 405 nm every 2 minutes with an EL808 Ultra Micro plate Reader (Bio-Tek instruments Inc.). The clot lysis time was defined as the time between maximum turbidity and the midpoint of maximum turbid to clear transition.

**Detection of TAFI Fragments by Western Blotting on Immunoprecipitation**

TAFI-TI (1 μmol/L) was activated by thrombin (20 μmol/L), thrombolamin (5 μmol/L), and CaCl2 (5 μmol/L) in 60 μL. Tris-buffer (20 μmol/L Tris, 0.1 mol/L NaCl, pH 7.4) at 37°C for 30 minutes. The reaction was stopped by addition of PPACK (30 μmol/L).

The sample was diluted to a final concentration of 0.1 μmol/L in 500 μL PBS buffer (140 μmol/L NaCl, 8 mmol/L Na2HPO4, 2.7 mmol/L KCl, 15.5 mmol/L KH2PO4, pH 7.3; start sample) and incubated for 2 hours at room temperature with 50 μL of MA-T18A8-sepharose beads. The supernatants (nobilizing fraction) was removed and the beads were washed 3 times with 500 μL of PBS. Subsequently, 200 μL of PBS with SDS was added to the beads (final concentration of 1% SDS) and the mixture was heated at 100°C for 1 minute (binding fraction). The start sample, the binding fraction, and the nonbinding fraction were subjected to Western blotting analysis using either MA-T18A8 or MA-T3DS as primary antibody and Goat-Anti-Mouse-HRP as secondary antibody followed by ECL staining.

**Statistical Analysis**

Quantitative data were summarized by the mean and standard deviation. Statistical analyses were performed with Graph Pad Prism 4.01 with the unpaired t test (Graph Pad Software Inc). Probability values less than 0.05 were considered statistically significant.

**Results**

**Generation of TAFI Mutants**

To study the biochemical importance of the N-glycosylation of the activation peptide of TAFI, initially 4 single mutants were generated in which a glycosylation site was removed by replacing Asn by Gln (TAFI-N22Q, TAFI-N51Q, TAFI-N63Q, and TAFI-N86Q). Furthermore, 6 double mutants (TAFI-N22Q-N51Q, TAFI-N22Q-N63Q, TAFI-N22Q-N86Q, TAFI-N51Q-N63Q, TAFI-N51Q-N86Q, TAFI-N63Q-N86Q), 4 triple mutants (TAFI-N51Q-N63Q-N86Q, TAFI-N22Q-N63Q-N86Q, TAFI-N22Q-N51Q-N86Q, TAFI-N22Q-N51Q-N63Q, TAFI-N51Q-N63Q-N86Q) and 1 quadruple mutant (TAFI-N22Q-N51Q-N63Q-N86Q) were generated.

**Quantification of the TAFI Antigen and TAFIa Activity Levels on the Conditioned Medium**

On transfection of the single, double, triple, and quadruple mutants, both TAFI antigen and TAFIa activity levels were measured on the conditioned medium and expressed as percentage of TAFI antigen and TAFIa activity levels relative to those observed in conditioned medium of TAFI-wt. The single mutants revealed TAFI antigen levels between 63% to 146% and TAFIa activity levels between 29% to 119%. Among the double mutants 2 groups could be distinguished. TAFI-N22Q-N51Q, TAFI-N22Q-N63Q, and TAFI-N51Q-N63Q revealed antigen levels between 65% to 78% and activity levels between 80% to 87% whereas TAFI-N22Q-N86Q, TAFI-N51Q-N86Q, and TAFI-N63Q-N86Q revealed antigen levels between 21% to 49% and activity levels between 31% to 35%. Among the triple mutants, TAFI-N22Q-N51Q-N63Q had the highest TAFI antigen (57%) and TAFIa activity level (56%), whereas TAFI-N51Q-N63Q-N86Q and TAFI-N22Q-N51Q-N63Q revealed antigen levels between 32% to 38% and activity levels between 28% to 37%. For TAFI-N22Q-N63Q-N86Q and TAFI-N22Q-N51Q-N63Q-N86Q neither TAFI antigen (≤3%) nor TAFIa activity (≤7%) could be measured.

Based on these results, the 4 single mutants and the triple mutant TAFI-N22Q-N51Q-N63Q were purified. All further experiments were performed on purified material.

