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Role of Heparin Binding in Plasmin-mediated Activation of TAFI and Thermal Stability of TAFIa

Anastassia Filipieva

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Role of Heparin Binding in Plasmin-mediated Activation of TAFI and Thermal Stability of TAFIa

by

Anastassia Filipieva

A thesis submitted to the Faculty of Graduate Studies through the Department of Chemistry and Biochemistry in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

University of Windsor

Windsor, Ontario, Canada

2012

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“Role of Heparin Binding in Plasmin-mediated Activation of TAFI and Thermal Stability of TAFIa”

by

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Declaration of Originality

I hereby certify that I am the sole author of this thesis and that no part of this thesis has been published or submitted for publication.

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**Abstract**

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a molecular link between fibrinolysis and coagulation. TAFI gets activated either by thrombin, thrombin-thrombomodulin complex, or plasmin. Once activated, TAFIa removes carboxyl-terminal lysines from partially degraded fibrin, thus attenuating fibrinolysis. It has been previously reported that heparin accelerates plasmin-mediated activation of TAFI and increases thermal stability of TAFIa. In the present study we set out to identify heparin binding sites on TAFI. We constructed TAFI variants that contained mutations in the regions proposed to participate in the interaction with heparin. We identified several variants that showed impaired binding to heparin. Of these, K211Q/K212Q, R320A/K324A and K306A showed reduced acceleration by heparin of plasmin-mediated activation, and K327A, K327A/R330A, and R320A/K324A showed reduced stabilization of TAFIa by heparin. R320A/K324A bound very poorly to heparin, and hence might represent a useful variant with which to study the importance of glycosaminoglycans in regulating TAFI function.
For my grandparents,

Who courageously battled but succumbed to cardiovascular disease
Acknowledgements

First and foremost, I would like to thank Dr. Michael Boffa and Dr. Marlys Koschinsky for giving me this amazing opportunity and welcoming me into their lab. This experience was one of the most valuable experiences that I have acquired throughout my academic career. I also thank them for constant feedback, guidance, and patience.

I would also like to thank Tanya Marar for taking time to help me out throughout the last two years. I would also like to thank Corey, Rocco, Zainab, Branna, Dragana, Christina, and Tazeen for your help, support, advice and patience.

I have learned a lot, not only in terms of the academic knowledge, but also in terms of patience, self-discipline, and positive attitude. Thank you all for making this project possible.

I would like to thank members of my committee: Dr. Boffa, Dr. Koschinsky, Dr. Mutus, and Dr. Porter for their valuable input throughout this project.
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Abbreviations

AAFR  Anisylazoformylarginine
ADP  Adenosine triphosphate
ATP  Adenosine diphosphate
BHK  Baby hamster kidney cells
cDNA  Complement deoxyrinonucleic acid
DMEM  Dulbecco’s modified eagle medium
FDP  Fibrin degradation products
GAG  Glycosoaminoglycans
GEMSA  2-guanidinoethylmercaptosuccinic acid
HARE  Human hyaluronic acid receptor
HBS  Hapes buffed saline
HBST  Hapes buffered saline with 0.01% Tween 80
HIT  Heparin induced thrombocytopenia
$k_{cat}$  Turnover number
$k_{cat}/K_m$  Catalytic efficiency
$K_m$  Michaelis constant
Opti-MEM  Reduced serum Minimal essential medium
PAI-1  Plasminogen activator inhibitor-1
PF4  Platelet factor 4
PMSF  Phenylmethylsulfonyl fluoride
PPAck  D-phenylalanylprolylarginyl chloromethylketone
PTCI  Potato tuber carboxypeptidase inhibitor
SDS-PAGE  Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERPIN  Serine protease inhibitor
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<td>TAFI</td>
<td>Thrombin activatable fibrinolysis inhibitor</td>
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<td>TF</td>
<td>Tissue factor</td>
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<tr>
<td>TM</td>
<td>Thrombomodulin</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator</td>
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<tr>
<td>VFKck</td>
<td>Valylphenylalanyllysyl chloromethylketone</td>
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<td>vWF</td>
<td>von Willerbrand factor</td>
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Chapter 1

Introduction

1.1 Haemostasis

Maintaining the balance between the processes of coagulation and fibrinolysis is essential for proper haemostatic response. Once damage to a blood vessel occurs, a haemostatic response is initiated that involves soluble plasma proteins, tissue components, and receptors on platelets [1, 2]. This response is tightly regulated by the opposing coagulation and fibrinolytic cascades (Figure 1-1). The coagulation cascade is responsible for the formation of an insoluble fibrin clot at an injury site, while the fibrinolytic cascade degrades the fibrin clot [1]. Any imbalances in the regulation of these cascades can lead to excessive clot formation, which can lead to a heart attack or a stroke, or inadequate clot formation, which can result in bleeding disorders such as haemophilia [3]. If the two cascades are regulated properly, the haemostatic response is localized to the site of the injury, due to the involvement of the above mentioned plasma proteins, and cellular components.

The primary response to a vascular injury, which results in the exposure of blood to subendothelial tissue, is initiated with the formation of a platelet plug. Inert platelets circulate in close contact with the endothelium, but do not adhere to the endothelial cells [4]. However, upon injury to the endothelium, platelets come into contact with extracellular matrix components resulting in platelet activation and aggregation, leading to the formation of a platelet plug [5]. Initially, the interaction of platelets with the
extracellular matrix is mediated through the platelet von Willerbrand Factor (vWF) receptor Glycoprotein IB/V/IX [6] and collagen receptor Glycoprotein VI [7]. However, this interaction alone is insufficient to allow adhesion and instead facilitates intracellular signalling which activates integrins αIIbβ3 and α2β1 [7]. These integrins interact with the extracellular matrix to facilitate firm adhesion of platelets to the wall of an injured vessel resulting in the formation of a platelet monolayer. More platelets are rectuited to the injury site by a variety of mediators that are synthesized once platelets adhere, and limited activation of platelets takes place. These mediators are adenosine diphosphate (ADP), adenosine triphosphate (ATP), thromboxane A₂, and thrombin. Thrombin is produced on the surface of activated platelets, while, ADP, ATP and thromboxane A₂ are released from activated platelets [5]. Upon activation platelets change morphology. Cytoskeletal reorganization and secretion from storage granules takes place [8,9]. Among the secreted granule components are calcium ions, which are released into the cytoplasm, as well as vWF, and growth factors, which are released into the extracellular space. Another morphological change that takes place once the platelets are activated is the exposure of an ionic phospholipid, phosphatidylserine [9,10]. Phosphatidylserine provides a surface for the assembly of the enzymes and cofactors of the coagulation cascade to form functional complexes [9]. The secondary response to a vascular injury is the initiation of the coagulation cascade. Tissue Factor (TF) is a transmembrane protein that is expressed by various cells of the vascular wall, however, under normal conditions does not come into contact with blood [11]. Upon a vascular injury, TF is exposed to flowing blood and forms a complex with plasma protein factor VIIa (FVIIa). TF-FVIIa complex activates circulating factors IX and X into IXa and Xa respectively [11].FXa and its activated cofactor Va (FVa) form a complex on the activated platelet surface with the aid of the
aforementioned negatively charged exposed phospholipids. FXa-FVa complex on the platelet surface is termed the ‘prothrombinase’ complex, as it activates prothrombin to thrombin [12]. The majority of the circulating FV is activated by the thrombin that is generated in the initial stages of the coagulation process. TF-dependant coagulation pathway is often termed ‘extrinsic’ [2]. However, there is another pathway that can result in the activation of prothrombin to thrombin. This pathway is termed ‘intrinsic’, and, in vitro, can be initiated by the factor XII (FXII), high molecular weight kininogen, and prekallikrein, which results in the activated FXII (FXIIa), which in turn activates FXI, followed by activation of FIX [2]. However the current concept is that FXII, high molecular weight kininogen, and prekallikrein do not play a role in the activation of the intrinsic pathway in vivo but, rather, limited activation of FXI by thrombin initiates the pathway through activation of FIX [13,14]. FIXa forms a complex with thrombin-activated FVIIIa that is termed the ‘tenase’ complex [2]. The function of this complex is to convert FX to FXa. The assembly of the ‘tenase’ complex, just like the assembly of the ‘prothrombinase’ complex occurs on the negatively charged phospholipid surface of the activated platelets [2]. The ‘extrinsic’ pathway is the main activator of the coagulation cascade, while the ‘intrinsic’ pathway allows for amplification of the amount of thrombin generated [15]. In addition to providing a negatively charged surface for the assembly of the coagulation factors, activated platelets expose Glycoprotein IIb/IIIa receptors that can efficiently bind the soluble plasma protein fibrinogen. Thrombin generated from both ‘extrinsic’ and ‘intrinsic ‘cascades cleaves platelet-bound fibrinogen to form fibrin to further stabilize the platelet plug [16]. Thrombin then cleaves circulating fibrinogen to
Figure 1-1. The balance between the coagulation and fibrinolytic cascades. The extrinsic pathway of coagulation produces a small amount of thrombin that promotes fibrin formation and the activation of the intrinsic pathway. The large amount of thrombin produced by the intrinsic pathway consolidates the clot, in part through activation of TAFI. In complex with thrombomodulin, thrombin no longer recognizes fibrinogen as a substrate but its ability to activate protein C and TAFI is enhanced by three orders of magnitude. Fibrin dissolution is accomplished by plasmin, which is formed by cleavage of plasminogen by tPA with fibrin as an essential cofactor. Limited digestion of fibrin leads to a form of fibrin that contains exposed carboxyl-terminal lysines. This form of fibrin is a several-fold more effective cofactor for plasminogen activation. TAFIa attenuates plasminogen activation by removing the carboxyl-terminal lysines, thereby yielding a form of fibrin with no cofactor activity.
create a meshwork to form a fibrin plug over the aggregated platelets [17]. Furthermore, generated thrombin activates FXIII, a transglutaminase, that cross-links fibrin monomers to stabilize the fibrin clot [16].

Uncontrolled blood coagulation is dangerous, and is controlled by anticoagulant pathways. The serine protease inhibitor (SERPIN) antithrombin (AT) downregulates clot formation by inhibiting Factors Xa, XIa, and thrombin [18,19]. AT is much more efficient at binding and inhibiting free circulating enzymes, then the enzymes that are assembled in the ‘tenase’ or ‘prothrombinase’ complexes on the activated platelet surface [20] or fibrin, in the case of thrombin [21]. The preferential affinity of AT to the free enzymes assists with limiting the coagulation reactions to the sites of vascular injury. The inhibitory function of AT is highly stimulated by heparin [22,23]. Because of this function, heparin is used as anticoagulant therapy. The activated protein C pathway is another anticoagulant pathway which controls coagulation. Activated protein C functions by inactivating the glycoprotein cofactors FVIIIa, and FVa [24].

To maintain proper haemostasis once the healing to the injured vessel has occurred, the fibrinolytic system is responsible for dissolution of the clot. Plasmin serves as a central enzyme of the fibrinolytic cascade. Plasmin is a serine protease that cleaves fibrin to form soluble fibrin degradation products (FDPs), which ultimately leads to the dissolution of the clot. This protease is generated by the activation of plasminogen by plasminogen activators, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), with the former enzyme being more important to the lysis of intravascular thrombi [25]. As in the case with coagulation, enzyme inhibitors play a key role in controlling and localizing the fibrinolytic cascade. tPA and plasmin are bound
and inhibited by plasminogen activator inhibitor (PAI-1) and α-antiplasmin respectively [26,27]. Plasmin promotes activation of plasminogen to form more plasmin by cleaving fibrin to expose carboxyl-terminal lysines [28,29]. This partially degraded form of fibrin possesses exposed carboxyl-terminal lysine residues and acts as a more efficient cofactor for tPA-mediated activation of plasminogen, by providing binding sites for both plasminogen and tPA [25]. Additional roles of the carboxyl-terminal lysines will be discussed below.

Studies in the 1990’s defined a molecular mechanism that functionally links the coagulation and fibrinolytic cascades. Studies of clot lysis in vitro revealed the presence of a factor that was activated by the coagulation cascades and appeared to inhibit the fibrinolysis; accordingly, activation of protein C in the clots prevented the action of this factor. The factor was purified and found to be procarboxypeptidase B-related zymogen, and was termed thrombin activatable fibrinolysis inhibitor (TAFI) [30,31]. TAFI attenuates fibrinolysis by cleaving C-terminal lysine residues from partially degraded fibrin, yielding a form of fibrin that does not possess cofactor activity for tPA-mediated plasminogen activation [32,33].

