FUNCTIONAL STUDIES WITH DIRECT ORAL ANTICOAGULANTS: INVESTIGATION OF THE REGULATION OF KEY BLOOD COAGULATION PROTEASES

By

Calvin H. Yeh

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AUTHOR:	Calvin H. Yeh, B.H.Sc.(Hons)
SUPERVISOR:	Jeffrey I. Weitz, M.D.
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The end of one journey marks the beginning of another.

I can't wait to go on our next adventure together.

ABSTRACT

Intrinsic structural and conformational mechanisms regulate the functional specificity of the coagulation system. The study of these structure-function relationships is important for understanding the strategies used in the management of clinical thrombosis. Previous studies have shown that the central enzyme in clotting, thrombin, is sequestered inside of a clot, and protected from the natural downregulator antithrombin (AT). This is problematic for anticoagulants like heparin which depend on AT. Subsequently, it was found that the key upstream propagator of thrombin, the prothrombinase enzyme complex, is also resistant to the AT-heparin. Our data show that further upstream of prothrombinase, the intrinsic tenase is only moderately protected, while there is no protection at the level of the initiator complex, extrinsic tenase. This protection phenomenon possibly reflects steric and allosteric mechanisms that ensure maximal activation of the coagulation system once a threshold stimulus is achieved. These mechanisms likely evolved as a result of conformational rearrangement, as evidenced by the proteolytic activation of thrombin activity following proteolysis of prothrombin. Indeed, subtle differences in the structural interaction of ligands with the active site can lead to substantial differences in enzyme activity. The binding of rivaroxaban and apixaban to factor Xa is nearly identical; both interact with the active site with comparable affinity. Despite this, a 3-fold faster rate of the rivaroxaban on-rate yields significantly greater prolongation of the prothrombin time (PT) and activated partial thromboplastin time (aPTT), global tests of coagulation. These small differences in ligand interaction also have allosteric consequences. Structural differences between the direct thrombin inhibitors dabigatran and argatroban yield divergent exosite-mediated thrombin binding to physiologic ligands like γA-fibrin, γ′-fibrin, factor Va, and factor VIII, interactions that govern clot-mediated protection from AT inhibition, and the various functions of thrombin. These divergent effects were robust and ligand-dependent, suggesting conserved energetic scaffolds within the thrombin molecule that govern allosteric changes throughout the molecule. Because proteolysis of prothrombin yields significant allosteric and structural rearrangement that capacitates the active site for substrate recognition amd catalytic ability, we investigated the role of Ser195, a key residue in the thrombin catalytic triad in also regulating thrombin allostery. Site directed mutagenesis of Ser195 to Ala yielded a significant increase in the flexibility of the entire thrombin molecule, as evidenced by increased potency of dabigatran and

argatroban in terms of their capacity to modulate exosite binding through the active site, and increased interexosite cooperative and competitive allostery. Together, these studies represent an advance in our understanding of the consequences of both small molecule ligation of coagulation proteases, as well as the consequences of subtle structural modification for overall allosteric function.

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GENERAL INTRODUCTION

The circulatory system plays a crucial role in the maintenance of homeostasis by delivering oxygen and nutrients, shuttling metabolic waste, regulating temperature, and providing a conduit for the immune and inflammatory system by the circulating blood throughout the vasculature. Damage to a blood vessel triggers the hemostatic system; a rapid series of cellular and protein reactions in the blood that have evolved to prevent hemorrhage and seed and modulate the immune and healing response (Hoffman and Monroe, 2007). These reactions create a physical plug at the site of vessel damage, a blood clot, made of platelets, red blood cells, and leukocytes intertwined in an insoluble mesh of fibrin polymers. This mechanism is delicately balanced by cellular and protein safeguards to maintain blood flow in the normal state. Dysfunction or disruption of this equilibrium leads to inappropriate bleeding tendencies or thrombosis, which are the cause of hemophilias or venous and arterial thromboembolic diseases, respectively. These diseases are the root of significant morbidity and mortality worldwide and have prompted large efforts into understanding the mechanisms of hemostasis in hopes of translating this for therapeutic use. The studies presented in this thesis represent some of those efforts.

To generate a fibrin meshwork, blood is armed with circulating platelets and coagulation proteins. Damage to endothelial cells of the vascular lumen exposes a high local concentration of subendothelial collagen and tissue factor (TF), which serve as receptors and activators for binding clotting proteins and platelets. Platelets are circulating anuclear sentry cells that bind, activate, and aggregate at the site of exposed subendothelium, forming the initially weak physical plug. Activation of platelets leads to changes in the morphology and organization of their membranes, as well as the release of the contents of their α-granules and dense granules, all of which allow platelets to act as platforms that seed, direct, and localize the coagulation system.

The coagulation system is a set of proteins that participate in a finely regulated series of enzymatic reactions, culminating in the generation of the terminal clotting enzyme thrombin, which cleaves circulating soluble fibrinogen causing it to polymerize to form an insoluble fibrin clot (Figure 1). Coagulation is initiated when the circulating precursor zymogen FVII binds to its cofactor TF on the subendothelium and

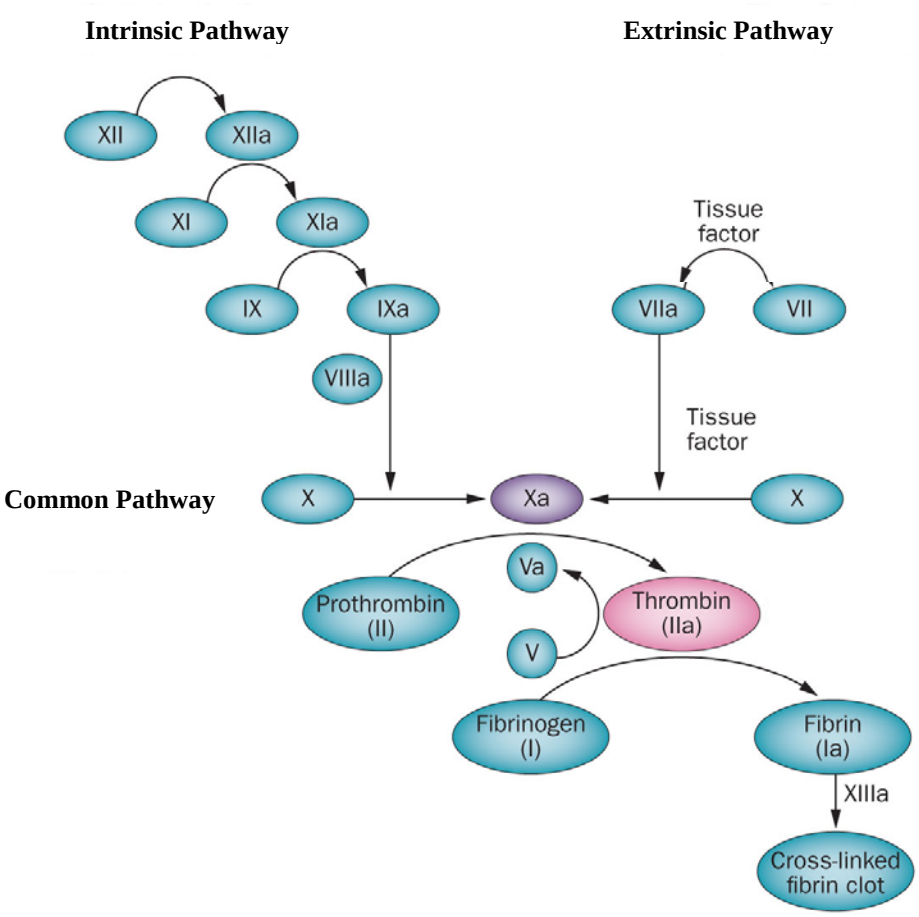

Figure 1. Schematic of the coagulation cascade.

The coagulation cascade is a series of enzymatic reactions culminating in the generation of thrombin. Coagulation may be initiated by the intrinsic pathway (*left*) and the extrinsic pathway (*right*). These two pathways converge at the common pathway (*center*), as both the intrinsic and extrinsic pathways generate FXa. The prothrombinase complex converts prothrombin to thrombin. Thrombin cleaves soluble fibrinogen, causing it to polymerize and form an insoluble fibrin meshes.

is activated, forming the enzyme complex of FVIIa/TF, known as extrinsic tenase. Extrinsic tenase is able to cleave and activate the zymogens FIX and FX. FXa binds to its cofactor FVa to form the prothrombinase complex on platelet surfaces, while FIXa binds to its cofactor FVIIIa to form the intrinsic tenase complex. During the initiation phase of coagulation, little if any of these cofactors are available, allowing only a small amount of thrombin to be generated from circulating prothrombin by FXa. This small amount of thrombin is not sufficient for clotting fibrinogen and instead serves to amplify the coagulation system by activating the procofactors FVIII and FV. This allows the assembly of intrinsic tenase complexes on platelets to generate large amounts of FXa, which in turn form a large amount of prothrombinase. This complex rapidly cleaves prothrombin, generating the "burst" of thrombin required for fast cleavage of fibrinogen and further activation of platelets to effect clotting. The final meshwork plug of platelets and fibrin also traps red and white blood cells, which are thought to aid in signaling wound repair and modulate immune and inflammatory activity.

Several mechanisms are in place to maintain the hemostatic system in a quiescent state, including endothelial cells that line the lumen of the vasculature, and circulating protease inhibitors. Endothelial cells act as a physical barrier from the procoagulant subendothelium, and also express anticoagulant receptors and modulators, such as thrombomodulin (TM) and the endothelial protein C receptor (ePCR), and heparan sulfate proteoglycan. Circulating inhibitors of activated coagulation factors include the antithrombin (AT) and tissue factor pathway inhibitor (TFPI). Indeed, deficiencies in the up- or down-regulators of hemostasis lead to hemorrhage or thrombosis in humans. In addition to the biological regulation mechanisms of protein specificity, biophysical variables such as flow, local concentration, and kinetics can determine the activity of the system. Hemostasis is therefore a highly potentiated positive feedback system for clotting that is delicately balanced to avoid inappropriate activation.

This thesis is a series of studies examining the mechanisms that imbue the coagulation system with explosive kinetic potency and impeccable specificity. We will explore these mechanisms in the context of natural and synthetic regulators, with the ultimate goal of expanding on the understanding of the treatment of clinical thrombosis.

THESIS OVERVIEW AND PROJECT AIMS

Direct oral anticoagulants (DOACs) have arrived for the prevention and treatment of thromboembolic disease. We have written extensively on this topic (Yeh *et al.*, 2015), including papers detailing the bioengineering and clinical development of these inhibitors (Yeh et al., 2012), their use in the treatment of venous thromboembolism (Yeh *et al.*, 2014), their clinical limitations with regards to the lack of an antidote (Yeh et al., 2013), and the management of complications that may result from the use of the DOACs (Yeh et al., 2013b). The DOACs are a shining example of targeted therapies that are the product of rationally designed small molecules using functional and structural information.

The DOACs can be used as tools to directly probe the structural determinants of specificity and their role in the regulation of the coagulation system. Previous methods to stop coagulation have revolved around disrupting the body's ability to generate functional clotting proteins, or depended on using molecules that act as cofactors for the body's natural hemostatic mechanism. The DOACs are synthetic small molecules that directly bind to and inhibit the active sites of thrombin or factor Xa, and have been designed using X-ray crystallographic insights from the binding of natural substrates. As a result we have tightly binding and highly specific active site substrates that may be used to further characterize the structure-function relationships of the coagulation system.

This dissertation examines (Chapter One) the intrinsic resistance to inhibition of the coagulation system and thus the requirement of direct inhibitors such as the DOACs, (Chapter Two) the effect of proteolytic activation on binding specificity of thrombin, (Chapter Three) how two DOACs differentially modulate factor Xa, (Chapter Four) the functional consequences for key enzyme-cofactor interactions when bound to DOACs, and (Chapter Five) the structural mechanisms that regulate the multiple functions of thrombin. These studies provide a framework for the development of direct small molecule inhibitors of coagulation enzymes, and insight into the intrinsic regulatory mechanisms of coagulation that may spur the development of new paradigms for future antithrombotic treatment strategies. Each of these studies are briefly described below.

Chapter One: The need for new direct anticoagulants

FVIIa, FIXa, and FXa are differentially protected when incorporated into their respective complexes

A limitation of existing heparinoid anticoagulants is that venous thrombi may continue to grow and extend in anticoagulated patients, and coronary re-thrombosis may occur even after thrombolytic therapy (Guerci *et al.*, 1987, Hull *et al.*, 1979, Lagerstedt *et al.*, 1985). These observations suggest that thrombi have residual coagulation potential due to active coagulation factors harboured within the thrombus. Indeed, active thrombin has been detected in pathological thrombi (Mutch et al., 2001), and this clot-bound thrombin was found to be protected from inhibition by the heparin-AT complex (Hogg and Jackson, 1989, Weitz *et al.*, 1990); an effect not seen with AT-independent inhibitors (Weitz *et al.*, 1998). Further studies showed that binding of FXa in the prothrombinase complex also protects it from AT inhibition (Rezaie, 2001). The presence of FXa within a clot may be highly prothrombotic, as one molecule of FXa in prothrombinase has the capacity to generate 1000 molecules of thrombin (Mann et al., 2003). Because the extrinsic and intrinsic tenase complexes are responsible for initiation and amplification of the procoagulant response, respectively, and are also likely localized within a clot, does complex assembly of FVIIa and FIXa protect them from AT inhibition? These and other studies provided the impetus to develop direct inhibitors which circumvent the need for AT, as they could inhibit free and clot-bound thrombin.

Chapter Two: The search for new targets for anticoagulation

Prothrombin activation sequentially capacitates exosites I and II for binding

Thrombin bears two anion binding exosites that flank its active site (Huntington, 2005). These exosites bind multiple ligands, allowing thrombin to effect numerous roles in hemostasis. Additionally, the exosites are thought to aid in prothrombin activation (Krishnaswamy, 2013, Krishnaswamy and Betz, 1997). These exosites are dynamic in their structure as the prothrombin molecule is cleaved by prothrombinase to form thrombin. Thus, these intermediate forms of prothrombin have been shown to have varying affinity to exosite ligands (Anderson *et al.*, 2003, Anderson and Bock, 2003). To better characterize the development of the thrombin exosites during prothrombin activation, we used surface plasmon resonance (SPR) to measure binding to numerous physiological ligands. These studies may identify novel targets for the chemical modulation of the specific physiological effects of thrombin.