**Fragmentation Pattern of TAFI Variants on Activation With Thrombin/Thrombomodulin**

On SDS-PAGE analysis, intact TAFI variants migrated at the expected molecular weight ie, $\sim$56 kDa for TAFI-wt, $\sim$54 kDa for the single mutants, and $\sim$49 kDa for the triple mutant (Figure 1A). On activation of the TAFI-wt and TAFI mutants by T/TM a conversion of intact TAFI to TAFIa (36 kDa) was observed for all variants (Figure 1B).

**TAFIa Activity**

After activation with T/TM, all purified single mutants (except TAFI-N86Q (29.0 U mg$^{-1}$)) revealed significantly lower TAFIa activities (10.2 to 15.0 U mg$^{-1}$), compared with TAFIa-wt (18.6 U mg$^{-1}$; P<0.001; Table 1). Removing the glycosylation site at Asn$^{51}$ had the highest impact on the TAFIa activity. Surprisingly, the triple mutant TAFI-N22Q-N51Q-N63Q showed an activity of 10.4 U mg$^{-1}$ which is still...
more than 55% of the TAFI-wt activity. These results indicate functional differences between the TAFI variants.

TAFI Zymogen Activity
Removing the glycosylation site at Asn22 resulted in a 7.6-fold increase of the intrinsic catalytic activity of the zymogen compared with TAFI-wt (P<0.001; Table 1). The removal of the glycosylation site at Asn86 and Asn93 resulted in a 1.7- and 2.3-fold increase of the intrinsic catalytic activity (P<0.005 and P<0.05, respectively), whereas removing the glycosylation site at Asn86 had no significant effect. Simultaneous removal of the glycosylation sites at Asn22, Asn86, and Asn93 led to a 4.4-fold increase in intrinsic catalytic activity compared with TAFI-wt (P<0.05).

TAFI Activation by Thrombin/Thrombomodulin
As the glycans might influence the accessibility to the thrombin cleavage site, the ability to activate the different TAFI variants by T (0.5 nmol/L) and TM (1 nmol/L) was evaluated in a kinetic assay with different TAFI concentrations (0.06 to 1 μmol/L). The initial rates of TAFIa formation were evaluated by measurement of Hip-Arg hydrolysis. Double reciprocal plots of activation rates versus substrate concentration were linear (r²=0.95), confirming Michaelis-Menten kinetics. The apparent catalytic rate (kcat), but is still leading to an increase in apparent catalytic efficiency (kcat/KM) (2.4- to 8-fold).

Table 1. TAFIa Activity Generated by T/TM, the Intrinsic TAFI Activity and TAFIa Stability

<table>
<thead>
<tr>
<th>TAFI Variant</th>
<th>TAFIa Activity, U mg⁻¹</th>
<th>Intrinsic TAFI Activity, U mg⁻¹</th>
<th>TAFIa Half-Life, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAFI-wt</td>
<td>18.6±0.7</td>
<td>0.11±0.01</td>
<td>9.3±0.7</td>
</tr>
<tr>
<td>TAFI-N22Q</td>
<td>15.0±0.9**</td>
<td>0.74±0.09**</td>
<td>8.0±0.6</td>
</tr>
<tr>
<td>TAFI-N51Q</td>
<td>10.2±1.5**</td>
<td>1.17±0.01**</td>
<td>8.2±0.5</td>
</tr>
<tr>
<td>TAFI-N63Q</td>
<td>11.3±1.2**</td>
<td>0.22±0.02*</td>
<td>7.4±0.4*</td>
</tr>
<tr>
<td>TAFI-N86Q</td>
<td>29.0±2.7**</td>
<td>0.10±0.01</td>
<td>9.6±1.0</td>
</tr>
<tr>
<td>TAFI-N22Q-N51Q-N63Q</td>
<td>10.4±2.1**</td>
<td>0.43±0.06*</td>
<td>8.8±0.8</td>
</tr>
</tbody>
</table>

Mean±SD, n=6.
**significantly different (*P<0.05 and **P<0.005) compared to TAFI-wt.

were determined by a nonlinear regression of the data to the Michaelis-Menten equation. All TAFIa-mutants, except TAFI-N86Q, revealed a significantly lower Kₘ value for Hip-Arg and a significantly lower catalytic rate (kcat), except for TAFIa-N22Q, compared with TAFI-wt, leading to a comparable catalytic efficiency (kcat/KM; Table 3). TAFI-N86Q showed a comparable Kₘ value, but showed a 2-fold higher catalytic rate, leading to a nearly 2-fold increase in catalytic efficiency.