1.2 TAFI

Thrombin-Activatable fibrinolysis Inhibitor (TAFI) is a 60-kDa procarboxypeptidase B-related glycoprotein zymogen that was originally thought to be expressed only in the liver, and secreted to circulate in the plasma [34]. However, recent studies have shown that TAFI is expressed, although in smaller amounts, in megakaryocytes, endothelial
cells, macrophages, and adipocytes [35-37]. The translated product consists of 423 amino acids, of which 22 make up a signal peptide, 92 amino acids make up an activation peptide, while the remaining 309-amino acids represent a catalytic domain. The signal peptide is cleaved before secretion from the liver [34]. The theoretical molecular weight of TAFI zymogen is approximately 46 kDa, however, the apparent molecular weight of TAFI on SDS-PAGE is 60 kDa, due to N-linked glycosalation of TAFI [38,39]. TAFI zymogen is inactive and consists of the activation peptide, and the catalytic domain. TAFI becomes activated upon the cleavage at Arg92 which results in release of the activation peptide, and exposure of the TAFIa active site [31,40]. Proteolysis of the activation peptide is catalyzed by thrombin, or plasmin (Figure 1-2) [34]. However, both activators are relatively inefficient in cleaving the activation peptide. Thrombin, in complex with the endothelial cell surface cofactor thrombomodulin (TM), is 1250-fold more effective at activating TAFI [41]. In similar fashion, plasmin, in the presence of glycosaminoglycans, such as heparin, exhibits a 16-fold increase in the activation efficiency [43]. Previously, it has been suggested that thrombin-TM complex is the physiological activator of TAFI, due to the apparent efficiency in activating TAFI compared to thrombin. In comparison to plasmin, even in the presence of glycosaminoglycans, thrombin-TM complex exhibits 10-fold greater catalytic efficiency [43]. However, it has been shown that half-maximal effect of TAFIa on inhibition of clot lysis time is achieved at 1nM in vitro [41]. This represents less than 2% of the total concentration of TAFI in plasma. Therefore, significant inhibition of fibrinolysis can be achieved via activation of TAFI by thrombin, or plasmin alone, and this has certainly been demonstrated both in vitro and in vivo in animal models [44,45,46,47]. It is important to note that the contribution of these individual activators is unknown; in fact,
the contribution of these activators may be dependent on the location of the activation of TAFI, as well as the presence of vascular injury [43].

Vascular injury exposes glycosaminoglycans that are present in the extracellular matrix [48]. Therefore, plasmin-mediated activation of TAFI may be a significant contributor to proper clot stability at the injury site. On the other hand, if the vessel is not injured, thrombin-TM may play a key role in activation of TAFI, and proper fibrinolysis control [43,44].

To date, there are no known endogenous inhibitors of TAFIa. However, it has been shown that the intrinsic thermal instability is responsible for the inactivation of the enzyme; the activity of TAFIa appears to spontaneously decay with a half-life of 8-15 minutes [49,55]. This phenomenon has been associated with a marked structural change in the enzyme as assessed by measurement of the intrinsic fluorescence [50]. Crystal structures of TAFI have shed some light on the structural basis for the intrinsic instability of this enzyme. Crystal structure analysis suggests that residues 296-350 of TAFI play a key role in the thermal stability. Before cleavage at Arg92, the activation peptide of TAFI stabilizes the above mentioned region. Once cleavage occurs, the activation peptide no longer interacts with this region. This is followed by increase in the dynamics of this region and unfolding of TAFIa, yielding exposed thrombin and plasmin cleavage sites of TAFIa [51,52,53]. Proteolytic cleavage of activated TAFI by thrombin, or plasmin has also been suggested to play a critical role in regulating levels of TAFIa, since this cleavage yields a form of TAFIa that is inactive. Thrombin cleaves TAFIa at Arg302, while plasmin cleaves TAFIa at Arg302, Lys327, and Arg330 [49,53]. (Figure 1-2)

However, it has been shown that this proteolytic cleavage by thrombin can occur only after thermal inactivation of TAFIa, while cleavage of TAFIa by plasmin can occur
before and after thermal inactivation. Proteolytic cleavage of TAFIa by plasmin is accelerated following thermal inactivation. This suggests that thermal inactivation is a primary method of regulating TAFIa levels [49,53] ; indeed, mutants of TAFI, both naturally occurring and those created in the 296-350 region, that alter the intrinsic stability of TAFIa, correspondingly alter the antifibrinolytic potential of the enzyme [54,55]. The importance of proteolytic cleavage of TAFIa, as a way of regulating TAFIa levels, remains unknown [49,53]. In vitro clot lysis studies have shown that TAFIa exhibits the antifibrinolytic effect in a threshold-dependant manner. Like every threshold system, this one is also very complex, as the threshold concentration of TAFIa is dependent on the concentrations of other components of the pathways, such as plasminogen, plasminogen activator, and antiplasmin. However, as long as TAFIa concentration remains above a certain level, fibrinolysis is attenuated [56]. However, due to its intrinsic instability, and the consequential short half-life, the concentration of TAFIa gets progressively lower, and falls below the threshold value. Therefore increased clot lysis time is highly dependent on the half-life of an enzyme, which dictates the concentration of TAFIa [56, 57], as a more stable variant of TAFI (Ile325) exhibits increased antifibrinolytic potential [58].

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Figure 1-2. Activation of TAFI and inactivation TAFIa. TAFI consists of a pro-domain (black), which contains four N-linked glycans, and a catalytic domain (grey). Cleavage at Arg92 releases the activation peptide and formation of TAFIa. TAFIa is thermally unstable, and undergoes a conformational change which is associated with loss of carboxypeptidase B activity and formations of TAFIai. This conformational change also exposes Arg302, Lys327 and Arg330. The figure was adapted and modified from ref.41. The inset shows the location of Arg92, the target of thrombin and plasmin cleavage. Crystallographic data are from ref. 39 (PDB # 3D66). The side chain of the P1 arginine recognized by thrombin and plasmin is shown in red. The side chains of residues putatively involved in pinning Arg92 to the side of the zymogen are shown in cyan.
Although there are no known endogenous inhibitors of TAFIa, the role that several reversible inhibitors have on fibrinolytic effect of TAFIa has been studied [58]. Two of these inhibitors are 2-guanidinoethylmercaptosussinate (GEMSA), and potato tuber carboxypeptidase inhibitor (PTCI). It was found that these inhibitors of TAFIa exhibit a biphasic effect on the antifibrinolytic potential of TAFIa. This biphasic effect was found to be concentration dependant, where the clot lysis was prolonged at lower concentrations of the inhibitor, while enhancing clot lysis at higher concentrations of the inhibitor. This effect is thought to be due to the dynamic equilibrium of TAFIa bound to the inhibitor, and free TAFIa. When TAFIa is not bound to the inhibitor, it displays carboxypeptidase activity [58]. However, at body temperature, it undergoes spontaneous thermal inactivation rapidly [49]. On the other hand, TAFIa that is bound to the inhibitor does not display carboxypeptidase activity, but the half-life of TAFIa is increased when it is bound to the reversible inhibitor [40]. Since there is a dynamic equilibrium between bound and unbound TAFIa, as unbound TAFIa becomes inactivated, the dynamic equilibrium shifts to replenish the pool of unbound TAFIa as the reversible inhibitor is released. This observation would only remain true when the concentration of the reversible inhibitor is low enough, or the concentration of TAFIa is high enough, to sustain a pool of TAFIa that is not bound to the inhibitor. Since it has been shown that thermal stability plays a great role in the antifibrinolytic activity, extension of the half-life of TAFIa, and therefore clot-lysis time via reversible inhibitors can be used therapeutically [58]. However, since the biphasic effect is greatly dependant on the concentrations of TAFIa and the inhibitor in question, the effect that various concentrations of the inhibitor have on each individual becomes more complicated. Therefore, the use of these reversible inhibitors as therapeutic agents may be problematic
A number of studies on have been done to evaluate whether administration of the reversible TAFIa inhibitor alone improves endogenous thrombolysis. However, to date, in vivo results for various synthetic and naturally occurring inhibitors have been contradictory, as the efficiency of the inhibitor was found to be dependent on a number of factors, such as: the type of the thrombosis model, the studied animal species, the type of inhibitor, and whether the administration of the inhibitor is before or after the thrombus formation [59]. Although the reason for such great variability in data is unknown, there is a possibility that the biphasic effect of TAFIa inhibitors may contribute to that variability. One of the TAFIa inhibitors, AZD9684 was administered to pulmonary embolism patients in a phase II study [59]. However, according to the report by Astra Zeneca, the company that was developing this inhibitor, it is now discontinued (60).

As mentioned above, plasmin is a central enzyme of the fibrinolytic cascade. Plasmin digests fibrin, which structures a fibrin clot, into soluble FDPs. This results in the dissolution of a fibrin clot [25,59]. Fibrin provides binding sites for t-PA and plasminogen, and thus accelerates activation of plasminogen by 500-fold. The formation of this complex also restricts the activation of plasminogen to the site of a fibrin clot [62]. Initial plasmin cleavage of fibrin, however, cleaves fibrin to yield fibrin with exposed C-terminal lysines. These lysines bind t-PA and plasminogen, yielding a form of fibrin that acts as a better cofactor for t-PA-mediated plasminogen activation. In fact the increased efficiency of the partially degraded fibrin as a cofactor for plasminogen activation is approximately 3-fold [32,63]. It should also be noted that plasmin can cleave plasminogen from the initial Glu-plasminogen form to Lys-plasminogen form, which is more readily activated by t-PA to form Lys-plasmin [62,64]. Once activated, TAFI
exhibits carboxypeptidase activity on the partially degraded fibrin, which possesses carboxyl-terminal lysines, by cleaving these residues from the partially degraded fibrin. TAFIa attenuates fibrinolysis through a number of mechanism, however, all based on its ability to cleave carboxyl-terminal lysines from partially degraded fibrin. As mentioned above, partially degraded fibrin exhibits greater cofactor activity for tPA-mediated plasminogen activation. TAFIa thus reduces the cofactor activity of the partially degraded fibrin, and therefore attenuates fibrinolysis [32]. In the same manner, TAFIa reduces the conversion of Glu-plasminogen to Lys-plasminogen by removing carboxyl-terminal lysine residues from partially-degraded fibrin, which also downregulates fibrinolysis [32]. Both downregulation mechanisms result in lower plasminogen activation, and therefore lower plasmin concentration. As mentioned previously, one of the inhibitors that regulates fibrinolysis is α₂-antiplasmin, which binds plasmin irreversibly, and therefore inhibits the function of plasmin [27]. Plasmin, however, has the ability to bind to the lysine residues of the partially degraded fibrin. When bound to fibrin, plasmin is protected from the inhibition by α₂-antiplasmin [65]. Once TAFIa cleaves the carboxyl-terminal lysines from partially degraded fibrin, plasmin can no longer bind to the fibrin, and therefore is more readily inhibited by α₂-antiplasmin [66]. This results in lower localized plasmin concentration, which in turn corresponds to attenuation of clot lysis.

1.3 Plasmin

As previously mentioned, plasmin is a central enzyme of the fibrinolytic cascade, which is produced via activation of the proenzyme plasminogen. Plasmin is a serine
protease that cleaves fibrin to form fibrin degradation products, which ultimately leads to
the dissolution of the clot [67-69]. Plasminogen is a single-chain glycoprotein that
circulates in plasma at an approximate concentration of 2 µM. Plasminogen activation to
plasmin occurs through cleavage of Arg561-Val562 by tPA or uPA [69-71]. Plasmin is a
two-chain serine protease, linked by disulfide bonds, with an approximate molecular
weight 85 kDa, the heavy chain and light chain being 60 kDa and 25 kDa respectively.
The heavy chain of plasmin is composed of five kringle domains, which are involved in
the interaction of plasmin with other proteins, including fibrin, and the inhibitor of
plasmin, α2-antiplasmin [69]. Although plasminogen activation is possible without being
bound to fibrin, once tPA and plasminogen are both bound to partially degraded fibrin,
via carboxyl-terminal lysines, plasminogen activation is greatly accelerated. The
carboxyl-terminal lysines are removed by TAFIa, thus reducing plasminogen activation,
and reducing plasmin concentration [69, 25]. Although plasmin is considered to be the
main enzyme of the fibrinolytic cascade, it can also act as an antifibrinolytic enzyme,
through activation of TAFI [34]. Plasmin activates TAFI to form TAFIa, which
ultimately attenuates fibrinolysis [32]. It has been reported that plasmin substrate
specificity has been subjected to negative selection during evolution to prevent auto-
activation of plasminogen by plasmin. Plasmin is a very poor activator of plasminogen,
which is important to prevent short-circuiting of the fibrinolytic system through auto-
activation of plasminogen by plasmin. Optimal substrate for catalysis by plasmin contain
a lysine or an arginine residue at the P1 position, an aromatic residue at the P2 position,
either arginine, lysine, or serine at the P1’ position, as well as arginine, lysine, or glycine
at the P2’ position. Plasmin does not have stringent specificity at the P3 position of the
substrates [72]. Plasminogen, however has a proline at the P2 position which prevents
cleavage by plasmin [73]. Of note, TAFI has a proline at the P2 position as well, Pro91, which may account for the relatively inefficient activation of TAFI by plasmin [74]. Therefore, plasmin’s role in fibrinolysis is dependent on the balance between activation of plasminogen, inhibition of plasmin by antiplasmin, and plasmin-mediated activation of TAFI [32,65].