Chapter Three: Functional differences of the direct oral anticoagulants

The mechanism of differential effects of the factor Xa inhibitors on global tests of coagulation

The DOACs that inhibit factor Xa have been licensed for use for various indications around the world following phase III clinical trials. Despite their similar mechanism of action, the inhibitors do not inhibit global tests of coagulation to the same manner (Dale et al., 2014). This complicates management of patients who are using these new drugs. This project is a head-to-head measurement of the kinetic characteristics of two direct oral anticoagulants, apixaban and rivaroxaban. These studies suggest that small differences in the structural contacts of the inhibitors with the active site of coagulation proteases may yield different kinetic parameters with potentially vastly different consequences for clotting.

Chapter Four: Secondary anticoagulant functions of dabigatran, a direct thrombin inhibitor

Engagement of the active site of thrombin by dabigatran or argatroban modulates its exosite-mediated interactions with fibrin, factor Va, and glycoprotein Ib α

With the consequences of subtle structural contacts on the kinetics of factor Xa activity observed in part three, we examined the consequences of active site ligation on thrombin activity. Unlike factor Xa, thrombin possesses two binding exosites that flank the active site. These sites allow thrombin to bind to various ligands and cofactors, and allow for action in thrombosis, fibrinolysis, inflammation, and immune modulation. These three sites of binding are interlinked through a complex energetic network that permits surface binding contacts to allosterically modulate binding in the entire molecule (Fuglestad *et al.*, 2013, Huntington, 2006). This allosteric network allows for competition or cooperation of the binding of multiple ligands and may be a central regulatory mechanism of thrombin function. Although ample evidence exists for allosteric communication between the exosites, and communication to the exosite to the active site, there is weak evidence that ligation of the active site leads to allosteric changes at the exosites. These studies reveal potential new secondary consequences of active site inhibition of thrombin.

Chapter Five: Regulation of the structure-function of binding interactions of thrombin

Mutation of the active site serine to alanine enhances the allosteric capacity of thrombin

To investigate the critical residues for regulating the allosteric capabilities of thrombin, we compared the catalytically inactive Ser195 to Ala mutant variant of thrombin (S195A) with native thrombin in their abilities to coordinate inter-exosite and active-site-to-exosite allosteric responses. We and others have observed that subtle surface changes in the molecule can have long range consequences for binding elsewhere in the molecule through the allosteric regulatory network (Croy *et al.*, 2004, Fredenburgh *et al.*, 2004, Kroh *et al.*, 2007, Petrera *et al.*, 2009). Despite this, the S195A-thrombin variant has been used to make a multitude of conclusions on the structure, function, and mechanism of interaction with numerous substrates of thrombin (Huntington, 2009, Johnson *et al.*, 2006, Lechtenberg *et al.*, 2010). This head-to-head study of reveals that Ser195 is a critical residue in not only the catalytic function of thrombin, but also in restricting the molecule for active-site-to-exosite and exosite-to-exosite allostery. This study provides insight into the structural factors for allosteric regulation of thrombin function.

ACTIVITY AND REGULATION OF THE COAGULATION SYSTEM

Overview of mechanism of serine proteases in coagulation

The coagulation cascade is the series of reactions whereby circulating zymogens are enzymatically activated in sequence at the site of vessel injury and platelet aggregation. The zymogen state prevents inappropriate activation of the coagulation system, an essential aspect of hemostasis. Each chymotrypsin-like molecule undergoes molecular rearrangement when cleaved, a process that is required for enzymatic activity. This molecular rearrangement is shared amongst all chymotrypsin-like serine proteases, endowing them with a catalytic site to carry out the hydrolysis reaction (Khan and James, 1998). The activated proteases thus all share the serine protease active site characteristics and hydrolysis mechanism.

Activation of serine proteases

To prevent inappropriate activity, serine proteases are kept quiescent by both a bond in the primary structure, as well as the requirement for allosteric rearrangement. The inactive zymogen actually harbours a pre-formed catalytic triad that is mechanistically inactive due to malformation of flanking functional sites (Bode *et al.*, 1978, Fehlhammer *et al.*, 1977). During activation, proteolysis of the arginine 15 – isoleucine 16 bond generates a new mobile N-terminus at Ile16, which forms an ion-pair with the carboxylate side chain of Asp194 in the pre-formed active site (all residues based on chymotrypsin numbering (Davie and Neurath, 1955, Stubbs and Bode, 1993). This insertion organizes both (1) the “oxyanion hole” required for stabilization of the unstable tetrahedral intermediate and (2) the substrate recognition sites flanking the nucleophillic serine residue in the catalytic triad. Subsequently, the critical event for enzymatic activity was found to be the ionic bond at Asp194, as non-proteolytic enzyme activation has been shown to activate coagulation zymogens using naturally occurring sequences that mimic the Ile16 N-terminus (Friedrich *et al.*, 2003).

Catalytic site and hydrolysis mechanism

The catalytic site of the serine proteases in coagulation contains the conserved triad of serine 195, histidine 57, and aspartic acid 102 (Figure 2). Binding of the substrate in the catalytic site cleft aligns the

scissile bond with the catalytic triad (Figure 3). The natural scissile bond of factor Xa occurs following the Glu/Asp-Gly-Arg sequence in the substrate and is guided by the substrate recognition residues and the flanking subsites (S subsites; Figure 3) complementary to the cleavage site (P subsites) (Bianchini *et al.*, 2002). In the hydrolysis reaction, the hydroxyl group of Ser195 serves as the nucleophile by attacking the carbonyl carbon of the scissile bond of the substrate, while His57 accepts the Ser195 hydrogen. The Asp102 carboxyl group assists the His57 nitrogen in accepting the hydrogen from Ser195. This breaks the peptide bond in the substrate and generates the tetrahedral substrate intermediate, which is stabilized by the oxyanion hole. A water molecule is moved in place and is coordinated by His57, which accepts the free hydrogen, a step that ejects the new N-terminus or the substrate. This step generates another tetrahedral intermediate within the enzyme that helps to eject the C-terminus and regenerate Ser195 for a new reaction.

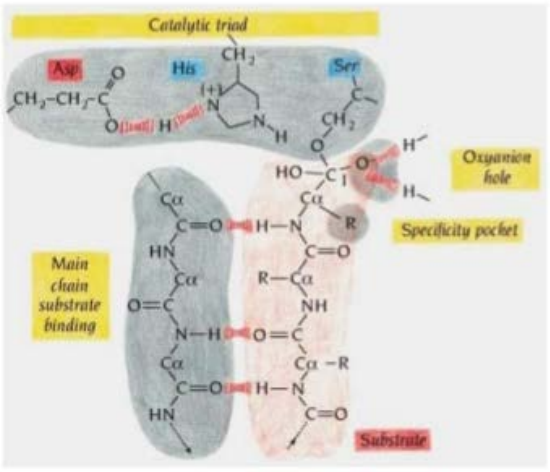

Figure 2 Serine Protease Active Site and Catalytic Triad.

In the hydrolysis reaction, the hydroxyl group of Ser195 serves as the nucleophile by attacking the carbonyl carbon of the scissile bond of the substrate, while His57 accepts the Ser195 hydrogen. The Asp102 carboxyl group assists the His57 nitrogen in accepting the hydrogen from Ser195. This breaks the peptide bond in the substrate and generates the tetrahedral substrate intermediate, which is stabilized by the oxyanion hole. A water molecule is moved in place and is coordinated by His57, which accepts the free hydrogen, a step that ejects the new N-terminus or the substrate. This step generates another tetrahedral intermediate within the enzyme that helps to eject the C-terminus and regenerate Ser195 for a new reaction.

Substrate specificity

Recognition of the substrate by the serine protease is insufficient to endow the enzymes with the narrow substrate specificity required for the coagulation cascade to proceed. Studies using small peptides that bind and are cleaved by the enzymes do not explain (1) how enzymes in the coagulation cascade distinguish among numerous other non-activation sequences on one substrate containing the scissile Glu/Asp-Gly-Arg sequence, (2) how multiple enzymes in the coagulation cascade with identical catalytic domains are able to express significantly different substrate specificity, and (3) the observations that the cleavages required for enzyme activation do not occur in the correct order or at an adequate rate in the absence of cofactors.

Specificity in the coagulation enzymes is thought to be controlled by (1) expression of unique substrate and cofactor binding exosites on the molecule flanking the active site, (2) subsequent specific binding of cofactors which in complex can express new binding sites for substrates as well as induce intermolecular allosteric modifications that promote catalysis, (3) steric restriction by loops surrounding the active site, and (4) the presence of exosites that provide additional binding interactions (Krishnaswamy, 2013) . These concepts are integral in the specific reactions at each step of the coagulation system.

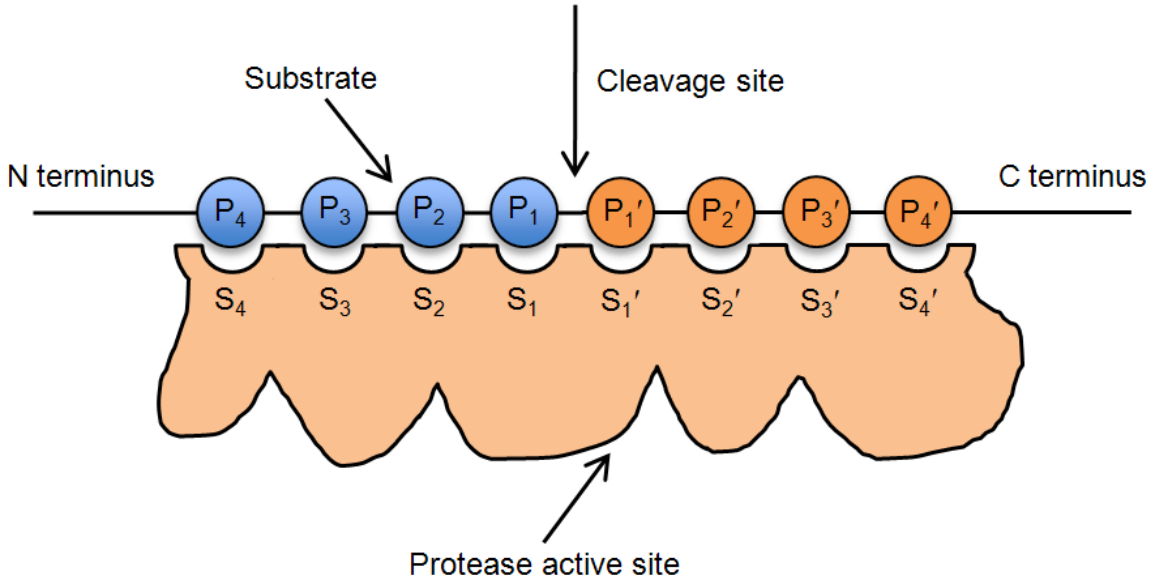

Figure 3 Nomenclature of substrate alignment in protease active site.

The nomenclature of the enzyme-substrate interaction at the active site is shown schematically. Numbering occurs away from the scissile bond, where *S* denotes the subsites of the enzyme active site on the N-terminal end, S_x' is the subsites toward the C-terminal end, and *P* denotes the complementary substrate residues.

Enzyme Complexes in Coagulation

Activated coagulation serine proteases such as FVIIa, FIXa and FXa are not able to activate their substrates at a rate that is relevant for clotting in vivo. In order to effect this, activated coagulation enzymes assemble with their respective cofactors, TF, FVIIIa, or FVa on negatively charged membrane surfaces in the presence of Ca^{2+}. Assembly of the enzyme complex increases its catalytic efficiency (k_{cat}/K_M) by several orders of magnitude. Membrane binding decreases the K_M by restricting the dimensions in which the enzyme, cofactor, and substrate component may interact, while the cofactor largely increases the k_{cat} of the enzyme by increasing its binding affinity to both the substrate and the phospholipid surface (thereby raising the local concentration) (Krishnaswamy *et al.*, 1988).

The outer layer of phospholipid cell membranes normally consists of phosphatidylcholine (PC), while the cytosolic layer consists of negatively charged phosphatidylethanolamine (PE) and phosphatidylserine (PS) residues. When platelets are activated, this asymmetrical phospholipid distribution is reversed, and negatively charged PS and PE residues are displayed on the cell surface to permit complex assembly (Krishnaswamy *et al.*, 1988). The coagulation zymogens and enzymes factors IX/IXa, X/Xa and II are able to bind to PE and PS exposed on cell surfaces through an N-terminal domain containing 9-12 γ-carboxylated glutamic acid (Gla) residues. Ca^{2+}-binding to the Gla-residues exposes hydrophobic side chains that permit binding to phospholipid surfaces (Sunnerhagen *et al.*, 1996). Whereas TF is a transmembrane protein constitutively expressed on the surface of subendothelial cells, binding of the cofactors FVIII and FV to phospholipids is facilitated by electrostatic and hydrophobic interactions of their light chains (Arai *et al.*, 1989, Lecompte *et al.*, 1994).

Extrinsic Pathway

Factor VII/VIIa

FVII (50 kDa) circulates as a single chain at a concentration of 500 ng/mL (10 nM) with a 3-6 hour half-life. About 1% of FVII is activated in plasma, and FVIIa has a half-life of 2 hours. FVIIa is composed of light and heavy chains linked by a disulfide bond. The light chain of FVIIa contains the Gla domain at the NH_2-terminus followed by a hydrophobic stack domain and two EGF-like domains. The heavy chain

contains the serine protease domain at the COOH-terminus. FVII is activated by proteolytic cleavage at the Arg^{152}-Ile^{153} bond by FIXa, FXa, FXIIa, and thrombin (Eigenbrot, 2002).

Tissue Factor

TF (40 kDa) is a membrane-bound protein expressed on subendothelial cells such as fibroblasts and smooth muscle cells. It can also be found on monocytes, macrophages, and microparticles in circulation. Soluble TF binds FVIIa and can increase its catalytic efficiency 10^4 fold, however, membrane anchoring of TF is required for optimal efficiency. TF can serve as a receptor for either FVII of FVIIa, although it binds FVIIa more tightly. TF/FVIIa can auto-catalyze the activation of TF-bound FVII by mechanisms that remain uncertain (Butenas *et al.*, 2008).