The Effect of the Removal of Glycans on TAFIa Stability
The TAFI stability of the TAFI variants was evaluated at 37°C (Table 1). The half-life of TAFIa-wt was 9.3±0.7 minutes. The half-life of the mutants was not significantly altered compared with TAFIa-wt, except for TAFI-N63Q, which revealed a slightly but significantly decreased half-life (ie, 7.4±0.4 minutes, P<0.05).

Effect of the TAFI Variants in a Clot Lysis Assay
The in vitro clot lysis assay was performed to evaluate the antifibrinolytic effect of the glycosylation knockout mutants. Addition of TAFI-wt (90 nmol/L) to TAFI-depleted plasma resulted in a significant increase of the 50% clot lysis time (ie, 7.3±4.8 minutes versus 11.9±2.6 minutes, P<0.005; Figure 2). Addition of TAFI-N51Q, TAFI-N63Q, and TAFI-N22Q-N51Q-N63Q mutants (90 nmol/L) to TAFI-depleted plasma resulted in a 50% clot lysis time of 70.7±4.9 minutes.

Table 2. Apparent Kinetic Parameters for the Activation of TAFI Variants by Thrombin/Thrombomodulin (0.5 nmol/L and 1 nmol/L)

<table>
<thead>
<tr>
<th>TAFI Variant</th>
<th>kcat (μM)</th>
<th>kcat (μM)</th>
<th>kcat/KM (μM⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAFI-wt</td>
<td>0.807±0.098</td>
<td>1.552±0.050</td>
<td>1.945±0.194</td>
</tr>
<tr>
<td>TAFI-N22Q</td>
<td>0.179±0.028*</td>
<td>0.611±0.046**</td>
<td>3.553±0.863</td>
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<tr>
<td>TAFI-N51Q</td>
<td>0.044±0.004*</td>
<td>0.452±0.007**</td>
<td>10.459±1.057*</td>
</tr>
<tr>
<td>TAFI-N63Q</td>
<td>0.066±0.009*</td>
<td>0.964±0.004**</td>
<td>14.996±2.288*</td>
</tr>
<tr>
<td>TAFI-N86Q</td>
<td>0.038±0.009*</td>
<td>0.489±0.078**</td>
<td>13.227±1.065**</td>
</tr>
<tr>
<td>TAFI-N22Q-N51Q-N63Q</td>
<td>0.560±0.064*</td>
<td>1.733±0.081</td>
<td>3.117±0.210**</td>
</tr>
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</table>

Mean±SD, n=3.
**significantly different (*P<0.05 and **P<0.005) compared to TAFI-wt.

Table 3. Kinetic Parameters for Hippuryl-L-Arginine Hydrolysis by TAFIa Variants

<table>
<thead>
<tr>
<th>TAFIa</th>
<th>kcat (μM)</th>
<th>kcat (μM)</th>
<th>kcat/KM (μM⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAFI-wt</td>
<td>1.92±0.07</td>
<td>13.6±0.6</td>
<td>7.13±0.54</td>
</tr>
<tr>
<td>TAFI-N22Q</td>
<td>1.29±0.10*</td>
<td>11.2±1.2</td>
<td>8.66±0.62</td>
</tr>
<tr>
<td>TAFI-N51Q</td>
<td>0.78±0.04**</td>
<td>5.86±0.27**</td>
<td>7.51±0.50</td>
</tr>
<tr>
<td>TAFI-N63Q</td>
<td>1.38±0.18*</td>
<td>9.87±0.78**</td>
<td>7.21±0.44</td>
</tr>
<tr>
<td>TAFI-N86Q</td>
<td>2.08±0.12</td>
<td>27.0±1.2**</td>
<td>13.0±0.2**</td>
</tr>
<tr>
<td>TAFI-N22Q-N51Q-N63Q</td>
<td>0.51±0.04**</td>
<td>4.10±0.35**</td>
<td>7.99±0.54</td>
</tr>
</tbody>
</table>

Mean±SD, n=3.
**significantly different (*P<0.05 and **P<0.005) compared to TAFI-wt.
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The isoelectric point (pI) of the cleavage of the glycosylated activation peptide from the zymogen TAFI varies between 4.8 and 5.5, which can be explained by the heterogeneity in glycans attached to TAFI. On activation of TAFI, the pI of TAFIa switches toward 8 and the solubility decreases.