1.4 Multifunctional Role of Glycosaminoglycans

Glycosaminoglycans play a central role in regulating the balance between coagulation and fibrinolysis. Heparin is a glycosaminoglycan that is synthesized in mast cells and consists of alternating 1→4 linked residues of D-glucuronic acid and D-glucosamine [75]. During its biosynthesis, heparin chains are attached to a core protein, serglycin, through serine residues. However, this peptidoglycan gets cleaved by β-endoglucuronidase to yield a number of heparin molecules [76]. Another molecule that is structurally related to heparin is heparan sulphate, which also contains repeating of D-glucuronic acid and D-glucosamine residues. Compared to heparin, however, heparan sulfate is less sulfonated. On average, heparin contains 2.7 sulfate groups per disaccharide, while heparan sulfate, contains one sulfate group per disaccharide [77]. Unlike heparin, heparan sulfate gets synthesized by most mammalian cells, and becomes incorporated into the cell surface and the extracellular matrix [78]. The molecular weight of heparin varies from 5kDa to 40 kDa, while the molecular weight of heparan sulphate varies between 5kDa to 50 kDa [77]. Due to its heterogeneity, heparin, as well as other glycosaminoglycans, can bind to a variety of proteins and therefore play an important role.
in the biological function of these proteins [75,79]. The specificity of the interactions of glycosaminoglycans and the binding protein in question depends on the ionic interactions of the negatively charged sulphate with positively charged basic residues. However, it has also been shown that hydrogen bonds may play a role in heparin binding to proteins. Arginine residues exhibit tighter binding to heparin than lysine residues. This phenomenon is largely attributed to the stronger hydrogen bond between the guanidine side chain of arginine and the sulphate group of heparin, compared to the ammonium side chain of lysine [80].

Heparin accelerates inhibition of a number of proteinases of the blood coagulation system by proteinase inhibitors antithrombin and heparin cofactor II. Antithrombin inhibits thrombin, as well as Factors IXa, and Xa, while heparin cofactor II inhibits thrombin only [22, 23]. It has been previously reported that heparin first binds to the inhibitor, either antithrombin or heparin cofactor II, forming a complex. The formation of this complex induces a conformational change, which yields a more rapid reaction of the inhibitor and the proteinase in question. Heparin accelerates inhibition of thrombin by antithrombin by a factor of 2000 [81,22]. However, there is a minimum length requirement for heparin to bind to certain proteins, and therefore exhibit its effect on inhibition. In the case of thrombin inhibition by antithrombin, heparin that is made up of at least 5 saccharide units, with a 3-O-sulfo group on an internal glucosamine residue, is specifically required for high affinity to antithrombin [82]. However, this pentasaccharide is a minimal requirement for binding. There is also a minimal length requirement, of at least 16 saccharides, needed to accelerate the inhibition of thrombin by antithrombin [83]. This requirement is not uniform for inhibition of other proteins by antithrombin, heparin
that is as small as a pentasaccharide is able to bind antithrombin and catalyze the inhibition of factor Xa [84]. This is the basis for the synthetic heparin-like anticoagulant drug Fondaparinux, that specifically stimulates inhibition of FXa by antithrombin. This specificity is thought to underlie the reduced bleeding complications that are a feature of Fondaparinux compared to unfractionated heparin or low molecular weight heparin [85].

In a similar fashion, the protein also has to have a specific sequence motif to facilitate heparin binding. Through analysis of a number of heparin binding proteins, initially two heparin binding consensus sequences were proposed: XBBXBX and XBBBBXXBX, where B is a basic residue and X is a hydrophobic residue. It is suggested that if the XBBXBX sequence is in a β-strand conformation, or the XBBBBXXBX sequence is folded into an α-helix, lysine and arginine residues would be exposed, while hydrophobic residues would be pointing into the protein core [86]. However, a third heparin binding consensus sequence has been proposed, XBBBXXBBBXXBBX, which is found in vWF [87]. Although consensus sequence is a valuable tool in screening for potential heparin binding sites, many heparin binding proteins do not contain these specific sequences. Folding of the protein may be the determining factor in heparin binding, as it can bring distant basic residues into close proximity and thus facilitate heparin binding [77]. It was also found that, regardless of the tertiary structure, a distance of 20 Å between basic residues was able to facilitate heparin binding [88].

Heparin has been used as an anticoagulant therapeutic agent for deep venous thrombosis in clinical settings since the 1930s. One of the main uses of heparin is to prevent thrombus growth and pulmonary embolism, though accelerated inhibition of the proteases involved in the coagulation cascade. Unfractionated heparin is administered
intravenously as a treatment for venous thrombosis. However, due to variable individual responses to unfractionated heparin, appropriate dosage has been one of the problems associated with this therapy [89,90]. Another problem associated with heparin therapy is heparin-induced thrombocytopenia (HIT). Several types of HIT have been defined. However, only one type is associated with an increased risk of thrombosis. This type of HIT is induced by an immune response. Platelet Factor 4 (PF4) is a molecule that gets released from platelet granules upon activation of platelets. PF4 is a positively charged molecule that binds to the negatively charged heparin molecule. Patients that are diagnosed with this type of HIT produce an IgG antibody against the PF4-heparin complex. This antibody has the ability to bind to platelets through an Fc receptor and induce activation of platelets, followed by activation of coagulation, and thrombus formation. This type of HIT is also associated with the decrease of platelets, as antibody-coated platelets get cleared by phagocytic cells [91]. Therapeutically introduced heparin, as well as endogenous heparin gets cleared from the body. It has been previously reported that liver sinusoidal endothelial cells of the lymph nodes, liver, and spleen are the principal site of clearance of heparin from the vascular and lymphatic circulation [92]. These cells express a hyaluronic acid receptor (HARE), also known as Stabilin-2, an endocytic receptor that is responsible for binding, internalizing, and degradation of heparin [93].

Interaction of heparin with antithrombin and heparin cofactor II is the basis of its anticoagulant activity. However, heparin also binds to TAFI, leading to increased activation of TAFI by plasmin, as well as increased stability of TAFIa [43]. Several forms of heparin-like glycosaminoglycans tested, such as heparan sulfate, low molecular weight
heparin, chondroitin sulfate, keratan sulfate, and dextran sulfate, have a similar effect on TAFIa activation by plasmin [43]. Like heparan sulfate, chondroitin sulfate and keratan sulfate are expressed by various mammalian cells [94]. Low molecular weight heparin is a derivative of the unfractionated heparin [95], while dextran sulfate is synthetic analogue of heparin [96]. Chondroitin sulfate contains the same major disaccharide repeat as heparin and heparan sulfate, D-glucuronic acid and D-glucosamine, while keratan sulfate contains a disaccharide repeat of D-galactose and D-glucosamine [94]. Since TAFIa attenuates fibrinolysis, heparin may now act as an antifibrinolytic molecule. Little is known about this function of heparin, as it was recently discovered. However, this may be an important aspect to consider when heparin is administered as an anticoagulant drug.

1.5 Potential TAFI-Heparin Interactions

Glycosaminoglycans, such as heparin, have been reported to accelerate activation of TAFI by plasmin and increase the half-life life of TAFIa [43]. However, the binding sites of heparin on TAFI are still unknown. Several reports of the crystal structure of TAFI, in the presence of sulfate ions, have shed some light on the potential interaction sites of TAFI and glycosaminoglycans (Figure 1-3) [50,51]. Mao and coworkers have reported that, based on the sequence analysis, Trp210-Ser221 is a potential binding site. This region possesses sequence similarity to that of other glycosaminoglycan binding proteins [43]. However, according to the crystal structure of bovine TAFI, in the presence of sulfate ions, only one out twenty two sulfate molecules was bound to that region of TAFI. The rest of the sulfate molecules were bound to the pro-domain region, the region from the instability region to the active site cleft, as well as the instability region.
However, it should be noted, that binding of heparin in the segment spanning between the instability region and the active site cleft would actually hinder the substrate binding and therefore interfere with the carboxypeptidase activity of TAFI. While binding of heparin in the pro-domain may be important for various biological activities of TAFI, it would not explain the increased stability of TAFIa. Binding of heparin to the instability region of TAFI would explain the increased half-life of TAFIa in the presence of heparin [51]. Since therapeutic manipulation of TAFI function by heparin is a potentially important goal, and since clinically used heparins and heparin analogues may be compromised by their effect on the balance between coagulation and fibrinolysis through modulation of TAFI, we undertook this study to define heparin binding sites on TAFI.
**Figure 1-3. Potential glycosaminoglycan binding sites on TAFI.** The ribbon representation of bovine TAFI (taken from ref. 50) is on the left. Bound sulfates, which represent potential glycosaminoglycan binding sites are clustered in three regions: at the activation peptide (blue sulfates), at the dynamic region (orange sulfates), and within the active site cleft (purple sulfates). Glycosaminoglycan binding to the active site would most likely hinder enzymatic activity of TAFIa, therefore it is unlikely to be a site of heparin binding. The ribbon representation on the right was prepared from the crystallographic data PDB #3D66 (Marx, 2008). A putative glycosaminoglycan-binding site spanning Trp210-Met215, contains −XBBXBX− motif where X represents hydrophobic residues (green side chains) and B represents basic residues (blue side chains). Also shown is the position of Arg92, which is the site of TAFI cleavage by thrombin or plasmin.
1.6 Overall Hypothesis and Specific Objectives

The overall goal of this study is to begin to understand the interactions between heparin and TAFI, and the resulting biological effects of these interactions in terms of plasmin-mediated activation of TAFI and thermal stability of TAFIa. It has been previously reported that the presence of heparin increases plasmin-mediated TAFI activation and TAFIa stability. However, the interactions that are responsible for these effects remain unknown. By mutating basic residues in the potential heparin binding sites, we will attempt to determine if these residues participate in heparin binding. Hence these TAFI variants will be used to study the overall hypothesis that the residues that potentially participate in heparin binding are responsible for either increased TAFI activation by plasmin or increased thermal stability of TAFI in the presence of heparin, or both. The aim of the current study is to develop TAFI mutants impaired in heparin binding, and to determine the effect of this impairment on plasmin-mediated activation of TAFI and thermal stability of TAFIa in the presence of heparin.