Intrinsic Pathway

Factor IX/IXa

The FIX zymogen (56 kDa) circulates in plasma at a concentration of 5 µg/mL and consists of a Gla domain at the NH_2-terminus that includes twelve Gla residues. This is followed by two EGF domains, an activation peptide, and a protease domain. A disulfide bond links EGF2 and the protease domain.

Activation of FIX is mediated by both the extrinsic tenase complex and the intrinsic pathway through FXIa. FIX activation by extrinsic tenase is defined by a K_M of 0.2 to 0.4µM and k_{cat} of 68 min^{-1}, whereas FXIa activates with a K_M of 2µM and k_{cat} of 10 min^{-1}. FXIa activates FIX in a Ca^{2+}-dependent fashion, but its kinetics are not affected by phospholipids, suggesting that this reaction occurs free in solution.

Factor VIII/VIIIa

FVIII procofactor (330 kDa) circulates in complex with von Willebrand factor at a concentration of 300 pM. Structurally, FVIII is homologous to FV, with a 200 kDa N-terminal heavy chain consisting of A1-A2 domains, a connecting region, B domain, and a 100 kDa light chain of A3-C1-C2 domains.

Thrombin activation of FVIII occurs by proteolytic cleavages at Arg^{372}, Arg^{740}, and Arg^{1689}. Initial cleavages by thrombin occur at Arg740 of the A2-B region of the FVIII heavy chain, which removes the heavily glycosylated B domain. Further cleavages include Arg372 that separate the A1 and A2 domains, and Arg1689 in the A3 domain to yield the final heterotrimer, where the A2 subunit is weakly associated with the stable A1/A3-C1-C2 dimer through electrostatic interactions. FXa also can activate FVIII by cleavage at the same sites as thrombin, however, FXa later inactivates FVIIIa by cleavage at amino acids 336 and 1721. The cleavage of Arg^{740} is not essential for maintenance of FVIIIa activity, but cleavage at Arg^{372} is essential. Once activated, FVIIIa has a half-life of less than 5 minutes (Fay and Jenkins, 2005).

Common Pathway

Factor X/Xa

FX is a 59 kDa zymogen that circulates in plasma at a concentration of 10 µg/mL (170 nM) and has a half-life of 36 h.[4] FX is secreted as a 2 chain polypeptide linked by a disulfide bond. Its Gla-domain at the NH_2-terminus of the light chain consists of 11 Gla residues. Two EGF-like domains, which prothrombin binding, follow the Gla-domain. The heavy chain contains a 52 amino acid activation peptide, as well as the catalytic triad contained in the chymotrypsin-like protease domain. Cleavage of Arg^{194}-Ile^{195} by either intrinsic or extrinsic tenase releases the activation peptide to generate FXa.

Factor V/Va

Factor V procofactor (330 kDa) circulates at a concentration of 6.6 µg/mL (20 nM) and has a half-life of 12 h.[4] The A1-A2 domains at the NH_2-terminus form the factor Va heavy chain, while the COOH-terminus A3-C1-C2 domains form the light chain. They are joined by a connecting region by the B domain.

Thrombin or FXa activates FV through three proteolytic cleavages. The first cleavage at Arg^{709} separates the A1-A2 heavy chain from the B domain. The second cleavage at Arg^{1018} is inside the B domain. Finally, a cleavage at Arg^{1545} removes the B domain from the A3 subunit of the light chain. The two chains are weakly linked by divalent cations, an association that is critical for the activity of FVa (Kalafatis and Mann, 2001).

Prothrombin

Prothrombin (72 kDa) circulates at a plasma concentration of 100 µg/mL (1.4 µM) and has a half-life of 2.5 days. Like the other vitamin K dependent proteins, it contains a Gla domain at its NH_2-terminus. Two kringle domains, F1 and F2, are followed by the A and B chains of the protease domain, which are joined by a disulfide bond.

Activation of prothrombin to thrombin depends on proteolytic cleavages at Arg^{272} and Arg^{320} by FXa. Because of the two sites of cleavage, two possible prothrombin activation intermediates exist. The pathway of activation is dependent on the assembly of prothrombinase. FXa, in the presence of fVa, cleaves II at Arg^{320} to form meizothrombin, while FXa in the absence of factor Va cleaves Arg^{271} to form Fragment 1.2 and prethrombin-2. Prethrombin-2 is an inactive precursor of thrombin, whereas meizo-thrombin is catalytically active. Cleavage at the remaining residue in each pathway yields the mature thrombin (Fig. 4).

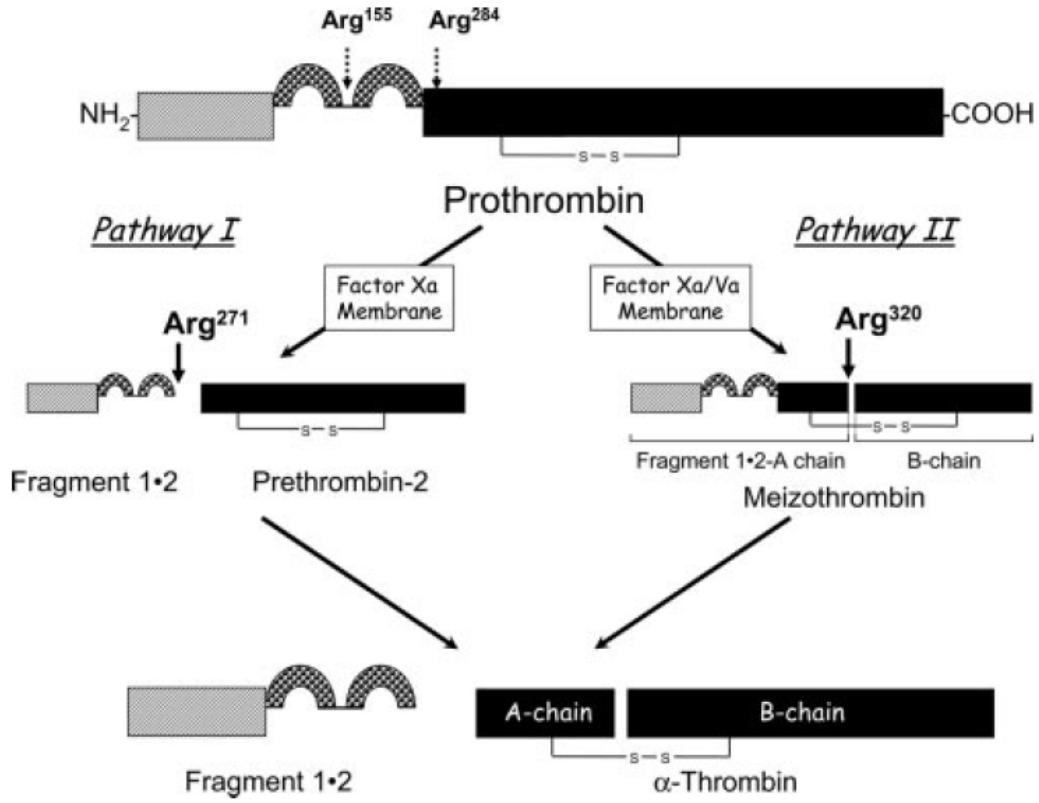

Figure 4 Pathways for the Activation of Human Prothrombin

Prothrombin contains 10 Gla residues at its NH_2-terminus (grey region), two kringle domains (checkered region), and a serine protease domain (black region). Cleavage by membrane bound FXa at Arg271 yields Fragment 1.2 and Prethrombin-2, while membrane bound FXa in the presence of fVa yields a cleavage at Arg^{320}, forming meizothrombin. Subsequent cleavage in each pathway yields Fragment 1.2 and thrombin. Once formed, thrombin truncates the A-chain without functional consequence at Arg^{284}, or negatively regulate its own activation by cleaving prothrombin at Arg^{155}. Adapted from (Bukys *et al.*, 2005).

Prothrombinase complex assembly and activation

The prothrombinase complex is the prototypical coagulation enzyme complex. Binding of factor Xa to factor Va in the presence of Ca^{2+} on the surface of negatively charged phospholipid surfaces (such as platelets) causes a 300,000-fold increase in the catalytic efficiency of the enzyme. This event is the most

critical part for the function of the coagulation cascade. The intrinsic and extrinsic pathways exist solely to generate sufficient factor Xa to meet the minimum threshold that is necessary for a massive positive feedback mechanism coordinated by prothrombinase. The threshold is achieved when sufficient thrombin is generated to feedback activate factor V and VIII.

Assembly of this complex increases the catalytic efficiency by (1) increasing the turn-over rate of the enzyme (k_{cat}), and (2) increasing the binding affinity for the substrate prothrombin (decreasing the K_m). The mechanisms by which these functional enhancements are achieved have been extensively investigated. Complex assembly is thought to both sterically and allosterically capacitate the active site of factor Xa via interaction with the membrane surface (Srivasatava *et al.,* 2014, Zhai *et al.*, 2002) and factor Va, potentiate exosites for cofactor and substrate binding, create new binding moieties for substrate binding on the macromolecular complex (Krishnaswamy, 2005, Krishnaswamy and Betz, 1997), and constrain substrate binding to favour the one pathway of thrombin generation (Boskovic and Krishnaswamy, 2000).

Thrombin

Exosites

Thrombin activity is modulated by exosites 1 and 2, positively charged domains that flank the active site. The exosites modulate thrombin specificity by providing initial binding sites for substrates, sterically hindering other interactions, and by allosterically modifying the active site.

Exosite 1 is the more versatile site, because it (a) acts as a pre-binding site for substrates such as fibrinogen, FV, FVIII, and the protease-activated receptors (PARs) on platelets, (b) redirects thrombin activity by binding the cofactor thrombomodulin (TM), and (c) mediates thrombin inhibition by hirudin and heparin cofactor II (HCII) (Crawley *et al.*, 2007, Lane *et al.*, 2005, Segers *et al.*, 2007). Exosite 2 serves a tethering role, where it functions to localize thrombin with antithrombin, fibrin, or platelets by binding to heparin, γ′-fibrin, or GpIbα, respectively. In these ways, the exosites serve to promote substrate hydrolysis and to restrict thrombin to select locations. A third way that the exosites influence thrombin is by modulating its activity.

Allostery

Numerous studies have demonstrated that thrombin is subject to allosteric regulation. Na^+ binding at a conserved binding site increases the catalytic activity of thrombin by altering the conformation of the active site (Gohara and Di Cera, 2011, Pozzi *et al.*, 2011). Ligand binding exosites 1 and 2 also have been shown to exert allosteric regulation over thrombin activity. Binding of the hirudin peptide (Fredenburgh *et al.*, 1997, Lechtenberg *et al.*, 2010, Verhamme *et al.*, 2002), platelet PAR-1 peptide (Gandhi *et al.*, 2008) and thrombomodulin (Adams *et al.*, 2009, Gasper *et al.*, 2012) to exosite 1 cause conformational and functional changes at the active site. Likewise, binding of prothrombin fragment 2 (Fredenburgh *et al.*, 1997, Kamath *et al.*, 2010), the fibrinogen γ′-chain peptide (Siebenlist *et al.*, 2005), and glycoprotein Ib (Li *et al.*, 2001) affect thrombin activity. These studies provide structural and functional evidence of connections between the active site and the exosites. Further evidence for the plasticity of thrombin is given by studies showing interexosite allostery (Fredenburgh *et al.*, 1997, Kamath *et al.*, 2010, Malovichko *et al.*, 2013, Petrera *et al.*, 2009, Sabo *et al.*, 2006, Treuheit *et al.*, 2011). Together, these studies demonstrate that the highly varied functions of thrombin may be tightly controlled by ligand-binding induced allosteric modulation. (Huntington, 2012).

Antithrombin

Antithrombin (AT; 58 kDa) circulates in plasma at about 140 ug/mL (2.5 μM) (van Boven and Lane, 1997), and is a serine protease inhibitor (serpin), that is able to irreversibly inhibit the numerous clotting enzymes, including FIXa, FXa, FVIIa, and thrombin. AT deficiency is associated with thrombophilia, and in mice causes spontaneous thrombosis and disseminated intravascular coagulation. AT knockout in mice is embryonically lethal, suggesting the developmental importance of AT in vivo (van Boven and Lane, 1997).

AT inactivates serine proteases through a “mouse trap” mechanism, which is comprised of three β-sheets, nine α-helices, and a conformationally charged 20-residue reactive center loop (RCL) (Huntington, 2013). The P1-P1′ (Arg393-Ser394) bond in the RCL acts as an exposed bait loop for serine proteases. Once cleavage of the RCL occurs to form the acyl-intermediate with the protease (Michaelis complex), the

S1 Ser195 residue of the protease becomes covalently bonded to P1 Arg393 of AT, and the catalytic triad and oxyanion hole of the protease are disrupted as the N-terminus of the RCL discharges toward the core of the serpin, flinging the protease to the opposite pole. This “mouse trap” action dissociates the NH2-terminal Ile16 from Asp194 in the activation pocket, thus disrupting the critical salt bridge responsible for the zymogen-to-protease activation of all chymotrypsin-like enzymes.

THROMBOSIS

Cardiovascular diseases arising from aberrant thrombosis and subsequent thromboembolism account for significant morbidity and mortality around the world. These include venous thromboembolism from deep vein thrombosis (DVT) and pulmonary embolism (PE), as well as arterial thromboembolism in patients with atrial fibrillation and acute coronary syndromes being the most common. Broadly, the triggers of thrombosis are conceptualized in *Virchow's triad*, (1) physical or biological damage to the vascular endothelium, (2) the intrinsic coagulability of the blood due to chemical and biologic factors, and (3) hemodynamics, in particular with non-laminar flow (turbulent flow and stasis). These conditions may be caused by various disease states, including in trauma, medical illness such as malignancy, or prolonged immobilization, respectively.

The central clinical challenges in the medical management of thrombosis revolve around designing strategies that maximize efficacy (antithrombotic activity) with an acceptable level of safety (prevention of hemorrhage). The antithrombotic strategies have focused around the use of antiplatelet and anticoagulant drugs, with many large multinational clinical trials investigating their use for various indications. Due to the pharmacologic properties of each drug and their safety profiles from phase III clinical trials, anticoagulant drugs have largely won favour over antiplatelet agents for the prevention and treatment of VTE, and ischemic stroke in patients with AF.