Evidence has been provided that, in general, this type of posttranslational modification may also influence many of the biochemical properties of the proteins such as stability, dynamics, and ligand binding. Assuming a crucial role of the glycans, we hypothesized a large impact on TAFI functionality on removal of glycosylation sites. Therefore the goal of the current study was to determine the impact of the glycans, attached to the activation peptide, on the biochemical properties of TAFI(a) by generating single, double, triple, and quadruple mutants in which glycosylation sites were removed. Surprisingly all single, double, and 3 of 4 triple mutants revealed a functional TAFI protein. Only TAFI-N22Q-N63Q-N86Q and TAFI-N22Q-N51Q-N63Q-N86Q showed no functional TAFI on expression. This is in agreement with the findings that on modification of multiple glycosylation sites, folding may be compromised even though none of the glycans are needed individually.

Recently, Valnickova et al discovered that TAFI has a potential glycosylation site in the TAFIa moiety at Asn219. Therefore we constructed and transfected TAFI-N219Q, but neither TAFI antigen (Western blotting and ELISA) nor TAFIa activity (chromogenic assay) could be measured on the conditioned medium. Because Asn also occurs in a nonglycosylated form, it is evident that glycosylation at Asn is not crucial and that the lack of functional TAFI is attributable to the substitution of the amino acid Asn into Gln rather than the absence of glycosylation.

On deletion of 1 or more glycosylation sites, the intact TAFI variants migrated at the expected molecular weight ie, ≈54 kDa for the single mutants and ≈49 kDa for the triple mutant. After activation of the TAFI mutants, a 36-kDa band appeared, corresponding to the TAFIa moiety. From these data, it can be concluded that the activation of the mutants is not hampered. Previously a panel of 42 inhibiting monoclonal antibodies (MA) was raised against human plasma-derived TAFI. Three activation inhibiting antibodies (ie, MA-T1C10, MA-T12D11, and MA-T94H3) with distinct epitopes (ie, Gln, Gly, and Val, respectively) were able to impair the activation of the TAFI variants (data not shown). This reveals that removal of the glycans does not affect the binding of the MA to the TAFI variants.

**Discussion**

Cleavage of TAFI by T/TM leads to the formation of an activation peptide (20 kDa) and a catalytic domain TAFIa (36 kDa). The activation peptide region contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa). The activation peptide region contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa). The activation peptide contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa). The activation peptide region contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa). The activation peptide region contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa). The activation peptide region contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa). The activation peptide region contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa). The activation peptide region contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa). The activation peptide region contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa). The activation peptide region contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa). The activation peptide region contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa). The activation peptide region contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa). The activation peptide region contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa). The activation peptide region contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa). The activation peptide region contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa). The activation peptide region contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa). The activation peptide region contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa). The activation peptide region contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa). The activation peptide region contains 4 potential glycosylation sites, and the attached glycans account for 48% (36 kDa).
confirmed by affinity measurements revealing similar affinity constants for all TAFI variants (data not shown).

Until now it was presumed that activation of TAFI, leading to the release of the activation peptide, was needed for its catalytic activity. Valnickova et al recently revealed that the zymogen also exerts an intrinsic catalytic activity. It is believed that the activation peptide controls the access to the active site and because this activation peptide is heavily glycosylated, the glycans attached to the activation peptide may be responsible to control the catalytic activity of the zymogen. Therefore we determined the intrinsic carboxypeptidase activity of the TAFI variants. As expected, TAFI mutants from which glycosylation site(s) had been removed, had a higher intrinsic carboxypeptidase activity than TAFI-wt. Especially the removal of the glycans at Asn increased the intrinsic activity whereas removing Asn had no effect.