The specific objectives of the current study are as follows:

2. Evaluation of binding of TAFI variants to heparin using an affinity chromatography approach.
3. Characterization of activation kinetics of the TAFI variants by plasmin in the presence and absence of heparin.
4. Characterization of the thermal stability of each TAFI variant in the presence and absence of heparin.
Chapter 2

Materials and Methods

Materials

The synthetic carboxypeptidase substrate anisylazoformyarginine (AAFR) was obtained from Bachem Americas Inc (Torrance, CA, US). The plasmin inhibitor D-valylphenylalanylllysyl chloromethylketone (VFKck), the TAFIa inhibitor DL-2-mercaptopmethyl-3-guanidinoethylthiopropanoic acid (Plummer’s inhibitor) and the thrombin inhibitor D-phenylalanlylprolylarginyl chloromethylketone (PPAck) were purchased from Calbiochem (San Diego, CA, US). Heparin sodium salt from porcine intestinal mucosa and heparin-agarose type I saline suspension were purchased from Sigma-Aldrich (Oakville, ON, CA). QuikChange mutagenesis kit was purchased from Stratagene (La Jolla, CA, US). Restriction enzymes were purchased from New England Biolabs (Mississauga, ON, CA). Oligonucleotides for mutagenesis were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, US). Dulbecco’s modified Eagle’s medium/nutrient mixture F-12, Opti-MEM, Trypsin-EDTA, and penicillin/streptomycin/fungizone (PSF) were obtained from Gibco/Invitrogen (Burlington, ON, CA). Newborn calf serum (NCS) was obtained from Sigma-Aldrich Canada Ltd (Oakville, ON, CA). Effectene transfection reagent was purchased from Qiagen (Toronto, ON, CA). Methotrexate was purchased from Mayne Pharma (Montreal, QC, CA). Amicon ultra-4 Centrifugal filter units, MWCO 10kDa were purchased from Millipore (Billerica, MA, US). Thrombin, rabbit-lung TM and plasmin were obtained from Haematologic Technologies (Essex Junction, VT, US). Polyclonal sheep anti-human TAFI antibody was purchased from Affinity Biologicales (Ancaster, ON, CA), polyclonal
rabbit anti-sheep antibody was purchased from ThermoFisher Scientific (Ottawa, ON, CA). Enhanced chemiluminescent western blotting substrate was purchased from Thermo Scientific Pierce Biology Products (Rockford, Illinois, US). Monoclonal anti-TAFI antibody MA-T4E3 (purchased from Dr. Ann Gils’ laboratory, Leuven, Belgium) was coupled to CNBr-activated Sepharose 4B (GE Healthcare Life Sciences, Mississauga, ON, CA) according to the manufacturer’s instructions (2-6 mg antibody/mL resin). Western blot imaging was done using Alpha Inotech Fluor Chem Q imager. Spectrophotometry experiments were performed using a SpectraMax Plus 384 spectrophotometer.

**Construction of TAFI Mutants**

Mutagenesis was carried out using the Quik-Change mutagenesis kit (Stratagene) as per the manufacturer’s standard protocol. In all cases, the template for the mutagenesis was TAFI-pNUT, which contains a cDNA encoding human TAFI that has threonine at positions 147 and 325. TAFI variants with threonine at 325 have a half-life of about 8 minutes whereas isoleucine at this position can increase half-life to about 15 minutes [55]. The presence of the mutations was verified by DNA sequence analysis. Creation of the variant R320A/K324A was carried out by Ashley Kellam prior to my arrival in the laboratory. Creation of R15A was carried out by Tanya Marar.
Table 2-1 Primer design used for construction of the variants used in this study.
Sense strand of each primer is shown. Location of the primer binding region is indicated by the number above the primer sequence. Nucleotides that are mismatched between the primer and template are underlined.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>K327A</td>
<td>1046</td>
</tr>
<tr>
<td></td>
<td>5’ TTCGTGCTATTGAGAAAACTAGTGCATAACCCAGGTATAACATGGCC3’</td>
</tr>
<tr>
<td>K327A/R330A</td>
<td>1046</td>
</tr>
<tr>
<td></td>
<td>5’ TTCGTGCTATTGAGAAAACACTAGTGCAATACCCAGGTATAACATGGCCCATGGCTCAG3’</td>
</tr>
<tr>
<td>H308F</td>
<td>991</td>
</tr>
<tr>
<td></td>
<td>5’ ACACGAAATAAAAGCAAGAAGATTTTGGAGAAGCTCTCAGTAGC3’</td>
</tr>
<tr>
<td>K306A</td>
<td>986</td>
</tr>
<tr>
<td></td>
<td>5’ CCTATACGAAATAAAAGCAAGCAGACCAGGAGAAACTGCTCTC3’</td>
</tr>
<tr>
<td>K212A</td>
<td>704</td>
</tr>
<tr>
<td></td>
<td>5’ GTTATGACTACTCATGGAAGAGCCAATCGAATGTGGGAAAG3’</td>
</tr>
<tr>
<td>K211Q/K212Q</td>
<td>697</td>
</tr>
<tr>
<td></td>
<td>5’ GTGGACGGTTATGACTACTCATGGAACACAGAATCGAATGTGGGAAAG3’</td>
</tr>
<tr>
<td>R320A/K324A</td>
<td>1020</td>
</tr>
<tr>
<td></td>
<td>5’ ACTGTCTCTAGTACGAAATGCAAGCATGCTCTTTGAGGGAATTAGCTAGTAAAATACC3’</td>
</tr>
<tr>
<td>R15A</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>5’ TAGCTGCTTTCTCATGAGACCCAACTCTGTGGCAGTTCAAAGTC-3’</td>
</tr>
<tr>
<td>K59A</td>
<td>240</td>
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<tr>
<td></td>
<td>5’ AAATGCATCTGATGCTGACAATGTGGGACGCCCATTTAAATGTGAGC3’</td>
</tr>
<tr>
<td>S316A</td>
<td>1017</td>
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<tr>
<td></td>
<td>5’ GGACTGCTCTCTAGTACGAAATGCTGCTATT-3’</td>
</tr>
<tr>
<td>S249A/S251A</td>
<td>218</td>
</tr>
<tr>
<td></td>
<td>5’ GTGAGGAAGGTGCATCCGCTTCGCGCATGCTCGGAACCTAC-3’</td>
</tr>
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</table>
Expression and Purification of Recombinant TAFI Variants

BHK cells were cultured in complete medium (DMEM/F12 containing 5% (v/v) NCS and 1% (v/v) PSF) in a humidified incubator at 37°C in a 95% air/5% CO₂ atmosphere. Expression plasmids encoding the respective TAFI variants were transfected into cells using Effectene reagent, employing 2 μg plasmid DNA and 60 μL of transfection reagent per 100 mm plate of cells. After 24 h incubation with the transfection mixture, the cells were then cultured in complete medium containing 400 μM methotrexate for approximately 2 weeks, with the medium changed every other day, for the selection of stable cell lines expressing the respective recombinant TAFI variants. For recombinant TAFI production, stably expressing lines were cultured in triple flasks (500 cm², Nunc, Roskilde, Denmark) in Opti-MEM containing 1% (v/v) PSF and 40 μM ZnCl₂. Medium was collected every 72 h and replaced with fresh medium. Harvested medium was centrifuged at 3000 × g for 5 minutes to pellet cell debris, supplemented with Tris-HCl, pH 8.0 (to 5 mM), reduced glutathione (to 0.5 mM) and phenylmethanesulphonylfluoride (PMSF) to (2 μM) and stored at -20°C. To purify the TAFI variants, conditioned medium was passed through a 0.22 μm filter and then over a 1 mL MA-T4E3-Sepharose column at 4°C. The column was washed with a 100 ml of HBST (20 mM HEPES pH 7.4, 150 mM NaCl, 0.01% (v/v) Tween 80). TAFI that was bound to the column was eluted with 1-mL aliquots of 0.2 M glycine pH 3. Fractions were collected into 0.5-mL aliquots of 1M Tris-HCl, pH 8. Protein-containing fractions were pooled, and then were concentrated, and exchanged into HBST using Amicon ultra-4 Centrifugal filter units, MWCO 10kDa. Purified and concentrated TAFI was quantified
by measurement of absorbance at 280 nm (ε1%, 280 = 26.4, $M_r = 60,000$), aliquoted and stored at -70°C.

**Binding of TAFI to Heparin**

A 1-ml heparin-agarose column was prepared by equilibrating the column with 10 column volumes of HBS. TAFI (0.00025 mg) was applied to the column. TAFI was then eluted with 12 fractions (200 µL each) of HBS, followed by 12 fractions of HBS containing heparin (0.177 U/µL). TAFI content in each fraction was determined through SDS-PAGE and western blotting.

**Thermal Stability of TAFI in the Presence and Absence of Heparin.**

Purified recombinant TAFI variants (1 µM) were activated by incubation with thrombin (25 nM). TM (100 nM), CaCl$_2$ (5mM) in HBST at 24°C for 15 min. The thrombin was then quenched by the addition of PPAck (to 1 µM). Activated TAFI was divided into aliquots corresponding to various time points and incubated at 37°C in the presence or absence of heparin (0.6 U/µL). At each time point, aliquots were placed on ice. Following the final time point, 15 µl of each aliquot was mixed with 35 µl HBST and 50 µl AAFR (120 µM final concentration) in a microtitre plate, and the initial rates of substrate hydrolysis were measured at 350 nm. The carboxypeptidase B function of TAFIa hydrolyzes AAFR according to the following reaction:
Before the anisylazoformyl moiety is removed, AAFR strongly absorbs at 350 nm. Upon the removal of this moiety, the resultant products do not absorb at this wavelength.

The hydrolysis rate was directly related to the activity of TAFIa and related to the initial TAFIa activity by dividing the initial hydrolysis rate by the hydrolysis rate at each respective time point. This relationship was plotted as a function of time. Non-linear regression to the equation for first-order exponential decay (SigmaPlot 11.0, SPSS Inc., Chicago, IL) was used to fit the data in order to obtain rate constants from which the half-lives of each variant could be calculated:

\[ [\text{TAFIa}] = [\text{TAFIa}]_0 e^{-k*t} \]  

(Equation 1)

Where \([\text{TAFIa}]_0\) is the initial TAFIa concentration, \([\text{TAFIa}]\) is the residual concentration at each time point, and \(k\) is the first order decay constant obtained from the non-linear regression in SigmaPlot.

Half-life is defined as the time when \([\text{TAFIa}] = 0.5* [\text{TAFIa}]_0\),

\[ 0.5*[\text{TAFI}]_0 = [\text{TAFIa}]_0 e^{-k*t} \]

\[ 0.5 = e^{-k*t} \]

\[ t = -\ln(0.5)/k \]  

(Equation 2)
The half-life of each variant is thus obtained by substituting the decay constant \( k \) into the equation (2).

**Kinetics of Activation of TAFI Mutants by Plasmin in the Presence or Absence of Heparin.**

For activation by plasmin, a range of concentrations of the TAFI variants (0.01-0.1 \( \mu M \)) was incubated with 25nM plasmin, and 5 \( \mu M \) Plummer’s inhibitor in the presence or absence of heparin 0.2 U/\( \mu L \) for 12 min at 24\(^\circ\)C. Reactions were stopped by the addition of VFKck (1\( \mu M \) final concentration). SDS-PAGE sample buffer (0.2 M Tris-HCl, 8% SDS, 40% Glycerol, 0.08% bromophenol blue) was immediately added to the samples. Samples were analyzed by SDS-PAGE and western blotting with the goat anti-human TAFI polyclonal antibody. A TAFIa standard curve was prepared along with each kinetic experiment. A TAFIa standard solution was made by activating 1 \( \mu M \) TAFI for 10 min at 24 \( ^\circ \)C in the presence of HBST, 25 nm thrombin, 100 nm thrombomodulin, 5 mM \( \text{CaCl}_2 \) to achieve complete activation of TAFI to TAFIa. TAFIa was then serially diluted to concentrations of 0.0053-0.04 \( \mu M \) and then subjected to SDS-PAGE and western blotting like the kinetic samples. For a given experiment, all blots containing standards and samples were processed simultaneously for visualization of immunoreactive bands or order for the standard curve to be applicable to the samples. The densities of the 35 kDa TAFIa and 25 kDa TAFIai bands were quantified using AlphaView SA software. A standard curve was constructed by plotting the density of each standard TAFIa sample against its concentration. The TAFIa concentrations formed in each kinetic run were thus
obtained by interpolating the densities on the standard curve. The concentration of TAFIa formed was then converted to the rate of TAFIa formation (moles of TAFIa formed per second per mole of plasmin). The data were fit to the Michaelis-Menten equation (Sigmaplot 11.0, SPSS Inc., Chicago, IL).

\[ V = \frac{k_{cat}[E]_T[S]}{(K_m+[S])} \]  

(Equation 3)

Although the data were fit to the Michaelis-Menten equation, the \( k_{cat} \) and \( K_m \) values that will be reported are apparent values, and referred to as \( k_{cat(app)} \) \( K_{m(app)} \) respectively. Since the substrate concentrations and the enzyme concentrations are comparable, the Michaelis-Menten assumption of a steady state \([ES]\) and saturations of \( E \) with \( S \) do not hold. In an attempt to account for this, the data were fit to the quadratic form of the Michaelis-Menten equation (Equation 4), but the data sets did not give a reasonable fit to this equation. Thus, the Michaelis-Menten equation was employed to give apparent constants that can at least be operationally compared to each other.

\[ V = k_{cat} \left( 0.5 \times (K_m + [E]_T + [S]_T - ((K_m + [S]_T)^2 - 4 \times ([E]_T[S]_T))^{1/2} \right) \]  

(Equation 4)
Chapter 3

Results

3.1 Construction and Expression of TAFI Variants

In order to assess potential heparin binding sites of TAFI, a number of TAFI variants were designed that contained mutations of potential residues involved in heparin binding. Based on the crystal structure of bovine TAFI in the presence of sulfate ions [50], where the negatively charged sulfate ions, in complex with TAFI, were suggested to represent potential heparin binding sites, Anand and coworkers identified four potential regions of heparin binding. Of these, we selected two of the regions for our mutagenesis (Fig 3-1; Table 3-1). The first region identified by Anand and coworkers was Trp210-Ser221. It also was previously reported that this region shows sequence similarity with other glycosaminoglycan binding proteins [43,51]. In the crystal structure of bovine TAFI, one out of 22 sulfate molecules was bound to this region at Lysine 212 [51]. The second region that had a set of bound sulfates is the region running from the instability region to the active site cleft. However, it was suggested that heparin binding in this region would actually hinder binding of TAFI substrates, and thus interfere with carboxypeptidase activity of TAFI. Therefore, we did not pursue mutagenesis in this region. The third region of interest was Ser305-His335, which is the instability region of TAFI. Binding of heparin to this region would explain the increased half-life of TAFI and therefore a number of mutations were made in this region. Five sulfates were also bound to the pro-domain. However, binding of heparin to this region would not explain the
increased stability of TAFI [51]. Regions Trp210-Ser211 and Ser305-His335 were therefore the focus of this study. Table 3-1 summarizes the variants that were pursued.