Pharmacologic properties and mechanisms of anticoagulants in the management of thrombosis

Warfarin

Warfarin acts as an anticoagulant by reducing the function of the vitamin K-dependent clotting proteins, factors II, VII, IX and X, thereby attenuating the extrinsic, intrinsic and common pathways of blood coagulation. Thus, warfarin disrupts the γ–carboxylation of glutamic acid residues on vitamin K-dependent proteases required for platelet membrane binding. This is achieved through reducing the available vitamin K cofactor for γ–carboxylation by γ-glutamyl carboxylase by warfarin inhibition of the VKORC1 subunit of vitamin K epoxide reductase, the enzyme responsible for regenerating oxidized

vitamin K epoxide. Because of its indirect mechanism of action, the onset and offset of action with warfarin take several days.

Heparins

Heparin is a highly sulfated glycosaminoglycan of heterogeneous chain length isolated from porcine intestinal mucosa (3 – 30 kDa, average 15 kDa). Heparin is closely related in structure to heparan sulfate on blood vessel walls and catalyzes the AT-dependent inhibition of coagulation enzymes via two mechanisms (Li *et al.*, 2004):

1. Conformational

   A unique pentasaccharide sequence, present in up to a third of commercially available heparin preparations, is responsible for binding to AT. This interaction confers a change to the tertiary structure of AT to inactivate coagulation enzymes up to 1000-fold faster. Thus, pentasaccharide binding expels the hinge region of the RCL from beta-sheet A, a critical event in allosteric activation of AT (Langdown *et al.*, 2004, Langdown *et al.*, 2009).

2. Ternary complex formation or "bridging"

   Heparin chains containing the pentasaccharide sequence and a minimum of 13 additional units (a mean molecular mass of 5400) are able to serve as a template to "bridge" the serpin to the protease. Inhibition of thrombin requires this bridging almost exclusively due to steric hindrance by the 60s and γ- (149s) insertion loops, whereas pentasaccharide induced changes in AT are sufficient for FXa, FIXa, and FVIIa inhibition since these enzymes lack the large β-barrels flanking the active site.

Heparinoids of shorter chain lengths have been exploited to avoid the unfavourable biologic and pharmacokinetic properties of unfractionated heparin. In particular, the highly negatively charged heparin chains binds to many tissues and cells, including macrophages, endothelial cells, and platelets and platelet factor 4. This non-specific binding leads to unpredictable bioavailability and the potential for adverse effects such as heparin induced thrombocytopenia. Reducing the chain length decreases the likelihood of non-specific binding and increases the bioavailability and predictability of the dose response. Low

molecular weight heparin (LMWH) is prepared by depolymerizing unfractionated heparin to a mean molecular weight of about 5000 Da; 50-75% of commercially available LMWH preparations contain 18 or fewer monosaccharide units, although the pentasaccharide sequence is found on ~one-fifth of the chains. Thus, LMWH exerts its anticoagulant effect by favouring FXa inhibition by AT rather than thrombin because of its limited bridging capacity. Synthetic pentasaccharide (PS; fondaparinux) was developed to further enhance the favourable pharmacokinetic features of LMWH. PS is the minimal heparin binding sequence for AT and accordingly exerts its anticoagulant effects through FXa.

Current reversal strategies for the reversal of heparin revolve around using highly cationic compounds such as protamine (injected as protamine sulfate salt), which neutralize the negative charge of the circulating glycosaminoglycans.

Aptamers

Aptamers are short RNA nucleotide sequences usually less than 50 subunits that fold into three-dimensional structures (Tasset *et al.*, 1997). By screening aptamers from large combinatorial libraries, sequences that bind with high affinity and specificity can be isolated for the target molecule (Eaton *et al.*, 1995, Tasset *et al.*, 1997). The benefits of aptamers as therapeutics include minimal immunogenicity and toxicity, as well as the potential for reversibility with antisense-oligonucleotides.

Aptamers have been developed for several key hemostatic proteins, including thrombin, factor IXa, von Willebrand factor, tissue factor pathway inhibitor, and type 1 plasminogen activator inhibitor (Povsic *et al.*, 2010). The thrombin specific aptamers, HD-1 and HD-22, bind to exosites I and II with high affinity (34 and 35 nM, respectively) and have been shown to perturb exosite-mediated ligand binding (Kretz *et al.*, 2006, Mosesson, 2007a). Because ligation of the thrombin exosites also leads to significant modulation in active site and inter-exosite function, bivalent binding aptamers that bind both exosites have also been investigated (Soule *et al.*, 2016).

Direct oral anticoagulants

In order to circumvent the pharmacologic limitations of warfarin and the heparins, a search for small molecules that directly bind to key coagulation proteases has been underway for thirty years. To

discover these new compounds, emphasis has been placed on understanding the binding modes and moieties of the active site of thrombin or factor Xa. Development of the DOACs has resulted in compounds with more favourable pharmacokinetics. Due to lower non-specific binding, fewer drug-drug interactions, predictable clearance, and rapid absorption, these drugs are easier to use, have more predictable anticoagulant effects, and less adverse effects compared with warfarin (Table 1).

Seminal work was based on dissecting the binding of thrombin to the key P1-P1' Glu/Asp scissile bond in fibrinogen to discover a competitive inhibitor. Indeed, use of the flanking Glu/Asp-Gly-Arg sequences attached to the chromophore para-nitroaniline (pNA) paved the way for decades of analysis of the activity of coagulation proteases through colorimetric assays (Claeson *et al.,* 1993). These compounds however, were ineffective for anticoagulation as they were quickly hydrolyzed. Future approaches focused on the structural analysis of binding with naturally occurring inhibitors such as hirudin (Figueiredo *et al.*, 2012), as well as non-peptidic structures based on benzamidine binding (Nar, 2012).

Characterization of the binding of a benzamidine moiety in the S1 pocket of the serine protease, the key substrate recognition site, provided initial clues toward the new generation of non-peptidic inhibitors. Thus, peptidomimetic inhibitors such as Nα-(2-naphthyl-sulphonyl-glycyl)-DL-p-amidinophenylalanyl-piperidine (NAPAP) significantly guided the structure-based approach to the generation of dabigatran (Brandstetter *et al.,* 1992, Hauel *et al.,* 2002). By using the NAPAP scaffold, modifications to the moieties binding S2 and S4 were used to improve affinity and selectivity for thrombin. Indeed, other inhibitors such as argatroban (MW 508.6, K_i = 39 nM (Fitzgerald and Murphy, 1996)) relying on hydrophobic binding to S2 and the aryl-binding S4 pocket were subsequently discovered (Banner and Hadvary, 1991). Interestingly, argatroban did not require an aromatic benzamidine-like moiety, and instead relied on arginine for S1 pocket interference (Claeson *et al.,* 1993).

	Warfarin	Dabigatran	Rivaroxaban	Apixaban	Edoxaban
Target	VKORC1	Thrombin	Factor Xa	Factor Xa	Factor Xa
Prodrug	No	Yes	No	No	No
Bioavailability (%)	100	7	80	60	62
Dosing	OD	BID	OD (BID)	BID	OD
Time to peak effect	4 - 5 d	1 - 3 h	2 - 4 h	1 - 2 h	1 - 2 h
Half-life (h)	40	14 - 17	7 - 11	8 - 14	5 - 11
Renal clearance as unchanged drug(%)	None	80	33	27	50
Monitoring	Yes	No	No	No	No
Interactions	Multiple	P-gp	3A4/P-gp	3A4/P-gp	P-gp

Table 1 Pharmacologic properties of the direct oral anticoagulants in comparison with warfarin.

OD, once-daily; BID, twice-daily; P-gp, P-glycoprotein; VKORC1, C1 subunit of the vitamin K epoxide reductase enzyme; 3A4, cytochrome P_{450} 3A4 isoenzyme. Adapted from (Yeh *et al.*, 2012).

Dabigatran binds to thrombin with high selectivity and in a reversible fashion with K_i of 4.5 nM (Figure 5) (Wienen *et al.*, 2007). The highly basic natural of the S1 pocket binding substrates benzamidine and arginine prevents small inhibitors like argatroban from being orally available. The creation of dabigatran circumvented this by masking the active drug as a prodrug called dabigatran etexilate (Van Ryn *et al.*, 2013). The prodrug is highly lipophilic and rapidly absorbed through the gastrointestinal tract, where it is then metabolically activated by esterases in the gut, plasma, and liver (Van Ryn *et al.*, 2013).

The first leads for factor Xa inhibitors contained isoxazoline or isoxazole derivatives, such as benzamidine, guanidine, or napthylamidine, which are thought to mimic Glu-Gly-Arg, the sequence in prothrombin that is recognized by FXa.(Lam *et al.*, 2003) The first such compound was DX-9065a (Daiichi), which contained highly basic amidine groups as non-peptidic mimetics of the Arg residue in the prothrombin recognition sequence.(Hara *et al.*, 1995) Because it exploits interactions with the S4 and S1 subsites of the active site (Figure 2), DX-9065a is specific for FXa and inhibits the enzyme with a K_i value of 46 nM.(Doolittle and Pandi, 2006) After establishing its antithrombotic activity in a variety of animal models of venous and arterial thrombosis(Hara *et al.*, 1995, Herbert *et al.*, 1996, Yamashita *et al.*, 1997), DX-9065a was compared with heparin in a small phase II dose-finding study in ACS patients (Becker *et al.*, 2006). Although the results of the study were promising, development of DX9065a was halted because the oral bioavailability of the drug in humans was only 2 to 3% because of its highly basic amidine content (Lam *et al.*, 2003). Nonetheless, DX9065a provided the groundwork for the development of orally active FXa inhibitors.

Using molecular modeling based on the x-ray crystallographic structure of FXa in complex with the first generation of FXa inhibitors, such as DX-9065a, quantitative structure-activity relationship analyses were employed to determine how modifications affected the potency of the leads (Kamata *et al.*, 1998, Maignan and Mikol, 2001, Matter *et al.*, 2005, Padmanabhan *et al.*, 1993b). These techniques revealed that the highly basic amidine moiety in the P1 position of isoxazoline derivatives participates in a two-component interaction with the carboxylate group of the Asp residue at position 189 in the floor of the S1 pocket that flanks the catalytic triad of FXa. The first component involved a Coulombic interaction between positive and negative charges (so called ion pairing), while the second reflected a bidentate

hydrogen bond(Matter *et al.*, 2005). Elimination of either these interactions increased the K_i by 1-2 orders of magnitude (Nazare *et al.*, 2005). Building on this information, the amidine group in the P1 position was replaced with non-basic moieties in an attempt to increase oral bioavailability (Quan *et al.*, 2003). Various linker or P4 elements were then added to overcome the subsequent loss in potency by maximizing interactions at the S4 site. A decade of development led to the synthesis of several orally active FXa inhibitors (Figure 5) (de Candia *et al.*, 2009). Of these, rivaroxaban (Bayer Healthcare) (Perzborn *et al.*, 2011), apixaban (Bristol-Myers Squibb) (Wong *et al.*, 2011) and edoxaban (Daiichi Sankyo) (2011) are licensed for various clinical indications. All of these agents contain non-basic moieties in the P1 position; chlorothiophene in rivaroxaban, methoxyaryl in apixaban and chloro-substituted pyridine rings in edoxaban (de Candia *et al.*, 2009).

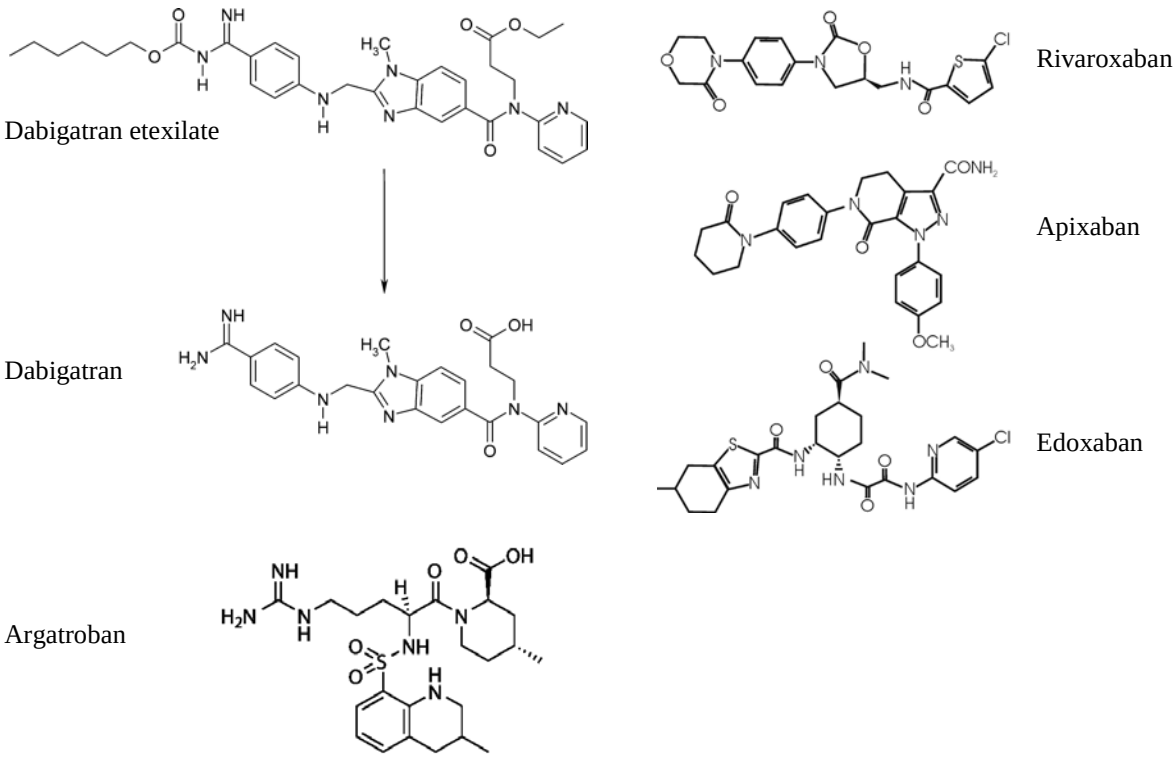

Figure 5 Structures of the direct oral anticoagulants.

The direct thrombin inhibitors are shown on the left, while the direct factor Xa inhibitors are shown on the right. The prodrug dabigatran etexilate may be processed in the liver and gut to form the active drug dabigatran by hydrolysis to expose the benzamidine moiety. Dabigatran (MW 471.5) binds to thrombin with a K_i of 4.5 nM (Wienen *et al.*, 2007), while argatroban (MW 508.6) binds with a K_i of 39 nM (Fitzgerald and Murphy, 1996). Rivaroxaban (MW 435.9), apixaban (MW 459.5), and edoxaban (MW 738.3) have Ki values of 1.0 (Perzborn *et al.*, 2005), 0.9 (Luettgen *et al.*, 2011), and 0.56 nM (Furugohri *et al.*, 2008) respectively.