By measuring the apparent kinetic parameters of TAFI activation using 0.5 nmol/L thrombin and 1 nmol/L thrombomodulin, it was observed that all glycosylation knockout mutants have a lower $K_M$ value, which is partially compensated by a lower activation rate but still results in a higher activation efficiency. The kinetic parameters of TAFIa activity revealed more surprising results. There were no significant differences in the catalytic efficiency between TAFI-wt and the TAFI mutants, except for TAFI-N22Q which revealed a slightly (but not significantly) increased catalytic efficiency and for TAFI-N86Q which revealed a significantly higher catalytic efficiency (Table 3). This is also in line with the data obtained in the clot lysis experiment in which both TAFI-N22Q and TAFI-N86Q revealed a higher antifibrinolytic potential than TAFI-wt. From these data it can be hypothesised that the catalytic efficiency for the hydrolysis of the physiological substrate (ie, fibrin surface) is also increased.

From the apparent kinetic parameters of the activation of the TAFI variants by thrombin (0.5 nmol/L) and thrombomodulin (1 nmol/L), it can be deduced that the overall efficiency of activation using this thrombomodulin concentration is much more efficient when 1 or more glycans are removed. This is attributable to decreased apparent $K_M$ value (Table 2) which compensates the lower apparent $k_{cat}$ values observed for all single mutants. We point out that the differences in kinetic parameters may reflect alterations in the interaction of TAFI with either thrombin or TM. We did not determine the thrombin or TM dependency of TAFI activation for the glycosylation knockout mutants in this study.

Although it is believed that glycans contribute to the thermal stability of glycoproteins, no significant differences were observed in the stability of the TAFI variants, except for TAFI-N63Q which revealed a slightly decreased half-life. At first glance, one should indeed not expect a strong influence of glycosylation on the stability of TAFI because on activation the activation peptide is cleaved from the active enzyme. To our knowledge, no group was either able to isolate the activation peptide from the TAFI moiety or to express recombinant TAFI separately. To provide additional evidence that the activation peptide and TAFI remain noncovalently associated after cleavage of the zymogen, we have activated TAFI using T/TM, immunoprecipitated this mixture using an activation peptide specific MA, and performed Western blotting experiments using MA-T3D8 which was previously shown to react with the TAFIa moiety. This Western blotting experiments revealed a clear band at 36 kDa revealing that the enzymatic moiety sticks to the aminoterminal part of TAFI (=activation peptide) on activation. Moreover, using electron microscopy Dr Marx has recently shown that activated TAFI and intact TAFI look structurally similar, suggesting that the activation peptide is cleaved but not released during activation. The importance of the activation peptide was also demonstrated by a TAFI variant from which the first 9 amino acids of the activation peptide were deleted but with an intact signalpeptide. No TAFI antigen (Western blotting and ELISA) or TAFIa activity (chromogenic assay) could be detected for this variant (data not shown).

A number of biochemical properties (eg, TAFIa activity, enzyme kinetics, and antifibrinolytic potential) of TAFI are solely attributed to the active moiety of TAFI (ie, TAFIa). However, this study demonstrates that these properties are altered when the glycosylation of the activation peptide is altered. Therefore, it can be deduced that on activation of TAFI, the activation peptide stays in close proximity to the TAFIa moiety and influences the properties of the TAFI a moiety.

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Disclosures

None.

References


Biochemical Importance of Glycosylation in Thrombin Activatable Fibrinolysis Inhibitor

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Online Figure I: Apparent activation kinetics of TAFI variants by T/TM.

TAFI variants (0.06-1 µmol/L) were activated for different time intervals at 25°C (range between 0-2 min) in the presence of CaCl₂ (5 mmol/L), thrombin (0.5 nmol/L) and thrombomodulin (1 nmol/L) in Hepes-BSA buffer. Activation was stopped by addition of PPACK (30 µmol/L). Samples were incubated for 20 minutes at 25 °C with hippuryl-arginine. The amount of TAFIa generated after each activation time was obtained using standard calibration curves (absorbance/min per nmol/L TAFIa). The apparent $k_{cat}$ and $K_m$ values for activation of each TAFI variant were determined by nonlinear regression of the data to the Michaelis-Menten equation using GraphPad Prism 4.01 (mean ± S.D. $n=3$).