Figure 3-1 shows an alignment of bovine and human TAFI.

**Figure 3-1. Alignment of bovine and human TAFI amino acid sequences.** Two regions of interest, that contained bound sulfates, are circled, with arrows indicating the residues that were mutated in this study.
Table 3-1. Recombinant TAFI variants used in this study. The majority of the mutations were made on based on the bound sulfates, with the exception of K211Q/K212Q, and R320A/K324A.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Region</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>K212A</td>
<td>Sequence similarity with GAG binding proteins</td>
<td>Bound sulfate</td>
</tr>
<tr>
<td>K211Q/K212Q</td>
<td>Sequence similarity with heparin GAG binding proteins</td>
<td>Sulfate may be bound to the neighbouring lysine in human TAFI</td>
</tr>
<tr>
<td>K327A</td>
<td>Instability region</td>
<td>Bound sulfate</td>
</tr>
<tr>
<td>K327A/R330A</td>
<td>Instability region</td>
<td>Bound sulfate</td>
</tr>
<tr>
<td>H308F</td>
<td>Instability region</td>
<td>Bound sulfate</td>
</tr>
<tr>
<td>K306A</td>
<td>Instability region</td>
<td>Bound sulfate</td>
</tr>
<tr>
<td>R320A/K324A</td>
<td>Instability region</td>
<td>Charged, exposed residues. In close proximity to sulfate-binding residues that are not conserved between bovine and human TAFI.</td>
</tr>
</tbody>
</table>
Variant K212A was pursued because a sulfate molecule was bound to lysine 212 in the bovine TAFI crystal structure. Since the three-dimensional shape of the bovine TAFI molecule slightly differs from that of the human TAFI, it is possible that that the sulfate molecule could bind to the neighbouring lysine residue, 211. Therefore, both lysines, 211 and 212, were mutated. Originally, the double mutation of the two lysine residues (211, 212) was intended to be to two alanine residues. However these mutations did not result in detectable secreted protein. Therefore both lysine residues were mutated to glutamine residues, as this polar residue might be better accommodated on the surface of the protein. Variants K327A, K327A/R330A, H308F, K306A were pursued due to the binding of the sulfate ions to these residues. The majority of these variants contained a mutation to an alanine, with the exception of the H308F, where the charged histidine residue was mutated to phenylalanine to preserve the presence of an aromatic ring. A number of sulfate ions were bound to residues, in the instability region, that are not conserved between human and bovine TAFI, therefore neighbouring exposed charged residues, such as arginine 320 and lysine 324 were mutated to an alanine.
3.2 Binding of TAFI variants to heparin-agarose

Both bovine and human TAFI have been shown to bind heparin, through the use of heparin-sepharose, as the majority of the protein was eluted with high salt concentrations [52]. We employed a similar method. However, after the protein was applied to the column, and following the HBS wash, bound proteins were eluted with HBS containing heparin, to more conclusively demonstrate specific binding of TAFI to heparin. Figure 3-2 summarizes the elution profile of wild-type TAFI and other TAFI variants. As predicted, wild-type TAFI remained bound to heparin throughout a series of wash fractions (2.4X column volumes), and was eluted with the addition of heparin. To demonstrate the specificity of TAFI binding to the immobilized heparin, rather than the agarose itself, a similar binding experiment was conducted using a column containing unconjugated agarose beads. TAFI eluted in the wash fractions and heparin did not result in any further elution of TAFI, suggesting that none of the applied TAFI bound nonspecifically to agarose. Some of the variants exhibit an elution profile similar to wild-type. However, K211A, K211Q/K212Q, K327A, as well as K327A/R330A exhibit a two peak elution pattern, indicating either a reduced affinity for heparin or that there may be both a binding and a non-binding fractions. Variant R320A/K324A appears to have lower affinity for heparin-agarose, as it is almost entirely eluted in the HBS was
Figure 3-2 Binding of TAFI variants to heparin-agarose. TAFI was applied to a heparin-agarose column, followed by an HBS wash, and heparin wash. The elution profile of each variant is representative of its affinity for heparin-agarose. Wild-type TAFI was also applied to an agarose column to account for heparin-independent binding to agarose. The detection of TAFI content in each fraction was done through western blotting.
3.3 Kinetics of activation of TAFI variants by plasmin in absence and presence of heparin

The activation of TAFI by plasmin is accelerated in the presence of heparin, an effect expressed by a 5.9-fold increase in $k_{\text{cat}}$ and a 2.7-fold decrease in $K_m$ [43]. The contribution that several potential heparin-binding residues have on the acceleration of TAFI activation by plasmin in the presence of heparin was analyzed by western blot analysis. Table 3-2 and Figure 3-3 summarize the kinetic parameters of activation of TAFI variants by plasmin in absence and presence of heparin. To varying degrees, activation of all of the variants by plasmin is accelerated when heparin is present, indicating that none of the mutated residues completely abolish the stimulating effect heparin has on TAFI activation by plasmin. Variants K212A, K327A/R330A, and H308F exhibit the greatest enhancement of catalytic efficiency in the presence of heparin, and in each case this reflects a lower catalytic efficiency in the absence of heparin. The degree of rate enhancement by heparin is considerably lower for variants K211Q/K212Q and R320A/K324A, with the fold difference in the catalytic efficiency being 1.3- and 1.2-fold respectively, compared to wild-type, which is 1.5-fold. Compared to wild-type TAFI and most of the other variants, a substantial increase in $K_m$ was observed for each of these variants both in the absence and presence of heparin, which indicates a reduced affinity of plasmin for these variants. Although variant K306A does not show a difference with wild-type with respect to fold increase in catalytic efficiency in the presence of heparin,
inspection of Figure 3-3f does show a clear decrease in the magnitude of the effect of heparin, compared to wild-type TAFI and most other variants. We suspect that the $K_{m(app)}$ of this variant in the absence of heparin has been over-estimated: at these low rates, we are approaching the limit of sensitivity of the western blot assay.
Table 3-2. Activation of TAFI variants by plasmin in the absence and presence of heparin. $k_{\text{cat(app)}}$ and $K_M(app)$ values were determined from the data in Figure 3-3. The standard error of $k_{\text{cat(app)}}$ and $K_M(app)$ were estimated by the regression algorithm in SigmaPlot 11.

<table>
<thead>
<tr>
<th>Variant</th>
<th>$k_{\text{cat(app)}}$ ($s^{-1}$)</th>
<th>$K_{\text{m(app)}}$ ($\mu M$)</th>
<th>$k_{\text{cat(app)}}/K_{\text{m(app)}}$ ($\mu M^{-1} s^{-1}$)</th>
<th>$k_{\text{cat(app)}}/K_{\text{m(app)}}$ fold increase</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+heparin</td>
<td>-heparin</td>
<td>+heparin</td>
<td>-heparin</td>
<td></td>
</tr>
<tr>
<td>TAFIwt</td>
<td>0.00190±0.0004</td>
<td>0.00070±0.0007</td>
<td>0.036±0.002</td>
<td>0.021±0.005</td>
<td>0.051</td>
</tr>
<tr>
<td>K327A</td>
<td>0.00220±0.0005</td>
<td>0.00060±0.0006</td>
<td>0.040±0.018</td>
<td>0.018±0.004</td>
<td>0.053</td>
</tr>
<tr>
<td>K327A/R330A</td>
<td>0.00190±0.0003</td>
<td>0.00100±0.0004</td>
<td>0.049±0.013</td>
<td>0.063±0.004</td>
<td>0.039</td>
</tr>
<tr>
<td>H308F</td>
<td>0.00190±0.0001</td>
<td>0.00100±0.0006</td>
<td>0.032±0.005</td>
<td>0.039±0.010</td>
<td>0.059</td>
</tr>
<tr>
<td>K212A</td>
<td>0.00280±0.0006</td>
<td>0.00110±0.0004</td>
<td>0.067±0.003</td>
<td>0.071±0.009</td>
<td>0.041</td>
</tr>
<tr>
<td>K211Q/K212Q</td>
<td>0.00150±0.0003</td>
<td>0.00090±0.0002</td>
<td>0.096±0.031</td>
<td>0.076±0.029</td>
<td>0.014</td>
</tr>
<tr>
<td>R320A/K324A</td>
<td>0.00720±0.0010</td>
<td>0.00330±0.0001</td>
<td>0.309±0.072</td>
<td>0.168±0.068</td>
<td>0.023</td>
</tr>
<tr>
<td>K306A</td>
<td>0.00230±0.0004</td>
<td>0.00190±0.0001</td>
<td>0.122±0.031</td>
<td>0.157±0.017</td>
<td>0.019</td>
</tr>
</tbody>
</table>
Figure 3-3 Activation of TAFI variants by plasmin in the presence or absence of heparin. Various concentrations of wild-type TAFI (a), K327A (b), K327A/R330A (c), H308F (d), K212A (e), K211Q/K212Q (f), R320A/K324A (g), K306A (h) were incubated with 25 nM plasmin, in the presence (circle) or absence (cross) of 0.02 U/µL heparin for 12 min at room temperature. The rates of activation were calculated and the data were fit to the Michaelis-Menten equation. Each point represents the mean of at least two experiments, with the error bars representing the standard error of the mean (for n≥3) or the range of the data (for n=2). The lines are regression lines calculated from the fit parameters.

a)

![Wild-type graph]

b)

![K327A graph]
c) K327A/R330A

\[ V (\text{nM TAFIa/sec/nM Plasmin}) \]

\[ [\text{TAFI}] \text{nM} \]

\[ 0 \quad 20 \quad 40 \quad 60 \quad 80 \quad 100 \]

\[ 0.0000 \quad 0.0002 \quad 0.0004 \quad 0.0006 \quad 0.0008 \quad 0.0010 \quad 0.0012 \quad 0.0014 \quad 0.0016 \quad 0.0018 \]

K211A

\[ V (\text{\text{nM TAFIa/sec/nM Plasmin}}) \]

\[ [\text{TAFI}] \text{uM} \]

\[ 0 \quad 20 \quad 40 \quad 60 \quad 80 \quad 100 \]

\[ 0.0000 \quad 0.0002 \quad 0.0004 \quad 0.0006 \quad 0.0008 \quad 0.0010 \quad 0.0012 \quad 0.0014 \quad 0.0016 \quad 0.0018 \]
g) $\text{TAFI} (\text{nM})$ vs. $V (\text{nM TAFI/sec/nM Plasmin})$

h) $\text{TAFI} (\text{nM})$ vs. $V (\text{nM TAFI/sec/nM Plasmin})$

R320A/K324A

K306A
3.4 Thermal Stability of TAFI variants in the Presence and Absence of Heparin