CHAPTER ONE

Factors VIIa, IXa, and Xa are differentially protected from inhibition by the heparin-antithrombin complex when incorporated into their respective activation complexes†

Running head: Protection of factors VIIa, IXa, and Xa from AT inhibition

Calvin H. Yeh[1‡], Hashem Alshurafa[1‡], James C. Fredenburgh[1], Alan R. Stafford[1], Paul Y. Kim[1], Jeffrey I. Weitz[*1,2]

Departments of Medicine[1] and Biochemistry and Biomedical Sciences[2], McMaster University, and the Thrombosis and Atherosclerosis Research Institute, Hamilton, Canada

[‡] These authors contributed equally to this work

[*]Address correspondence to: Dr. Jeffrey Weitz, Thrombosis and Atherosclerosis Research Institute, 237 Barton St. E., Hamilton, Ontario L8L 2X3, Canada. Phone: 905-574-8550 Fax: (905) 575-2646 Email: weitzj@taari.ca

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FOOTNOTES

The abbreviations used are: AT, antithrombin; F, factor; PCPS, phosphatidylcholine-phosphatidylserine vesicles; SPR, surface plasmon resonance; TAP, tick anticoagulant protein; TF, tissue factor;

ABSTRACT

Although previous studies have shown that prothrombinase assembly protects factor (F) Xa from inhibition by heparin-antithrombin, it is unclear whether FIXa and FVIIa, which separately generate FXa, also are protected when incorporated into the intrinsic and extrinsic tenase complexes, respectively, in the absence or presence of FX. To address this, we compared the effects of complex assembly on the heparin-catalyzed rates of FXa, FIXa and FVIIa inhibition by antithrombin. When prothrombin or FX was present, we used hirudin or tick anticoagulant protein, respectively, to inhibit the activity of the generated enzymes. The heparin-catalyzed rates of FXa and FIXa inhibition by antithrombin were reduced 11.3- and 4.3-fold when the enzymes were incorporated into their respective activation complexes. Although addition of prothrombin produced a further 100-fold decrease in the rate of FXa inhibition, FX addition did not alter the rate of FIXa inhibition. Tissue factor increased the heparin-catalyzed rate of FVIIa inhibition by antithrombin 13.3-fold; an effect unchanged by FX addition. Our results indicate that like FXa in prothrombinase, FIXa in intrinsic tenase is protected from inhibition by heparin-antithrombin, albeit to a lesser extent, whereas FVIIa in extrinsic tenase is not. Therefore, the inadequacy of heparin in limiting thrombus growth mainly reflects the protective effect of prothrombinase complex assembly, and not assembly of the intrinsic or extrinsic tenase complexes.

Rapid clot formation at sites of vascular injury depends on explosive thrombin generation. To achieve this, coagulation factors assemble on membrane surfaces to form catalytic complexes.(Mann et al., 1988) Each complex is composed of a vitamin K-dependent serine protease and a receptor/cofactor protein, both of which are bound to the membranes of damaged cells, activated platelets, or circulating leukocytes at the site of injury.(Hoffman and Monroe, 2007) The assembly of catalytic complexes increases the activity of the constituent serine proteases by 10^5- to 10^7-fold, and membrane surface binding localizes the reactions to the site of injury.(Mann et al., 1988)

Thrombin is the ultimate enzymatic product of the coagulation system and is responsible for clotting fibrinogen, activating platelets, and modulating hemostasis through numerous feedback reactions. Thrombin is generated from prothrombin by the coordinated action of three catalytic complexes. The process is initiated by extrinsic tenase, which is formed when factor (F) VIIa, which is biologically inactive, interacts with membrane-bound tissue factor (TF) exposed at sites of vascular injury. The FVIIa-TF complex activates two substrates, FX and FIX, to form FXa and FIXa, respectively. The first wave of FXa molecules binds to the membrane surface and activates prothrombin to generate a small amount of thrombin. This thrombin then amplifies its own generation by activating platelets and converting the procofactors, FV and FVIII, into their respective cofactor forms; FVa and FVIIIa. Once the cofactors are generated, intrinsic tenase, the FIXa-FVIIIa complex, becomes the predominant activator of FX.(Hockin *et al.*, 2002) The resultant FXa then binds to FVa on the activated platelet surface to form prothrombinase, which generates the burst of thrombin required for physiological clotting.(Ahmad *et al.*, 2003, Hoffman and Monroe, 2007)

Because of its position at the convergence of extrinsic and intrinsic tenase and its critical role in thrombin generation, regulation of FXa in prothrombinase is essential to prevent excessive fibrin formation. Although several inhibitors contribute to this process, antithrombin (AT) is critical because it inhibits all of the coagulation proteases and it circulates in plasma at a concentration of about 3 μM, which is higher than the zymogen concentration of any of the coagulation proteases.(Conard *et al.*, 1983, Izaguirre *et al.*, 2003, Wiebe *et al.*, 2003) The inhibitory activity of AT is enhanced by heparan sulfate proteoglycans expressed on the endothelium.(Huntington, 2003) Like endogenous heparan sulfate, medicinal heparin also accelerates

the inhibition of coagulation enzymes by AT. The interaction of heparin with AT is mediated by a unique pentasaccharide sequence that binds AT with high affinity and induces conformational changes that accelerate the rate at which AT inhibits coagulation proteases by up to 4 orders of magnitude.(Hirsh et al., 2001) These conformational changes are sufficient to accelerate the rate of FXa inhibition by AT by at least 2 orders of magnitude. By contrast, only heparin molecules of sufficient length to simultaneously bind both AT and thrombin are capable of enhancing the rate of thrombin inhibition by AT. This explains why low-molecular-weight heparin preparations, such as enoxaparin, have greater capacity to promote FXa inhibition by AT than thrombin inhibition and why fondaparinux, a synthetic analog of the pentasaccharide sequence, enhances the rate of FXa inhibition by AT, but has no effect on the rate of thrombin inhibition.(Izaguirre *et al.*, 2003)(Herbert et al., 1998)

Although heparin-AT rapidly inhibits free forms of the proteases, thrombin bound to fibrin and FXa incorporated into the prothrombinase complex are relatively protected from inhibition.(Billy *et al.*, 1995, Brufatto *et al.*, 2003, Brufatto and Nesheim, 2001, Ellis *et al.*, 1986, Rezaie, 2001, Weitz *et al.*, 1990) This phenomenon may explain why thrombus accretion can occur despite heparin treatment in animal models, and why there is reactivation of coagulation and thrombus extension after heparin therapy is stopped in patients with venous or arterial thrombosis.(Granger *et al.*, 1995, Hull *et al.*, 1979, Lagerstedt *et al.*, 1985)

Like prothrombinase assembly, formation of intrinsic and extrinsic tenase involves numerous protein-protein and protein-membrane interactions.(Krishnaswamy *et al.*, 1993, Mann *et al.*, 1988) Although Rezaie(Rezaie, 2001) and Brufatto *et al.*(Brufatto and Nesheim, 2001) have shown that the heparin-catalyzed rate of FXa inhibition by AT is reduced over 100-fold upon prothrombinase complex assembly, it is unclear whether incorporation of FIXa or FVIIa into intrinsic and extrinsic tenase, respectively, and the addition of the macromolecular substrates afford these enzymes similar protection.(Pieters *et al.*, 1988, Rao *et al.*, 1993, Rao *et al.*, 1995, Schoen and Lindhout, 1991) Given the structural homology between the intrinsic tenase and prothrombinase complexes, and the fact that the FX activation by the intrinsic tenase complex appears to be the rate-limiting step in the propagation of coagulation,(Hockin *et al.*, 2002) we were interested in comparing the extent of protection provided by

assembly of these two complexes. Accordingly, we compared the heparin-catalyzed rates of FXa, FIXa and FVIIa inhibition by AT in the absence or presence of the components of their respective activation complexes and substrates.

To explore the contribution of heparin chain length to our findings, studies were done using heparin, enoxaparin, or fondaparinux as catalysts. Studies of FXa protection have been limited due to the inability to directly monitor the AT inhibition in the presence of prothrombin. Thus, newly-generated thrombin in prothrombinase binds to AT, and also cleaves substrates used to quantify FXa. To circumvent this problem, previous studies used mutant AT that does not bind thrombin, or catalytically inert prothrombin to avoid consumption of AT by newly-generated thrombin.(Brufatto *et al.*, 2003, Rezaie, 2001) In order to measure AT inhibition rates of prothrombinase and intrinsic and extrinsic tenase using native proteins, we used hirudin to inhibit thrombin generated in the prothrombinase assays and tick anticoagulant protein (TAP), a potent inhibitor of FXa, to block FXa generated in the intrinsic and extrinsic tenase assays.(Krishnaswamy *et al.*, 1994, Stone and Hofsteenge, 1991) In addition, we confirmed our findings using apixaban, a potent FXa inhibitor that only interacts with the FXa active site, and a catalytically inert S195A FX mutant.

EXPERIMENTAL PROCEDURES

Materials – Human FX, FIX, FXa, FIXaβ, prothrombin, and α-thrombin were purchased from Enzyme Research Laboratories (South Bend, IN), AT was from Affinity Biologicals (Ancaster, ON), while human FVa was from Haematologic Technologies Inc. (Essex Junction, VT). Human recombinant FVIII (Kogenate FS; Bayer) was generously provided by Hamilton General Hospital (Ontario, Canada). Human recombinant FVIIa (eptacog alfa activated) was acquired from Novo Nordisk. Porcine intestinal mucosa-derived heparin and polybrene (hexadimethrine bromide) were from Sigma. Enoxaparin, with a mean molecular weight of 4500, was from Sanofi-Aventis, whereas fondaparinux was from GlaxoSmithKline. Prionex, Pefachrome FVIIa (Pefa-5979), Pefafluor 10148, and Russell's viper venom FX activator (RVV-X) were purchased from Pentapharm Ltd. (Basel, Switzerland). Chromogenic substrates S-2765 and S-2238 were from Chromogenix (Milano, Italy), whereas Chromozyme-thrombin (Chz-Th) was from Hyphen

BioMed (Neuville sur Oise, France). Criterion and Mini-PROTEAN precast 4-15% polyacrylamide gradient gels and Precision Plus Protein Dual Xtra molecular weight standards were obtained from Bio-Rad. Phospholipid vesicles were prepared in a 3:1 ratio of phosphatidylcholine-phosphatidylserine (PCPS) and stored in 10% sucrose at -80°C, as described previously.(Barenholz et al., 1977) Hirudin was acquired from Bachem Bioscience, Inc. TAP and full-length human recombinant TF were generous gifts from Dr. Sriram Krishnaswamy and Dr. George Vlasuk, respectively. Apixaban was purchased from Suzhou Howsine Biological Technology Company (Suzhou, China) and dissolved in 100% dimethyl sulfoxide (DMSO) to a concentration of 10 mg/mL and stored at -80°C. Full-length recombinant S195A human factor X expressed in HEK293 cells was purchased from Cambridge Protein Works (Cambridgeshire, UK). Reactions were performed in clear or black flat-bottom 96-well polystyrene plates (Corning Inc) pre-treated with 1% Tween in HBS.

FVIII activation – Kogenate FS was reconstituted in 20 mM HEPES-NaOH, 150 mM NaCl, pH 7.4 containing 0.5% Prionex and 0.01% Tween-80 (HBST) and stored at 4ºC. FVIII concentration was based on a specific activity of 2000 U/mg and verified by monitoring thrombin-mediated FVIII cleavage by SDS-PAGE and activity in parallel with a factor Xa generation assay. Activation was achieved by incubating 3 µM FVIII with 10 nM thrombin in the presence of 5 mM $CaCl_2$.(Newell and Fay, 2009) At intervals, reactions were terminated by hirudin addition to 30 nM and 15-µl aliquots were added to an equal volume of 2 x gel sample buffer containing 0.1% SDS and 5% β-mercaptoethanol and subjected to SDS-PAGE analysis on 4-15% polyacrylamide gradient gels to assess the extent of FVIII activation. The separated protein bands were then visualized with Fast Stain (Zoion Research, Newton, MA). FVIIIa activity was quantified using a discontinuous FXa generation assay by adding 10 nM FVIIIa to a reaction mixture consisting of 1 nM FIXa, 50 µM PCPS, and 2 µM FX diluted in HBS containing 5 mM $CaCl_2$. The extent of FXa generation was determined by monitoring S-2765 hydrolysis. Under these conditions, a 3-min incubation of FVIII with thrombin resulted in maximum enhancement in the rate of FXa generation; activity that was maintained for 15 min when FVIIIa was incubated with FIXa and PCPS in HBST containing 5 mM $CaCl_2$ at 25ºC. These conditions were used in subsequent studies.

TF relipidation and characterization – TF was relipidated in small unilamellar PCPS vesicles using the method of Barenholz *et al*.(Barenholz et al., 1977) Briefly, 6 µM TF and 5 mM PCPS, diluted in HBS containing 5 mM $CaCl_2$, were subjected to sonication for 6 h at 4ºC in a FS14 Fisher Water Bath Sonicator. Soluble PCPS vesicles were purified by ultracentrifugation at 40,000 x g for 3 h at 10ºC using a SW55 rotor in a Beckman Optima L90 ultracentrifuge. The PCPS concentration was determined by quantifying the concentration of phosphate.(Ames, 1966) To quantify the functional TF concentration in the PCPS vesicles, hydrolysis of Pefachrome-VIIa by 50 nM FVIIa was monitored as it was titrated with TF-PCPS vesicles. Maximum enhancement in chromogenic activity was achieved with a nominal TF concentration of 200 nM. Based on the assumptions that (a) TF is evenly distributed between the inner and outer leaflets of the PCPS vesicle surface, thereby reducing the effective concentration by 50%,(Bach et al., 1986) and (b) TF binds FVIIa with 1:1 stoichiometry, the yield of TF in PCPS vesicles was 25%. At this TF concentration, the PCPS concentration was 66.6 µM. The TF-PCPS vesicle preparation was made to 10% sucrose and stored at -80ºC.