Activated TAFI is a highly unstable enzyme. Depending on the isoform, the half-life of TAFI is 8-15 minutes [55]. It has been previously shown that thermal instability of TAFI is responsible for the inactivation of the enzyme and influences its antifibrinolytic potential [48]. Heparin has been shown to increase the half-life of TAFIa [43]. Although the study of the effect of heparin on the stability of TAFIa was previously done at 25˚C, increasing the half-life from 74 min to 170 min [43], we hypothesized that heparin would have a similar stabilizing effect at 37˚C. It was determined that heparin does stabilize TAFIa at body temperature, increasing the half-life from 6.4 minutes to 10.8 minutes (Table 3-4). The specific enzymatic activity of all of the variants ranged between 68 % of wild-type (K211Q/K212Q) and 124% of wild-type (R320A/R330A), trends that were not markedly altered by the presence of heparin (data not shown). The contribution of the various residues to the TAFIa stabilizing effect of heparin was determined. Table 3-3 and Figure 3-4 summarise the half-lives of wild-type TAFIa compared to the TAFIa variants. Several variants were less stable than wild-type TAFI in the absence and presence of heparin, while the R320A/K324A variant was more stable than wild-type TAFIa in the absence of heparin. Some of the variants exhibited a considerably reduced stabilizing effect of heparin, when compared to the wild-type enzyme: specifically K327A, K327A/R330A, K212A, and R320A/K324A, which corresponds to the lowest fold increase in the half-life in the presence and absence of heparin in these variants.
Table 3-3. The intrinsic stability of TAFIa variants in the absence and presence of heparin at 37°C. Decay constants estimated by the regression of the data in Figure 3-4 were used to calculate the half-lives of the TAFIa mutant in the absence and presence of heparin. The data represent the mean of at least 2 experiments. With error representing the standard error of the mean (for n≥3) or the range of the data for (n=2). Half-life fold increase is a ratio between the half-life of TAFI in the presence and absence of heparin.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Half-life</th>
<th>Half-life fold increase</th>
<th># of replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+heparin(min)</td>
<td>-heparin(min)</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>10.80 ±0.11</td>
<td>6.41±0.53</td>
<td>1.7</td>
</tr>
<tr>
<td>K327A</td>
<td>5.87±0.14</td>
<td>4.73±0.42</td>
<td>1.3</td>
</tr>
<tr>
<td>K327A/R330A</td>
<td>5.58±0.83</td>
<td>4.29±0.26</td>
<td>1.3</td>
</tr>
<tr>
<td>H308F</td>
<td>11.80±0.61</td>
<td>6.64±1.52</td>
<td>1.7</td>
</tr>
<tr>
<td>K212A</td>
<td>6.81±0.44</td>
<td>5.08±0.49</td>
<td>1.3</td>
</tr>
<tr>
<td>K211Q/K212Q</td>
<td>8.07 ±1.16</td>
<td>5.11±1.10</td>
<td>1.6</td>
</tr>
<tr>
<td>K306A</td>
<td>8.19±2.3</td>
<td>4.6±0.59</td>
<td>1.8</td>
</tr>
<tr>
<td>R320A/K324A</td>
<td>9.89±0.60</td>
<td>8.51±0.97</td>
<td>1.2</td>
</tr>
</tbody>
</table>
Figure 3-4 The intrinsic stability of TAFIa variants in the absence and presence of heparin at 37°C. WT(a), K327A(b), K327A/R330A(c), H308F(d), K212A(e), K211Q/K212Q(f), R320A/K324A(g), K306A(h) were activated in the presence of thrombin-TM, and incubated at 37°C in the presence (circle) or absence (cross) of heparin. TAFIa activity was measured at different time points. The data were fit to the equation describing first-order exponential decay in order to determine the half-lives of TAFIa. Each point represents the mean of at least two experiments with the error bars representing the standard error of the mean (for n≥3) or the range of the data for (n=2). The lines are regression lines calculated from the fit parameters.
e) Time (min)
0 10 20 30 40 50
% Residual activity
0
20
40
60
80
100
120

K211A

f) Time (min)
0 10 20 30 40 50
% Residual activity
0
20
40
60
80
100
120

K211Q/K212Q
g) K306A

h) R320A/K324A
Chapter 4
Discussion and Conclusion

4.1 Role of Glycosaminoglycans in the TAFI Pathway

TAFI pathway has been shown to play a significant role in fibrinolysis by removing carboxyl-terminal lysines from partially degraded fibrin thus reducing the ability of this material to promote the activation of plasminogen and the conversion of Glu-plasminogen to Lys-plasminogen, as well as to protect plasmin from consumption by antiplasmin [32,66]. To elicit these effects, TAFI zymogen has to be activated by thrombin, thrombin/thrombomodulin complex, or plasmin [34,41]. Even though the TAFI pathway has been studied extensively, the contribution of these activators on the localized activation of TAFI remains unknown. There is a possibility that none of these activators are the exclusive, physiologically relevant, activator of TAFI. Even though the catalytic efficiency of thrombin-thrombomodulin activation of TAFI is much greater than that for thrombin alone or plasmin [41], the characteristics of the site where the activation takes place may be the determining factor of what is the physiologically-relevant activator of TAFI at that site. High concentrations of glycosaminoglycans can be found at a site of a vascular injury, due to the exposure of the extracellular matrix components. Due to an increase in catalytic efficiency of plasmin-mediated activation of TAFI in the presence of glycosaminoglycans, it is fair to hypothesize that plasmin is an important activator at a site where there is a high concentration of glycosaminoglycans [43]. The majority of the endothelial cell cofactor thrombomodulin, however, would be absent at the site of a vascular injury, further emphasizing the importance of plasmin-mediated mechanism of
TAFI activation [43]. On the other hand, in the situation of an intact endothelial cell surface, thrombin/thrombomodulin may be main activator of TAFI [44].

Due to the function of TAFI in regulation of the balance between the coagulation and the fibrinolysis, understanding the TAFI pathway would aid in the development of either thrombosis or bleeding disorder therapeutics. Current therapy for thrombotic disorders heavily relies on the use of heparin, and other heparin-related drugs [89,90]. However, heparin therapy has been complicated with the determination of appropriate dosage, as well as heparin-induced thrombocytopenia (HIT), resulting in increased thrombosis [90,91]. Another potential problem that could arise from heparin therapy is increased plasmin-mediated activation of TAFI, as well as increased stability of TAFIa [43], which would result in decrease in fibrinolysis and therefore antagonize the anticoagulant properties of heparin.

The focus of the present study was to develop TAFI variants that are resistant to heparin binding and could be used to explore contribution of heparin on activation and stability of TAFI in vitro, and eventually in vivo. The number and location of sites on TAFI that bind heparin are completely unknown, as is the relationship between these sites and the observed effects of heparin on TAFI activation and TAFIa stability. Guided by X-ray crystallographic data on the structure of TAFI, in the presence of sulfate ions, as well as by similarity of certain sequences in TAFI to heparin-binding proteins, we have mutated selected residues in the Trp210-Ser221, and Ser305-His335 regions. However, it should be noted that the binding of individual sulfates may not necessarily represent bona fide heparin-protein interactions, as heparin itself is a polymer of sulfated glycosidic units. Therefore it is possible that some of the residues that were associated with a sulfate
ion, are not physiologically relevant as heparin binding sites. This might explain why mutation of some of the sulfate-bound residues had no effect on heparin binding. Molecular docking of a single heparin molecule to TAFI may be a better model for TAFI interaction with heparin. Nonetheless we anticipated this model would be indicative of some of the heparin binding sites and would lead us to variants resistant to heparin binding and thus resistant to enhanced activation by plasmin and to increased thermal stability of TAFIa due to heparin. As elaborated below, the results reveal a rather complex scenario where no one site definitively eliminated both heparin binding as well as the effects of heparin on TAFI activation and/or TAFIa stability. Several of the variants displayed impaired heparin binding, as evidenced by a two-peak elution pattern where some of the TAFI eluted in the wash fractions of the heparin-agarose column with the remainder being specifically eluted by heparin. Some of these variants showed reduced plasmin activation acceleration by heparin, while others did not. Likewise, some of these variants impaired in heparin binding showed decreased stabilization by heparin but others did not. One variant, R320A/K324A, did exhibit a drastically reduced affinity for heparin, with almost all of the protein eluting in the wash fractions. This variant was impaired in both acceleration of activation as well as in enzyme stabilization by heparin, yet some residual effects of heparin remained. Altogether, these findings shed new light on the nature of the TAFI-heparin interaction, and will aid us in future understanding of significance of TAFI binding to heparin and other glycosaminoglycans, and the effect of the binding on the activation and stability of the enzyme. This knowledge will lead to further understanding of modulation of the TAFI pathway via heparin and other glycosaminoglycans.
4.2 Binding of TAFI variants to heparin-agarose.

To estimate the contribution of various residues on the binding of TAFI to heparin, we employed a heparin-agarose column. We hypothesized that if the mutated residue is critical to heparin binding of TAFI, the protein will be eluted from the column more readily than the wild-type protein. In fact, wild-type TAFI remained bound to the column until the addition of heparin, while one variant, R320A/K324A, was almost entirely eluted from the column in the wash fractions with very little remaining for elution by heparin. On the other hand, several of variants, namely K211A, K211Q/K212Q, K327A, and K327A/R330A, exhibited a two peak elution profile with differing characteristic amounts of TAFI eluted in the wash fractions. Each of these mutants displayed a relatively consistent elution pattern, when compared between repeated experiments. The reason for the two peak elution pattern is unknown. However, it can be hypothesized that it results from either a reduced affinity of the variant for the column such that a fraction remains unbound under these conditions, or the presence of two fractions of TAFI with different affinities for the heparin-agarose.

The source of these two fractions may be the existence of two difference different glycoforms of TAFI. A previous report on recombinant antithrombin showed that there are two major glycoforms of antithrombin present due to both differential usage of the one of the glycosylation sites, as well heterogeneous glycosylation at the remaining sites. Those glycoforms varied in the affinity to the heparin-agarose up to 10-fold [97]. TAFI contains four sites of N-linked glycosylation, all of which are in the activation domain of TAFI and all of which appear to be used in all molecules of both recombinant and plasma-derived TAFI [39]. One group reported that there is an additional N-linked
glycosylation site, not utilized in all molecules of TAFI, in the catalytic domain of TAFI[38]. However, subsequent crystallographic data [40] comprehensively debunked this notion as the Asn219 residue implicated is buried within the core of the catalytic domain. It has been shown that there is microheterogeneity in three of the N-linked glycans while the fourth is homogeneous [38]. Mutation of each of four glycosylation sites on TAFI could potentially determine which of the sites might contribute to potential microheterogeneity affecting heparin binding. Alternatively, mass spectrometric analysis of the glycans present in the binding and non-binding TAFI fractions could be performed. However, it is difficult at this time to rationalize how mutations in the catalytic domain of TAFI could influence the extent of microheterogeneity in the N-linked glycans on the activation peptide.

We did rule out the third possibility for the presence of two binding fractions: that the TAFI-binding capacity of the heparin column was saturated by the amount of TAFI applied. Two factors suggest against this possibility. First, several of the variants including wild-type TAFI bound to the column, and eluted in a single peak, despite all variants being applied in the same amounts to same-sized columns. In addition, during the optimization of this experiment, even after lowering the amount of applied TAFI by nearly 10-fold, we still observed a two peak elution pattern for some of the variants. To further establish that the column is not saturated, the amount of TAFI for variants in question was lower by 1.5-fold. However, a similar elution pattern was observed (data not shown).

The next step in comparing the contribution of various residues on binding of TAFI to heparin, would be to perform equilibrium binding experiments to determine the
dissociation constant ($K_d$) of the TAFI variants for binding to heparin. We did investigate two different approaches to measuring this value. First, we purchased fluorescently-labeled heparin and titrated this material with TAFI while measuring the fluorescence intensity. If binding to TAFI increased or decreased the quantum yield of the heparin-bound fluorophore, the data could have been used to arrive at a $K_d$. However, no change in fluorescence was observed, suggesting that binding of TAFI did not alter the chemical environment of the fluorophore. Similar solution-phase experiments can be envisaged, such as using fluorescently-labeling TAFI, measuring changes in fluorescence anisotropy, or measuring changes in light-scattering intensity.

We also attempted an equilibrium binding analysis using a ligand binding assay where heparin-agarose was incubated with various concentrations of TAFI; following pelleting of the heparin-agarose by brief centrifugation, the TAFI concentration in the supernatant (representing the unbound fraction) was measured using either western blotting or enzyme-linked immunosorbent assay (ELISA). The results we observed are summarized in the Appendix. However, clear evidence for specific, saturable binding could not be obtained within the limited timeframe available; clearly, more optimization for the experiment is needed.