Characterization of the prothrombinase and extrinsic and intrinsic tenase complexes – All kinetic experiments were conducted at 25°C in HBST containing 5 mM $CaCl_2$. FX and prothrombin were activated by their respective activation complexes for determination of k_{cat}, K_M, and K_d values. The kinetic parameters for the prothrombinase complex were determined by titrating a solution containing 5 nM FVa and 20 µM PCPS with prothrombin in concentrations ranging from 0.025 to 10 µM. Reactions were initiated by addition of FXa to 50 pM in a total volume of 100 µl. At 10-s intervals, 10-µl aliquots were transferred to wells of a 96-well microplate containing an equal volume of 8 mM EDTA to terminate the reaction. After the time course, 80 µl of a 500 µM solution of Chz-Th or S-2238 was added to each well, and absorbance was then monitored at 405 nm using a SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, CA). Rates of substrate hydrolysis were converted to thrombin concentrations using a specific activity value of 17.3 mOD min^{-1} nM^{-1}, which was determined in a separate experiment. Initial rates of thrombin generation (nM/s) were then plotted versus prothrombin concentration and the data were fit to the Michaelis-Menten equation by nonlinear regression analysis using TableCurve (Jandel Scientific). The k_{cat} was calculated by

dividing V_{max} by the FXa concentration. The K_M and k_{cat} values were 157.4 ± 55.7 nM and 31.7 ± 3.0 s^{-1}, respectively; values in agreement with those reported previously.(Bukys *et al.*, 2005, Kretz *et al.*, 2010)

For studies with intrinsic tenase, solutions contained 1 nM FIXa, 10 nM FVIIIa, and 50 µM PCPS in HBS with 5 mM $CaCl_2$, whereas for studies with extrinsic tenase, solutions contained 1 nM FVIIa, 18.8 nM TF, and 25 µM PCPS in HBS with 5 mM $CaCl_2$. In both cases, reaction mixtures were titrated with FX in concentrations ranging from 0.05 to 10 µM. At 10-s intervals, 10-µl aliquots were removed and transferred to wells of a 96-well microplate containing an equal volume of 8 mM EDTA to terminate the reaction. To each well, 80-µl of 400 µM S-2765 was added and absorbance was monitored at 405 nm. Rates of substrate hydrolysis were converted to FXa concentrations using a specific activity value of 46.9 mOD min^{-1} nM^{-1}, which was determined in a separate experiment. For intrinsic tenase, the K_M and k_{cat} values were 57.9 ± 16.5 nM and 2.5 ± 0.2 s^{-1}, respectively, whereas the corresponding values for extrinsic tenase were 228.8 ± 62.3 nM and 4.4 ± 0.5 s^{-1}, respectively; values in agreement with previously published results.(Komiyama et al., 1990) Extrinsic tenase was also titrated with FIX, monitoring hydrolysis of 1 mM Pefafluor 10148 by FIXa in the presence of 40% ethylene glycol(Sturzebecher *et al.*, 1997) using a M3 fluorescence plate reader (Molecular Devices) at excitation and emission wavelengths of 342 and 440 nm, respectively, and an emission cut-off filter set at 420 nm. Rates of substrate hydrolysis were converted to FIXa concentrations using a specific activity value of 0.41 RFU s^{-1} nM^{-1}. FIX up to 4 µM was used, producing K_M and k_{cat} values of 1646.3 ± 379.2 nM and 1.54 ± 0.24 s^{-1}, respectively, analyzed as described above.

Determination of the heparin-catalyzed second order rate constants for FXa, FIXa and FVIIa inhibition by AT – Studies were performed as described by Olson *et al.* using a discontinuous assay under pseudo-first order conditions.(Olson et al., 1993) Reactions were performed at 25ºC in flat-bottom 96-well polystyrene plates pre-washed with HBS containing 1% Tween-80. AT concentrations were maintained at a minimum of a 10:1 molar ratio relative to the concentration of FXa, FIXa, or FVIIa and all reactions were conducted in HBS containing 5 mM $CaCl_2$. After pre-incubation of AT with heparin, enoxaparin, or fondaparinux, reactions were initiated by addition of an equal volume of solution containing FXa, FIXa, or FVIIa with or without their respective activation complex components. For prothrombinase, reactions were

performed in 20-µl volumes containing 2.5 nM FXa and 25 nM AT with 33 nM heparin, 2.6 µM enoxaparin, or 667 nM fondaparinux in the absence or presence of 25 nM FVa and/or 20 µM PCPS. For intrinsic tenase, reactions were performed in 50-µl volumes containing 50 nM FIXa and 0.5 to 1 µM AT with 40 nM heparin, 2.6 µM enoxaparin, or 667 nM fondaparinux in the absence or presence of 500 nM FVIIIa and/or 50 µM PCPS. For extrinsic tenase, reactions were performed in 50-µl volumes containing 25 nM FVIIa, 10 µM AT with 1.6 µM heparin, 13.3 µM enoxaparin, or 66.7 µM fondaparinux in the absence or presence of 500 nM TF and/or 50 µM PCPS, or relipidated TF vesicles containing 200 nM TF and 66.6 µM PCPS.

Reaction intervals ranged from 0.5 to 60 s and all reactions were terminated simultaneously by addition of a solution containing chromogenic or fluorogenic substrate, 8 mM EDTA and 10 mg/ml polybrene to each well. Residual FVIIa or FXa activity was quantified by monitoring hydrolysis of 1 mM Pefachrome VIIa or 400 µM S-2765, respectively, whereas residual FIXa activity was quantified by monitoring hydrolysis of 1 mM Pefafluor 10148 in the presence of 40% ethylene glycol.(Sturzebecher *et al.*, 1997) .

Pseudo-first order rate constants of inhibition (k_1) were determined by plotting ln (V/V_0) *versus* time, where V is the residual enzyme activity at a particular time interval, V_0 is the enzyme activity at time zero (in the absence of AT), and the slope is k_1. The second-order rate constants of inhibition (k_2) were determined by dividing the k_1 values by the AT concentration.

The same discontinuous assay system was used to determine the heparin-catalyzed rates of enzyme inhibition by AT in the presence of their respective macromolecular substrates; prothrombin (0.5 to 10 µM) for prothrombinase and FX (10 µM) for intrinsic and extrinsic tenase. Prothrombin or FX was first pre-incubated with the AT inhibitor solution. Because thrombin generated by prothrombinase reacts with AT and cleaves the FXa-directed chromogenic substrate, hirudin was added in a 10-fold molar excess over prothrombin to inhibit the generated thrombin. We expected complete inhibition of thrombin because hirudin inhibits thrombin with a K_i of 250 fM.(Antuch *et al.*, 1994, Knapp *et al.*, 1992) To exclude the possibility that prothrombin was consumed before assessments of AT inhibition were completed, SDS-PAGE analysis was used to assess the extent of prothrombin activation. By 1 min, 2 µM prothrombin was

completely activated by 2.5 nM FXa in the presence of 25 nM FVa and 20 μM PCPS, consistent with a k_{cat} of about 30 s^{-1}. The linearity of plots of pseudo-first order inhibition rates confirms that the effect of prothrombin remained constant during the inhibition time courses (not shown). Based on the results of SDS-PAGE analysis, 2 μM hirudin inhibited the formation of thrombin-AT complexes when 2 μM thrombin was incubated with an equimolar concentration of AT for 1 min in the presence of 33 nM heparin (Fig. 1A). Under the conditions of the experiments, hirudin prevented thrombin-mediated S-2765 hydrolysis (not shown).

Likewise, because FXa generated by intrinsic or extrinsic tenase not only reacts with AT, but also cleaves the FIXa- or FVIIa-directed fluorogenic or chromogenic substrates, we added TAP, a specific, tight-binding inhibitor of FXa (K_i of 370 pM)(Jordan et al., 1990), in a 3 – 10 -fold molar excess over FX. To exclude the possibility that FX was consumed before assessments of AT inhibition were completed, SDS-PAGE analysis was used to examine the extent of its activation. By 1 min, 2 μM FX was completely activated by 50 nM FIXa in the presence of 100 nM FVIIIa and 20 μM PCPS (not shown), or by 50 nM FVIIa in the presence of 200 nM TF, 66.6 μM PCPS and 5 mM $CaCl_2$ (not shown). When FX was present, the time courses of inhibition reactions were performed in less than 60 s; prior to complete FX activation. The linearity of plots of the pseudo-first order inhibition rates confirms that the effect of FX remained constant during the inhibition time courses (not shown). To ensure that the generated FXa did not interact with AT, experiments containing FX included TAP up to 100 μM. Based on the results of SDS-PAGE analysis, 3 μM TAP inhibited the formation of FXa-AT complexes when 2 μM FXa was incubated with an equimolar concentration of AT for 60 s in the presence of 40 nM heparin (Fig. 1B). TAP also prevented FXa-mediated Pefafluor 10148 hydrolysis (not shown). Because TAP binds to FX zymogen with low affinity (K_d > 20 μM; not shown), high concentrations of TAP decreased the rate of FX cleavage by the intrinsic tenase complex. Thus, 40 μM TAP inhibited the cleavage of 2 μM FX by 50 nM FIXa, 100 nM FVIIIa, 50 μM PCPS, 5 mM CaCl2 in HBST by up to 2-fold as evidenced by SDS-PAGE analysis (not shown). To complement this approach, apixaban up to 100 μM was used as a surrogate to TAP that does not bind to FX, but still inhibits FXa activity with high affinity (K_i = 0.25 ± 0.11 nM)(Berger et al., 2008),

as evidenced by blocking FXa-AT complex formation and FXa-mediated Pefafluor 10148 hydrolysis (not shown).

Surface plasmon resonance (SPR) – Hirudin and TAP were included in activation assays to neutralize thrombin and FXa, respectively. To investigate potential interference with activation, their respective interactions with zymogens prothrombin and FX, as well as thrombin and FXa, were quantified. Hirudin and TAP were modified with biotin using HOOK Sulfo-NHS-LC-biotin reagent (G-Biosciences, St. Louis, MO). Briefly, 0.5 mg of protein was dissolved in 0.5 ml of HBS. The biotinylation reagent was added at a 2-fold molar excess and after incubation at room temperature for 1h, 20 µl of 1M ethanolamine was added. After 20 min, samples were passed over a PD10 column (GE Life Sciences) equilibrated with HBS and 0.5 ml samples were collected. SPR was performed on a BIAcore T200 (GE Life Sciences), as reported previously.(Vu *et al.*, 2011) To investigate the binding of hirudin to thrombin or prothrombin, biotin-hirudin was immobilized on a streptavidin-coated biosensor chip at a concentration of 0.1 mg/ml in HBS containing 0.05% Tween 20 (HBS-Tw) to 200 response units. Active site blocked thrombin, prepared with Phe-Pro-Arg chloromethyl ketone as previously described,(Vu *et al.*, 2011) or prothrombin was injected into flow cells at concentrations of 15.6 nM to 1 µM or 625 nM to 40 µM, respectively. To examine the interaction of TAP with FX or FXa, biotin-TAP was immobilized on a streptavadin-coated biosensor chip at a concentration of 0.1 mg/ml in HBS-Tw to 700 response units. FXa or FX was injected into flow cells containing immobilized TAP at concentrations of 7.8 to 500 nM or 78 – 5000 nM, respectively. Where necessary, regeneration was performed using a 0.5% SDS solution for 20 s. Data were quantified by steady state analyses to determine K_d values.

Statistical analyses – All data are expressed as mean ± SD. Inhibition rates were compared using one-way ANOVA and Tukey's HSD post-hoc test. Analyses were performed using IBM SPSS software (version 17) and p-values < 0.05 were considered statistically significant.

RESULTS

Effect of FXa incorporation into prothrombinase on its heparin-catalyzed rate of inhibition by AT – The heparin-catalyzed rate of FXa inhibition by AT was determined in the absence or presence of the

intact prothrombinase complex or components thereof. With 33 nM heparin, the basal rate of FXa inhibition by AT was 100.5 ± 12.0 x 10^5 M^{-1} s^{-1} (Fig. 2A; Table 1). Similar rates were obtained in the presence of either FVa or PCPS alone. In contrast, when FXa was incorporated into the prothrombinase complex by the addition of both FVa and PCPS, there was a significant 11.3-fold decrease in the heparin-catalyzed rate of FXa inhibition by AT ($p < 0.0001$) to 8.9 ± 6.0 x 10^5 M^{-1} s^{-1}; a reduction similar in magnitude to the 10-fold lower value reported by Rezaie,(Rezaie, 2001) but more than the 2-fold reduction reported by Brufatto and colleagues.(Brufatto *et al.*, 2003)

Next, we set out to determine whether addition of prothrombin, the substrate for prothrombinase, influenced protection. Hirudin was added in these experiments to inhibit the generated thrombin, thereby preventing thrombin from competing with FXa for substrate hydrolysis and from consuming AT. In control experiments, there was complete inhibition of thrombin with the hirudin concentration used in these experiments because no thrombin-AT complexes were detected on SDS-PAGE analysis (Fig. 1A) and there was no thrombin-mediated S-2765 hydrolysis (not shown).

Addition of prothrombin in combination with either FVa or PCPS alone had no effect on the heparin-catalyzed rate of FXa inhibition by AT (Fig. 2A; Table 1). In contrast, when 2 μM prothrombin was added in combination with both FVa and PCPS (prothrombinase), the heparin-catalyzed rate of FXa inhibition was reduced 111-fold ($p < 0.0001$). We explored whether this reduction could be attributed to the presence of hirudin because hirugen, a peptide analog of the COOH-terminal of hirudin, retains weak affinity for exosite 1 in prothrombin.(Anderson *et al.*, 2000a, Kretz *et al.*, 2006) Based on SPR analysis, hirudin binds prothrombin with a K_d value of 20 μM (not shown). As determined by SDS-PAGE analysis, prothrombin activation was reduced by only 1.6-fold with 100 μM hirudin (not shown); a value much lower than the 111-fold reduction in the rate of inhibition observed with 2 μM prothrombin, indicating that hirudin did not alter the protection provided by prothrombin. These results show that prothrombin magnifies the protection of FXa afforded by prothrombinase.

The dose-dependence of prothrombin-enhanced protection was assessed. With 10 μM prothrombin, the rate was 3.1 ± 0.1 x 10^3 M^{-1} s^{-1}, which is 3,260-fold lower than that of FXa alone (Fig. 3). Thus, prothrombin reduced the heparin-catalyzed rate of FXa inhibition by AT in a concentration-

dependent and saturable fashion with an apparent K_i of 527 nM, a value comparable with the K_M for prothrombin of 157 nM. When incorporated into the prothrombinase complex, therefore, FXa is protected from inhibition by the heparin-AT complex and prothrombin augments this protection.

To determine whether the extent of protection varies depending on the length of the heparin molecules, we repeated the studies using enoxaparin or fondaparinux in place of heparin. With 667 nM fondaparinux (Fig. 2B) or 2.6 μM enoxaparin (Table 1), the rates of FXa inhibition by AT were reduced 6.3- or 5.1- fold, respectively, in the presence of both FVa and PCPS (from $7.3 \pm 0.2 \times 10^5$ to $1.2 \pm 0.4 \times 10^5$ M^{-1} s^{-1}, and from $14.3 \pm 0.7 \times 10^5$ to $2.8 \pm 1.3 \times 10^5$ M^{-1} s^{-1}, respectively). With both fondaparinux and enoxaparin, addition of prothrombin up to 10 μM further reduced the rates by 83-fold and 38-fold in a concentration-dependent and saturable fashion, with apparent $K_{i,\,app}$ values of 173 and 562 nM, respectively (Fig. 3).

Effect of FIXa incorporation into intrinsic tenase on its heparin-catalyzed rate of inhibition by AT – To determine whether FIXa incorporation into intrinsic tenase also protects the enzyme from inhibition, the heparin-catalyzed rate of FIXa inhibition by AT was determined in the absence or presence of the intact intrinsic tenase complex or components thereof. With 40 nM heparin, the basal rate of FIXa inhibition by AT was $27.1 \pm 6.9 \times 10^4$ M^{-1} s^{-1} (Fig. 4A; Table 1). Addition of either FVIIIa or PCPS had no significant effect on the rate. When FVIIIa and PCPS were added together, the rate was significantly ($p < 0.0001$) reduced by 4.3-fold to $6.3 \pm 2.0 \times 10^4$ M^{-1} s^{-1}. Because the rate of FIXa inhibition by AT varies by more than 3 orders of magnitude depending on the heparin concentration, we repeated the experiment using 667 nM heparin (not shown). When FVIIIa and PCPS were added, there was a statistically significant ($p < 0.0001$) 3.7-fold decrease in the rate of FIXa inhibition by AT; from $5.8 \pm 1.0 \times 10^6$ to $1.4 \pm 0.003 \times 10^6$ M^{-1} s^{-1}. Thus, even though the rate of FIXa inhibition by AT was 2 orders of magnitude faster with 667 nM heparin than with 40 nM heparin, the relative reduction in rate after addition of FVIIIa and PCPS was similar with both heparin concentrations. Therefore, like FXa in prothrombinase, in the presence of cofactor and PCPS, FIXa also is protected from inhibition by the heparin-AT complex, albeit to a lesser extent than FXa.

To examine the effect of FX on the extent of FIXa protection, we used TAP to inhibit the generated FXa, thereby preventing it from competing with FIXa for fluorogenic substrate hydrolysis or from interacting with AT. Under these conditions, addition of FX up to 10 μM produced no further reduction in the heparin-catalyzed rate of FIXa inhibition beyond that elicited by the combination of FVIIIa and PCPS (Fig. 4A). Numerous control experiments were performed to confirm the effectiveness of TAP. With the concentration of TAP used in these experiments, there was no evidence of FXa-mediated hydrolysis of the FIXa-directed fluorogenic substrate (not shown), nor were FXa-AT complexes detected by SDS-PAGE analysis (Fig. 1B). The interaction of TAP with FXa and FX was quantified using SPR. FXa bound immobilized TAP with high affinity (K_d of 2 nM), whereas the zymogen FX bound TAP with low affinity (K_d value >20 μM; not shown). Consistent with its low affinity for FX, 40 μM TAP produced only a 2-fold reduction in the rate of FX activation by intrinsic tenase as determined by SDS-PAGE analysis. To verify that FX was activated under the conditions of the inhibition assays, SDS-PAGE was used to visualize FXa generation. The extent of FX activation was unaffected by the presence of AT (Fig. 5), confirming that tenase was functional in the inhibition assay. These results suggest that although assembly of the intrinsic tenase complex protects FIXa from inhibition by the heparin-AT complex, unlike the situation with prothrombinase, FX does not augment the protection.

Enoxaparin and fondaparinux were used in place of heparin to explore the influence of heparin chain length on protection. When 2.6 μM enoxaparin was used instead of heparin, we observed a similar 5.4-fold reduction in the second-order rate constant of FIXa inhibition with addition of both FVIIIa and PCPS (from $20.4 \pm 2.0 \times 10^4$ to $3.8 \pm 2.6 \times 10^4$ $M^{-1} s^{-1}$) and no further reduction with FX addition to 10 μM (Table 1). In contrast, with 667 nM fondaparinux, the second-order rate constant of FIXa inhibition was similar in the absence or presence of FVIIIa and/or PCPS and FX addition to 10 μM had no effect on the rate (Fig. 4B; Table 1).

Effect of FVIIa incorporation into extrinsic tenase on its heparin-catalyzed rate of inhibition by AT – To determine whether extrinsic tenase complex assembly protects FVIIa from inhibition, the heparin-catalyzed rate of FVIIa inhibition by AT was determined in the absence or presence of TF, PCPS, or TF-PCPS vesicles. In the presence of 1.6 μM heparin, addition of TF produced a significant 13.3-fold increase

($p < 0.0001$) in the rate of FVIIa inhibition by AT (from $10.4 \pm 5.9 \times 10^1$ to $138.5 \pm 17.0 \times 10^1$ $M^{-1} s^{-1}$; Fig. 6A; Table 1). Addition of TF-PCPS vesicles yielded an 8.2-fold increase to $85.3 \pm 10.7 \times 10^1$ $M^{-1} s^{-1}$ compared with FVIIa alone, while PCPS alone had no effect. The observation that the rate of FVIIa inhibition by AT is increased in the presence of TF or TF-PCPS vesicles is consistent with the findings of previous studies.(Rao *et al.*, 1993, Rao *et al.*, 1995)

Next, we examined the influence of FX on the heparin-catalyzed rate of FVIIa inhibition by AT. Like the studies with intrinsic tenase, TAP was added to inhibit the FXa generated by extrinsic tenase. At the concentration used, TAP had no effect on the extent of FX activation by extrinsic tenase as determined by SDS-PAGE analysis (not shown). FX addition up to 10 μM had minimal effect on the heparin-catalyzed rate of FVIIa inhibition by AT in the presence of TF-PCPS vesicles (Fig. 6A).

When 13.3 μM enoxaparin was used in place of heparin, there was a 48-fold increase in rate of FVIIa inhibition by AT in the presence of TF (from $12.0 \pm 1.0 \times 10^1$ to $570.6 \pm 194.0 \times 10^1$ $M^{-1} s^{-1}$), but there were minimal changes in rates with subsequent additions of PCPS and/or FX (Table 1). With 66.7 μM fondaparinux, TF addition increased the rate of FVIIa inhibition by AT by 19-fold (from $25.3 \pm 2.9 \times 10^1$ to $495.7 \pm 51.1 \times 10^1$ $M^{-1} s^{-1}$). Addition of FX to 10 μM resulted in a 2-fold reduction in the rate to $212.9 \pm 105.4 \times 10^1$ $M^{-1} s^{-1}$ (Fig. 6B; Table 1). As observed with intrinsic tenase, AT inactivation of FVIIa in the extrinsic tenase complex was unaffected by the presence of FX and TAP (not shown).

Investigation of competition between FX and AT in the intrinsic and extrinsic tenase complexes – To verify the lack of protection from AT inhibition by intrinsic or extrinsic tenase was not an artifact due to the use of TAP, other approaches to avoid interference by generated FXa were examined.

Apixaban, a high affinity, active site inhibitor of FXa, was used in place TAP to block enzymatic activity. To verify it specificity, apixaban did not affect inhibition of FVIIa or FIXa by AT, but did block formation of FXa-AT complexes (not shown). In the presence of 100 μM apixaban, addition of FX to 10 μM did not confer protection to FIXa in intrinsic tenase from heparin-AT ($k_2 = 8.5 \pm 5.1 \times 10^4$ $M^{-1}s^{-1}$), similar to the results obtained with TAP. The rates of FIXa inhibition by AT catalyzed by enoxaparin or fondaparinux also mirrored those found when using TAP. With extrinsic tenase, addition of FX to 10 μM also did not protect FVIIa from AT inhibition catalyzed by heparin, enoxaparin, or fondaparinux (k_2 =

178.3 ± 50.5, 166.1 ± 11.9, and 618.8 ± 21.9 x 10^1 $M^{-1}s^{-1}$, respectively). Apixaban up to 3-fold molar excess of AT prevented the formation of FXa-AT complexes assayed by SDS-PAGE and did not affect the AT inhibition rate of FIXa alone or in intrinsic tenase, or FVIIa alone or in extrinsic tenase, nor did apixaban affect the rate of FX cleavage by the tenase complexes up to a concentration of 250 μM.

As an added control, we used S195A FX, a variant form that has no activity when converted to FXa, to obviate the need for TAP or apixaban.To test the observation that FX and AT do not compete for access to FVIIa in the extrinsic tenase complex, we used S195A FX as a surrogate for both FX and TAP. Cleavage of S195A FX was 2-fold slower than native FX with RVV-X, and greater than 10-fold slower with extrinsic tenase (not shown). Addition of S195A FX up to 5 μM did not substantially decrease the rate of FVIIa inhibition by AT catalyzed by heparin (116.8 ± 32.9, 175.7 ± 17.4 x 10^1 $M^{-1}s^{-1}$, respectively). Similar results were obtained in the presence of fondaparinux (not shown).

DISCUSSION

Previous studies have shown that FXa is protected from inhibition by the heparin-AT complex when it is incorporated into the prothrombinase complex.(Brufatto and Nesheim, 2001, Rezaie, 2001) Consistent with these findings, we observed an approximate 10-fold reduction in the rate of FXa inhibition by the heparin-AT complex in the presence of FVa-PCPS that was compounded 100-fold in the presence of prothrombin. Likewise, FIXa incorporated into the intrinsic tenase complex also is protected from AT inhibition, albeit to a lesser extent. However, unlike prothrombin, FX does not augment the protection afforded to FIXa by FVIIIa-PCPS. With extrinsic tenase, both the assembly of the complex and the subsequent addition of FX failed to protect FVIIa from inhibition by the heparin-AT complex. Therefore, the protection phenomenon that occurs upon activation complex assembly appears to be largely restricted to prothrombinase, which is strategically positioned in the common pathway of coagulation downstream to intrinsic and extrinsic tenase.

The mechanism by which catalytic complex assembly confers protection likely reflects both allosteric and steric elements. Capacitation of prothrombinase involves allosteric effects because complex assembly dramatically alters the kinetic parameters of prothrombin activation.(Krishnaswamy *et al.*, 1994,

Nesheim *et al.*, 1979b, Toso *et al.*, 2008) Consequently, complex assembly enables the protection of FXa from inhibition by the heparin-AT complex as evidenced by the fact that neither FVa nor PCPS alone conveys any protection. Consistent with this concept, it is hypothesized that heparin-activated AT utilizes exosites proximal to the active site of FXa to interact with the enzyme.(Izaguirre *et al.*, 2003) Allosteric changes in FXa induced by binding to FVa-PCPS may render these exosites less accessible, thereby compromising the interaction of FXa with AT. Thus, the protection phenomenon provides further evidence for cofactor-dependent alteration in the environs of the active site of FXa.

Prothrombin confers additional protection to FXa through steric inhibition, which is only evident in the presence of both FVa and PCPS. This phenomenon likely reflects competition between prothrombin and AT for FXa; a concept supported by the similarity of the K_M and K_i values for prothrombin as a substrate and as a competitor of AT. Consequently, prothrombin only attenuates FXa inhibition when the enzyme is incorporated in prothrombinase, where it is capacitated for substrate binding. The ability of prothrombin to attenuate inhibition is consistent with the findings of Rezaie, but departs from those reported by Brufatto and Nesheim.(Brufatto and Nesheim, 2001, Rezaie, 2001) The lack of protection observed by the latter group may reflect their use of a mutant form of prothrombin to report residual FXa activity.

Although the addition of prothrombin to prothrombinase reduces the heparin-catalyzed rate of FXa inhibition by three orders of magnitude, the rate remains up to two orders of magnitude higher than the uncatalyzed rate of FXa inhibition by AT. This finding makes it unlikely that prothrombinase protects FXa from inhibition by sequestering heparin from AT, which was a possibility because heparin binds to prothrombin and thrombin, and both proteins were in molar excess over heparin in our experiments. Further evidence that heparin sequestration is not responsible for the observed protection comes from the results with fondaparinux. The degree of protection with fondaparinux is similar to that with heparin despite the fact that, unlike heparin, fondaparinux only binds to AT. Therefore, the protection evoked by prothrombinase formation likely results from both allosteric modulation of FXa, which directly reduces AT binding, and steric hindrance of AT access by favoring prothrombin binding.

Because of its homology to prothrombinase and its critical role as an amplifier of coagulation, the major goal of this study was to determine whether intrinsic tenase assembly conferred protection to FIXa

from inhibition by the heparin-AT complex. Like the situation with prothrombinase, protection of FIXa was observed in the presence of FVIIIa and PCPS. The degree of protection was 5-fold, compared with 11-fold for prothrombinase, and was observed with heparin and LMWH, but not with fondaparinux. In contrast to prothrombinase, however, inclusion of FX afforded no additional protection. The attenuated protection of FIXa by complex assembly relative to FXa may be related to differences in cofactor and/or enzyme. Despite the similarities between FVIIIa and FVa, FVIIIa is less stable than FVa, which may render intrinsic tenase unstable, thereby attenuating the protective effects of complex assembly.(Fay, 2004) However, the integrity of the tenase complex was confirmed by monitoring concomitant activation of FX throughout the time course of inhibition. FIXa is distinguished from FXa by differences in its active site environment.(Hartmann *et al.*, 2009, Zogg and Brandstetter, 2009) AT is uniquely capable of accommodating these differences,(Johnson *et al.*, 2010) possibly explaining why, unlike prothrombin, FX is unable to attenuate inhibition. An alternative mechanism by which complex assembly could attenuate inhibition by AT is through FVIIIa competition for heparin binding to FIXa. Because heparin and FVIIIa share a common binding site on FIXa, access to this site is impaired when FIXa binds to FVIIIa, thereby limiting heparin catalysis.(Anderson *et al.*, 2001, Barrow *et al.*, 1994, Misenheimer *et al.*, 2007, Sheehan *et al.*, 2003) This would explain why there was attenuation with heparin and enoxaparin, but not with fondaparinux, which is too short to bridge AT to FIXa.(Wiebe *et al.*, 2003) Similarly, it is unlikely that heparin disrupted the FVIIIa-FIXa interaction because the concentration of FVIIIa exceeded that of heparin. Despite the homology between intrinsic tenase and prothrombinase, therefore, FIXa incorporation into intrinsic tenase does not protect the enzyme to the same extent as FXa incorporation into prothrombinase.