Overall, it can be concluded that variant R320A/R324A exhibited the lowest affinity for heparin agarose, as it was almost entirely eluted in a single peak, during the wash. Variants K211A, K211Q/K212Q, K327A, and K327A/R330A eluted in a two-peak manner.
4.3 Kinetics of activation of TAFI variants by plasmin in absence and presence of heparin

Plasmin is an active form of the proenzyme plasminogen, that results from the cleavage of plasminogen Arg561-Val562 peptide bond [68]. It was previously reported that plasmin-mediated activation of TAFI is accelerated in the presence of heparin [43]. Mao and coworkers activated TAFI with either thrombin or plasmin in the presence and absence of heparin. They found that heparin had no effect on activation of TAFI by thrombin, while enhancing catalytic efficiency of plasmin-mediated activation of TAFI 16 fold [43]. In the current study we examined the contribution of various residues on plasmin-mediated activation in the presence or absence of heparin. We found that all of the TAFI variants exhibited accelerated activation by plasmin in the presence of heparin, to a certain degree. While Mao and coworkers observed a 16-fold increase in catalytic efficiency in the presence of heparin in the case of the wild-type enzyme, we determined the catalytic fold increase to be 1.5. However we observed $k_{\text{cat(app)}}$ values in the presence and absence of heparin to be 0.00190 s$^{-1}$ and 0.00070 s$^{-1}$ respectively. These values are similar to those reported by Mao and coworkers, 0.0026 s$^{-1}$ and 0.00044 s$^{-1}$ in the presence and absence of heparin respectively. In the current study, we found that $K_m(\text{app})$ values were similar, within error, in the presence and absence and heparin. Mao and coworkers found a decrease in $K_m$ in the presence of heparin, while we tended to observe an increase, accounting for the differences in fold increase in catalytic efficiency between the two studies. It is possible, from inspection of our data, especially in the absence of heparin, that we were approaching the limits of sensitivity of our western blot assay and thus may have overestimated the extent of TAFI activation at low
concentrations of substrate, thus artificially decreasing \( K_{m(app)} \). Indeed, our \( K_m \) in the absence of heparin for wild-type TAFI was 0.021 \( \mu M \), versus 0.055 \( \mu M \) reported by Mao and coworkers. To more accurately estimate \( K_m \), we would have had to use even lower concentrations of TAFI which would have further compromised the accuracy of our assay. Moreover, we were not able to fit our data to the quadratic form of the Michaelis-Menten equation, which may have altered our estimates of \( k_{cat} \) and \( K_m \). Nonetheless, our kinetic data at higher TAFI concentrations clearly show a marked effect of heparin on the rate of TAFI activation. By examining the kinetic data (Fig 3-3) and the obtained kinetic parameters (Table 3-2) for plasmin-mediated activation of different variants, it is evident that the lowest degree of rate enhancement is exhibited by variants K211Q/K212Q and R320A/K324A. However, this is not well represented in a reduced fold \( k_{cat}/K_m \) difference likely due, as argued above, that the true extent of enhancement of catalytic efficiency of wild-type TAFI is underestimated. Nonetheless, variants K211Q/K212Q and R320A/K324A exhibit the lowest fold difference in \( k_{cat}/K_m \) between plasmin-mediated activation in the presence or absence of heparin. Although the fold difference in \( k_{cat}/K_m \) in the presence and absence of heparin in the case of mutant K306A is similar to that of wild-type, through the analysis of the Figure 3-3f, it appears that heparin has a reduced effect on plasmin-mediated activation of this variant. This could be due to the aforementioned inaccuracy of \( K_{m(app)} \) estimation. It is evident that the rate of plasmin-mediated activation of variants K211Q/K212Q and R320A/K324A, as well as K306A, is substantially lower, an effect attributable mostly to higher \( K_{m(app)} \) values for these variants. These data suggest that these residues play a role in interacting with plasmin.
Plasmin consists of two disulfide-bonded chains. The light chain consists of a trypsin-like protease domain; plasmin displays comparatively broad substrate specificity and the protease domain itself makes limited contacts with the substrate outside of the residues surrounding the scissile bond. The heavy chain, however, consists of five kringle domains. Kringle domains are tri-looped structures, containing six invariant disulfide bonds, that are thought to mediate protein-protein interactions. Indeed, kringles 1 and 4 of plasmin contain high affinity lysine-binding sites that participate in maintaining the closed conformation of native plasminogen and in binding interactions with basic residues on substrates such as fibrin and antiplasmin [69,99]. Although there are few reports characterizing plasmin-TAFI interactions, several residues have been identified to be important in the formation of the TAFI-plasmin complex, and therefore activation of TAFI by plasmin. These residues are lysine 268, serine 272, arginine 276, threonine 147, and phenylalanine 113. Lysine 268, serine 272, arginine 276 are important exclusively in plasmin binding to TAFI, while the rest of these residues are implicated in activation of TAFI by both plasmin and thrombin [100]. The fact that not all of these residues are lysines is explained by the fact that the mutations were made on the basis of binding sites mapped for antibodies against TAFI that specifically impaired activation by plasmin or thrombin; the effect of the mutations on recognition by these proteases may be due to local alterations in protein structure. Figure 4-1a exhibits the positioning of these residues in three-dimensional model of TAFI. Since variants K211Q/K212Q, R320A/K324A, and K306A exhibit a larger \( K_m(\text{app}) \), there is a possibility that these residues participate in plasmin binding to TAFI. Figure 4-1b displays the positioning of these residues in relation to each other, while Figure 4-1 c, d, and e display the positioning of these residues in relation to the residues that have been shown to participate in the binding of
TAFI to plasmin. Although these K211, K212, R320, K324, and K306 are not positioned in close proximity to the residues that have been implicated in plasmin binding, nor are the previously-described residues important for plasmin binding, in respect to each other (Fig 4-1). From Fig 4-1 it is also evident that K211, K212, R320, K324, and K306 are exposed charged residues that could potentially interact with the kringle domains of plasmin.
Figure 4-1. Location of residues implicated in decreased activation of TAFI by plasmin. Images were generated using Cn3D software (pdb file:3D66 of human TAFI) Activation peptide is demonstrated in cyan, catalytic domain is in purple. TAFI cleavage site by thrombin and plasmin, Arg92, is represented in red, indicated with an arrow. a) Location of residues previously reported to be critical for plasmin binding to TAFI (yellow). b) Location of the residues mutated in variants that exhibit lower affinity to plasmin (green). c)-e) Location of each individual variant in b) in relation to residues in a)
b) Lys 211/Lys 212

c) Lys 211/Lys 212
d) Lys 306

e) Arg320/Lys324
There was not complete concordance between impaired heparin binding and an effect on heparin-accelerated activation by plasmin. The variants K212A, K327A, and K327A/R330A show impaired heparin binding, yet essentially normal responses to heparin in the plasmin activation assay. The K211Q/K212Q and R320A/K324A variants did exhibit a lower fold difference in $k_{cat}/K_M$ in the presence or absence of heparin, which could potentially be due to the lower affinity of these variants for heparin. Notably, the K211Q/K212Q, R320A/K324A, and K306A variants were also impaired in respect to plasmin activation, when compared to wild-type TAFI, which complicates interpretation of the results. Is the defect in heparin binding responsible for the lower stimulation of activation, or does the apparently impaired interaction of the variants with plasmin impair the ability of heparin to have its stimulatory effect? In the case of thrombin inhibition by antithrombin, heparin catalyzes this reaction by binding to both thrombin and antithrombin [22,85]. It is highly likely that heparin binds to both plasmin and TAFI, thus eliciting its accelerating effect on TAFI activation by plasmin. In that case, impaired binding of TAFI to plasmin, would also affect heparin binding to TAFI. To further investigate this possibility, a truncated form of plasmin can be used. Microplasmin lacks all 5 krings, but has an active catalytic domain [98], which, in theory, could still activate TAFI. This approach may give an insight on whether heparin can still elicit its catalyzing effect on plasmin-mediated activation of TAFI in a kringle-independent manner.

In addition to the above mentioned variants, a number of additional variants were studied in respect to plasmin activation in the presence or absence of heparin. These
variants are K59A, R12A, R15A, S316A, and S249A/S251A. Variants K59A, R12A, and R15A were pursued based on the report that a number of sulfates were bound to the exposed charged residues in the pro-domain [51]. The identity of these residues was not provided, therefore, we used the Cn3D software (pdb:3D66) to explore the three-dimensional shape of the TAFI molecule, and to identify exposed charged residues in the pro-domain. Variants S316A, S249A/S251A were pursued based on another report of the TAFI crystal structure, where sulfates were found to be associated with these residues [52]. The data for these variants are preliminary, and therefore is reported in the Appendix; however, the replacement of these residues with an alanine residue did not abolish the accelerating effect of heparin on TAFI activation. Further characterization of these variants is required to determine if the accelerating effect of heparin is reduced to a certain degree, in comparison to the wild-type TAFI.


Heparin, has been shown to increase thermal stability of TAFIa [43]. Mao and coworkers established that half-life of TAFI at 25°C was increased from 74 min to 170 min in the presence of TAFI [43]. Although they did not examine the role of heparin on the stability of TAFIa at body temperature, we expected to see a similar effect of heparin on the stability of TAFIa at 37°C. Indeed, it was found that presence of heparin increased the half-life of the wild-type enzyme from 6.41±0.53 minutes to 10.8 ±0.11 minutes. To further examine the contribution of various residues on the stability of TAFIa in the presence and absence of heparin, we subjected the variants in question to the same assay. As expected, the majority of the mutations in the instability region decreased the stability of TAFIa both in the presence and absence of heparin, when compared to the wild-type
enzyme. Stability of the variant H308F was similar to the wild-type TAFIa, within experimental error. The variant R320A/K324A was found to be more stable in the absence of heparin, when compared to the wild-type TAFIa. The stability of TAFIa depends on the dynamics of the instability region, and this double mutation could potentially reduce the movement of this region, thus increasing stability. Lysine 324 is also neighbouring threonine 325, which is the site of a naturally occurring single nucleotide polymorphism. When isoleucine is substituted for threonine at this position, the half-life of TAFIa is increased from ~8 min to ~15 min [55]. Therefore, it is possible that the substitution of a charged residue with an uncharged alanine could potentially have a stabilizing effect. Variant K327A, K327A/R330A, and K212A seem to be slightly less stable in the presence and absence of heparin. When examining the effect of heparin on the half-lives of all of the above variants, half-life fold increase is the lowest for K327A, K327A/R330A, and K212A, and R320A/K324A. Half-life fold increase of these variants in the presence of heparin is 1.3, compared to the wild-type enzyme, where the half-life fold increase is 1.7. Therefore, these residues could potentially be important in the modulation of TAFIa stability by heparin. As with the plasmin activation experiment, there was no perfect agreement between the heparin binding data and the stability data. While all of the variants with impaired stabilization by heparin also show reduced binding to the heparin column, the K211Q/K212Q variant shows nearly normal stabilization by heparin.

As in the case of plasmin activation, a number of variants were introduced in the later stages of this study. Since variant S316A is in the instability region, while S249A/S251A is close to the instability region, the effect of heparin on the thermal
stability was analyzed. Preliminary results are reported in the Appendix. Although, the data is preliminary, there was a clear effect of heparin on the stability of TAFIa in both variants. Further studies will be needed to examine if the stability increase in the presence of heparin is reduced compared to the wild-type enzyme.

4.5 Conclusion

In the current study we set out to examine the effect of heparin binding on the plasmin-mediated TAFI activation and thermal stability of TAFIa. A number of TAFI residues suspected in the participation of heparin binding were mutated with an ultimate goal of determining variants that are resistant to heparin binding. Such variants would be useful for assessing the importance of heparin modulation of TAFI activation and TAFIa stability, for example in the setting of anticoagulation with heparin or in the presence extracellular matrix. Although variants that are completely resistant to heparin binding were not identified, we established a number of variants that are moderately impaired in respect to heparin binding, and one, R320A/K324A that is very nearly completely impaired in heparin binding. In addition, there was a strong, but not absolute, relationship between the degree of impairment in heparin binding and reduction in activation or stabilization by heparin. Therefore, it is reasonable to conclude that there are multiple sites on TAFI for heparin binding, and that heparin binding at these different sites may be important for different functions of heparin in respect to TAFI. Variants such as K211A, K327A, and K327A/R330A displayed reduced binding to the heparin-agarose column (dramatically so in the case of K327A/R330A), while still showing similar kinetic parameters in respect to plasmin-mediated activation. The same variants exhibited the lowest difference in the half-life of TAFIa in the presence and absence of heparin.
However, these variants with the addition of K211Q/K212Q eluted from the heparin-agarose column in two peaks, which could either be the result of lower affinity (for example because of the elimination of one heparin binding site), or presence of two glycoforms of TAFI. Different glycoforms may exhibit differential affinity for heparin. Further studies are required to analyze the role of potential differential glycosalation on the binding of these variants to heparin. Since the activation parameters were not affected by the reduced affinity to heparin for some variants, these residues may play a role in the stabilization of TAFI in the presence of heparin. Variants K306A, K211Q/K212Q, and R320A/K324A seem to exhibit both a reduced effect of heparin on the activation of TAFI by plasmin, as well as lower affinity for plasmin, due to large $K_m$ (app) of this variants. Further investigation is required to establish whether the lower affinity of these variants to plasmin interferes with proper TAFI binding to heparin, and prevents heparin from eliciting its accelerating function. It will be interesting to find out if combining sets of mutations, such as R320A/R324A and K327A/R330A is able to yield a variant of TAFI that is resistant to both heparin-mediated stimulation of activation by plasmin, as well as stabilization.

Although we identified certain residues that may participate in the binding of TAFI to heparin, there are still a number of remaining questions. Since both activation of TAFI and thermal stability of TAFIa will ultimately affect the antifibrinolytic potential of TAFI, the next step would be to determine the effect of heparin on the antifibrinolytic effect of TAFI, and to analyze the contribution of the studied residues on the attenuation of fibrinolysis in the presence and absence of heparin.
Another issue that still remains unresolved is the contribution of the accelerated activation of TAFI by plasmin versus the increased stability of TAFI in the presence of heparin. Mao and coworkers suggested that heparin binding changes TAFI conformation, making it a better substrate for plasmin activation [43]. However, once activated, TAFI is extremely unstable. Heparin, however, also stabilizes TAFIa. As a result, heparin contributes to increased effective TAFIa concentration in both ways. To distinguish between the two functions of heparin, and to determine the relative contribution of these functions on the concentration of TAFIa, a stable TAFI variant can be used. Recombinant TAFI variant, with mutations S305C, and T329I, and an isoleucine at the 325 position, has a half-life of 70 min [54]. Since this variant is more stable, the increase in TAFIa activity within a clot will mostly be a result of accelerated activation of TAFI by plasmin. Alternatively, we could use a mutant such as K327A that is susceptible to heparin acceleration of activation but not heparin stabilization.

Finally, to understand the full effect of heparin on the TAFI pathway, its contribution on TAFI activation by thrombin/thrombomodulin complex has to be investigated. It was reported that presence of heparin actually reduced activation by thrombin/thrombomodulin complex [44]. However, it is not known whether heparin binding to TAFI prevents binding of the thrombin/thrombomodulin complex to TAFI, or if heparin binds directly to thrombin/thrombomodulin complex. To investigate that, TAFI variants that are resistant to heparin binding would have to be used.

Overall, the goal of this study was to identify TAFI residues that participate in heparin binding, and their contribution on the plasmin-mediated activation and stability of TAFI. Understanding of the heparin function in respect to TAFI would provide answers for the overall picture of the role of TAFI in hemostasis of a healthy individual, as well as
that of an individual that is undergoing anticoagulation with heparin or a related compound. The anticoagulant properties of heparin may be antagonized by the TAFI pathway. Understanding the binding sites, and possibly length requirements of heparin-like molecule in respect to TAFI could give rise to a development of a treatment that is unable to either stabilize TAFI, or accelerate plasmin-mediated activation of TAFI, or both. Similar approach was taken during the development of the drug Fondaparinux which exclusively stimulates inhibition of FXa by antithrombin, while having no effect on inhibition of other proteases of the coagulation pathway. This exclusivity is due to the fact that a short sequence, present in this heparin-like molecule, is sufficient to stimulate inhibition of FXa but not other targets, such as thrombin [85]. This potentially could be the case with plasmin-mediated TAFI activation.

Understanding glycosaminoglycan binding to TAFI is extremely important under both natural and heparin treatment-induced circumstances. Understanding TAFI interaction with heparin, heparan sulfate, and other glycosaminoglycans will provide us with more insight into the contribution of thrombin, the thrombin/thrombomodulin complex, and plasmin on the localized concentration of TAFIa. In the case of the anticoagulant treatments with heparin-like drugs, understanding the interaction of heparin with TAFI and the consequence of this interaction will aid in the development of more tailored and targeted drugs.
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Appendix

This section summarizes the optimization process for quantification of heparin binding to TAFI. This section also summarizes the preliminary results for plasmin-mediated activation and thermal stability of recently developed variants. These variants were pursued in the later stages of this study and thus complete characterization of these variants could not be completed. The rationale for the development of variants K59A, R15A was based on the fact that in a crystal structure of bovine TAFI, a number of sulfates were bound to charged residues in the pro-domain [1]. Due to the fact that heparin binding in the pro-domain would not explain the increased stability of TAFI in the presence of heparin, Anand and coworkers suggested that the pro-domain is not the most likely heparin binding region. However, binding of heparin to the pro-domain can have other consequences in respect to TAFI function. Although, the identity of these residues was not disclosed, using a crystal structure of human TAFI (pdb: 3D66) we identified residues R15A and K59A to be exposed, and thus they could potentially facilitate binding to heparin. Another report of a bovine TAFI crustal structure, in the presence of sulfates, resulted in the development of S316A and S249A/S251A [2]. Preliminary thermal stability experiments were only done on variants S316A and S249A/S251A since the variants with the mutation in the pro-domain are not likely to have an effect on the thermal stability in respect to heparin binding.
A-1 Materials and Methods

Analysis of the dissociation constant between heparin-agarose and TAFI

20µL of Heparin-agarose resin (Sigma-Aldrich) or 4-% agarose resin (MPbiomedicals) was incubated with increasing concentrations of TAFI (0.02 µM-0.14 µM) for 30 min in HBS buffer. The mixture was then centrifuged at 3000 rpm, and the supernatant was removed. The supernatant is thought to contain unbound TAFI. To analyze the concentration of unbound TAFI versus the concentration of total TAFI, samples were first subjected to the western blot analysis. Using a standard curve for each experiment, band analysis was used to quantify the concentration of unbound TAFI, and thus the concentration of bound TAFI, through subtraction. The values were used to plot total TAFI concentration, or unbound TAFI concentration versus bound TAFI concentration. A scatchard plot was also constructed. Due to the scatter in the points, a dissociation constant could not be obtained. The experiment was repeated a number of times, varying either the concentrations of TAFI, or the volume of the resin, without an improvement in the quality of the data. We then investigated whether a different method of TAFI detection would be more appropriate for this experiment, and thus used enzyme-linked immunosorbent assay (ELISA). ELISA was carried out as follows. The binding experiment was performed in the same manner as above, samples representing unbound TAFI or total TAFI were diluted in coating buffer (0.012M sodium carbonate, 0.028M sodium bicarbonate pH 9.6) and applied on polystyrene 96-well plates (Corning) overnight at 4°C. The wells were washed 3 times with phosphate buffered saline (PBS) (137 mM NaCl, 1,4 mM KH₂PO₄, 2.7 mM KCl, 3mM Na₂HPO₄ pH 7.4) containing 0.1%(v/v)Tween-20. The wells were blocked with blocking solution (PBS containing 3%
(w/v) bovine serum albumin) for 2 hours at room temperature. The wells were washed again, and then incubated with 1 μg/mL primary polyclonal sheep anti-TAFI antibody (Affinity Biologicals) in dilution buffer (HBS containing 1% (w/v) bovine serum albumin) on a shaker for 1 hour at room temperature. After another wash of the wells, they were incubated with 1 μg/mL secondary hydrogen peroxide-conjugated anti-sheep IgG (Thermo Scientific) in the dilution buffer, on a shaker for 1 hour at room temperature. The wells were then washed again and colour development solution was then applied, consisting of phenylenediamine dihydrochloride tablet (Sigma-Aldrich) dissolved in a 12 mL of substrate buffer (0.05 M citric acid/sodium-phosphate buffer, pH 5.0); each well received 100 μL of the solution. Colour development was stopped by the addition of 50 μL of 2M H₂SO₄ and the absorbance of each well was measured at 490 nm (and corrected for absorbance at 560 nm) to quantify TAFI present in each well. The total TAFI wells were used as a standard curve. The dilution of the sample in coating buffer varied between 2μL-50μL of the sample in total volume of 100μL of coating buffer. The best standard curve was obtained with the lowest range of 2μL. Calculations of bound and unbound TAFI were done in the same manner as described above.

A-2 Results

A-2.1 Analysis of the dissociation constant between heparin-agarose and TAFI

Following the elution experiment of TAFI binding to heparin agarose, we set out to quantify the binding affinity of heparin to TAFI, employing a ligand binding assay. After varying different aspects of the experimental approach (volume of the resin, concentration of TAFI, and the detection technique), the dissociation constant could not be determined, as there was no clear relationship between the increasing concentration of TAFI, and the
concentration of bound TAFI. Figure A-1 c) and d) shows that there was no linear relationship, and the slope, which is used to determine the dissociation constant, could not be obtained. Due to time constraints, this experiment could not be further optimized, and it cannot be excluded that the dissociation constant can be quantified with this method. However, since the number of binding sites and the contribution of these sites to the binding of heparin to TAFI are unknown, this method may not result in a linear scatchard plot.
Figure A-1. Quantitative analysis of the dissociation constant between heparin-agarose and TAFI. Panels a) and b) represent the concentration of bound TAFI as a function of total TAFI for WT and variant R320A/K324A. Panels c) and d) exhibit the corresponding Scatchard plots of WT and variant R320A/K324A.
A-2.2 Activation of TAFI variants by plasmin in absence and presence of heparin

In respect to TAFI variants R15A, K59A, S316A, and S249A/S251A, preliminary results show that there is an effect of heparin on plasmin mediated activation of TAFI. Figure A-2 shows that all of the variants in question displayed increased activation of TAFI in the presence of heparin. However, whether or not the effect of heparin on activation of these variants is the same or reduced when compared to wild-type cannot be concluded using these data, as apparent $k_{cat}$ and $K_m$ values cannot be derived with the same confidence from these single experiments. Due to the nature of the experiment, and the dependency on the standard curve, the reduced rates of activation for some of these variants may not be representative of the actual activation kinetics, but rather an artifact of a single experiment. Therefore, the activation data cannot be compared between variants. These data can only be used to suggest that mutation of these residues does not abolish heparin’s effect on plasmin-mediated activation of TAFI.
Figure A-2 Plasmin-mediated activation of TAFI variants in presence and absence of heparin. Variants S249A/S251A(a), S316A(b), K59A(c), and R15A(d) were incubated with 25 nM plasmin, in the presence (circle) or absence (cross) of 0.02 U/μL heparin for 12 minutes at room temperature. Results are representative of one experiment.

a)

b)
A-2.3 Thermal stability of TAFI variants in the presence and absence of heparin

Contribution of serine 316, serine 249, and serine 251 on the stability of TAFI in the presence and absence of heparin was estimated using a thermal stability assay. Figure A-3 and table A-1 display that there is a clear difference in the thermal stability of these variants in the presence and absence of heparin. From these preliminary results it can be concluded that mutation of these residues did not abolish the effect of heparin on thermal stability. However, since these experiments were only done once, it cannot be concluded if effect of heparin on the stability of these variants is reduced, or the same when compared to wild-type.
Figure A-3 Intrinsic stability of TAFIa variants in the absence and presence of heparin at 37°C. Variants S316A and S249A were activated in the presence of thrombin-TM, and incubated at 37°C in the presence (circle) or absence (cross) of heparin. TAFIa activity was measured at different time points. The data were fit to the equation describing first-order exponential decay in order to determine the half-lives of TAFIa.
Table A-1  Intrinsic stability of TAFIa variants in the absence and presence of heparin at 37°C.

Decay constants estimated by the regression of the data in Fig. A-3 were used to calculate the half-lives of the TAFIa mutant in the absence and presence of heparin.

<table>
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<tr>
<th>Variant</th>
<th>Half-life</th>
<th>n</th>
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<tbody>
<tr>
<td></td>
<td>+ heparin (min)</td>
<td>-heparin (min)</td>
</tr>
<tr>
<td>S316A</td>
<td>14.2</td>
<td>10.1</td>
</tr>
<tr>
<td>S249A/S251A</td>
<td>11.7</td>
<td>7.2</td>
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References for the Appendix


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