Consistent with previous accounts, TF enhances the rate of FVIIa inhibition by AT by an order of magnitude in the presence of heparin or fondaparinux.(Rao et al., 1993) Because TF also promotes FVIIa chromogenic activity, the mechanistic role of this cofactor differs from those of FVa and FVIIIa. Like the situation with intrinsic tenase, FX fails to protect FVIIa incorporated into extrinsic tenase from inhibition by the heparin-AT complex. Although it has been proposed that, like FXa and prothrombin, FIXa and FVIIa also utilize exosites on FX to enhance their interaction, it is possible that only prothrombin occupies an exosite that is shared with AT.(Baugh *et al.*, 2000, Betz and Krishnaswamy, 1998, Johnson *et al.*, 2010)

Our results suggest that complex assembly plays no role in the regulation of FVIIa inhibition by AT. In fact, the relatively slow rate of FVIIa inhibition by AT, even in the presence of heparin, raises the possibility that AT is not a physiologically relevant inhibitor of TF/FVIIa. However, FVIIa-AT complexes have been detected in plasma samples collected from patients with acute ischemic stroke, suggesting that AT is an important regulator of FVIIa.(Lawson *et al.*, 1993, Spiezia *et al.*, 2010)

In conclusion, our results confirm the protective effects of catalytic complex assembly in prothrombinase and demonstrate an attenuated effect in intrinsic tenase. These results suggest that the protection phenomenon is largely limited to the common pathway at the level of prothrombinase. It is noteworthy that a protection phenomenon also occurs with the subsequent step of coagulation because thrombin is protected from inhibition by the heparin-AT complex once it binds to fibrin or fibrin degradation products.(Becker *et al.*, 1999, Hogg and Jackson, 1989, Weitz *et al.*, 1990, Weitz *et al.*, 1998) Because these protection phenomena are localized to the final stages of coagulation, they are well positioned to regulate the extent of thrombin generation and thrombin activity. In contrast, with little or no protection of coagulation enzymes incorporated into the upstream catalytic activation complexes, the system ensures that the initiation and propagation phases of coagulation only persist under conditions of robust stimulation. Because of protection, both FXa and thrombin can remain active despite heparin therapy. Unopposed thrombin can promote thrombus growth by activating platelets and through feedback activation of FV, FVIII, and FXI. Therefore, our findings reveal an additional limitation of heparin and help to explain why thrombus extension can occur even in the face of heparin therapy.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. Michael Nesheim for helpful discussions and this paper is dedicated to his memory. The authors also would like to thank Beverly Leslie for technical assistance and Lauren Lazowski for statistical analyses.

TABLE 1: Second order rate constants for FXa, FIXa, and FVIIa inhibition by antithrombin in the absence or presence of cofactors and heparin, enoxaparin, or fondaparinux.

Prothrombinase									
				Heparin		Enoxaparin		Fondaparinux	
FXa	FVa	PCPS	FII	k_2 ($x10^5$ $M^{-1}s^{-1}$)	Fold Protection	k_2 ($x10^5$ $M^{-1}s^{-1}$)	Fold Protection	k_2 ($x10^5$ $M^{-1}s^{-1}$)	Fold Protection
+				100.5 ± 12.0	1.0	14.3 ± 0.7	1.0	7.3 ± 0.2	1.0
+			+	86.6 ± 1.0	1.2	14.2 ± 1.3	1.0	8.4 ± 0.8	0.9
+	+			107.9 ± 26.8	0.9	13.6 ± 1.0	1.1	8.1 ± 0.4	0.9
+	+		+	105.3 ± 0.1	1.0	14.3 ± 0.8	1.0	7.8 ± 0.2	0.9
+		+		116.0 ± 34.6	0.9	11.0 ± 0.6	1.3	7.4 ± 0.3	1.0
+		+	+	83.9 ± 13.2	1.2	10.2 ± 0.4	1.4	6.8 ± 0.3	1.1
+	+	+		8.9 ± 6.0	11.3	2.8 ± 1.3	5.2	1.2 ± 0.4	6.3
+	+	+	+	0.9 ± 0.6	112.0	0.6 ± 0.4	22.9	0.2 ± 0.01	41.8

Intrinsic Tenase									
				Heparin		Enoxaparin		Fondaparinux	
FIXa	FVIIIa	PCPS	FX	k_2 ($x10^4$ $M^{-1}s^{-1}$)	Fold Protection	k_2 ($x10^4$ $M^{-1}s^{-1}$)	Fold Protection	k_2 ($x10^4$ $M^{-1}s^{-1}$)	Fold Protection
+				27.1 ± 6.9	1.0	20.4 ± 2.0	1.0	30.5 ± 14.0	1.0
+	+			13.0 ± 5.9	2.1	7.5 ± 1.5	2.7	14.0 ± 3.6	2.2
+		+		17.7 ± 4.3	1.5	17.1 ± 1.1	1.2	33.9 ± 8.2	0.9
+	+	+		6.3 ± 2.0	4.3	3.8 ± 2.6	5.4	19.3 ± 8.5	1.6
+	+	+	+	10.9 ± 1.3	2.5	4.0 ± 1.3	5.1	10.4 ± 4.9	2.9

Extrinsic Tenase									
				Heparin		Enoxaparin		Fondaparinux	
FVIIa	rTF	PCPS	FX	k_2 ($x10^1$ $M^{-1}s^{-1}$)	Fold Protection	k_2 ($x10^1$ $M^{-1}s^{-1}$)	Fold Protection	k_2 ($x10^1$ $M^{-1}s^{-1}$)	Fold Protection
+				10.4 ± 5.9	-	12.0 ± 1.0	-	25.3 ± 2.9	-
+		+		7.8 ± 4.3	-	14.2 ± 5.6	-	23.7 ± 6.2	-
+	+			138.5 ± 17.0	1.0	570.6 ± 194.0	1.0	495.7 ± 51.1	1.0
+	+	+		85.3 ± 10.7	1.6	215.5 ± 51.0	2.6	403.8 ± 53.2	1.2
+	+	+	+	146.0 ± 13.5	0.9	132.3 ± 21.0	4.3	212.9 ± 105.4	2.3

Values are mean ± SD. Fold protection values are relative to FXa alone, FIXa alone, or FVIIa/rTF for prothrombinase, intrinsic tenase, and extrinsic tenase, respectively.

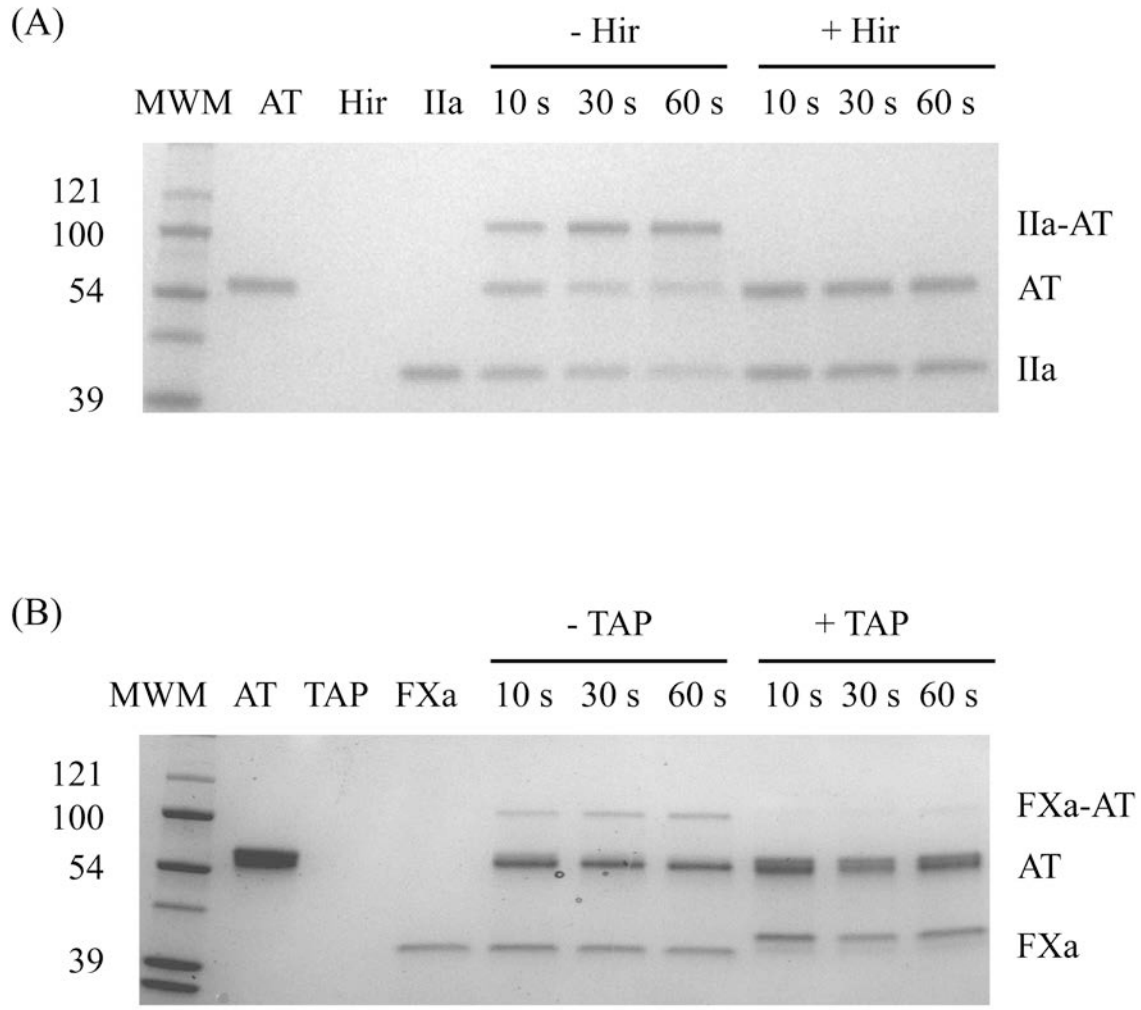
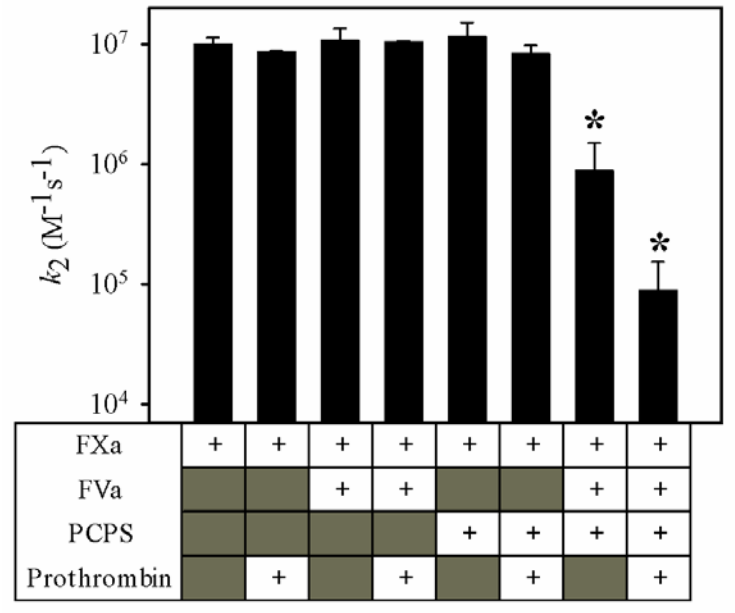


FIGURE 1. Effect of hirudin (Hir) or tick anticoagulant protein (TAP) on thrombin (IIa) or FXa inhibition by AT. IIa (2 µM) was incubated with 2 µM AT and 33 nM heparin for 60 s in the absence or presence of 3 µM Hir (panel A), whereas 2 µM FXa was incubated with 2 µM AT and 40 nM heparin for 60 s in the absence or presence of 3 µM TAP (panel B). At the times indicated, 15-µL aliquots were removed and subjected to SDS-PAGE analysis under reducing conditions to detect IIa-AT or FXa-AT complexes. Molecular weight markers (MWM) are labeled on the left (kDa) and the identities of the protein bands are provided on the right.

(A)



(B)

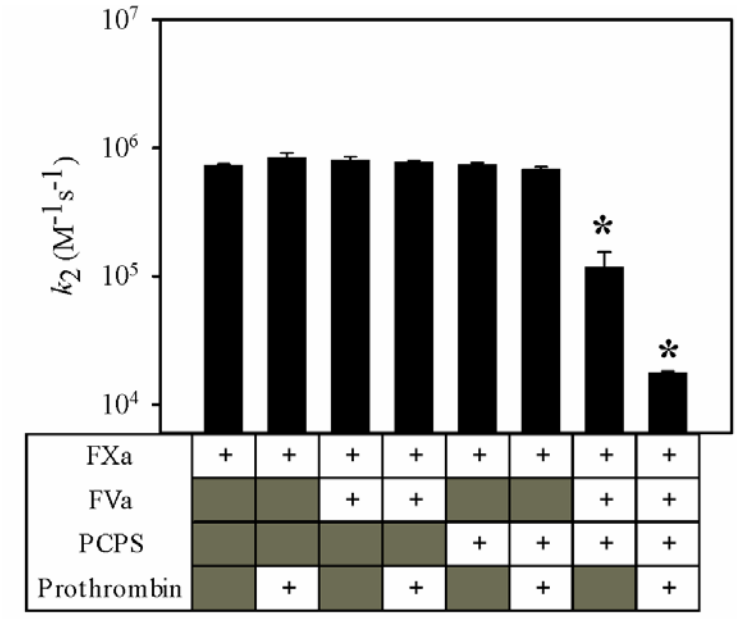


FIGURE 2. Effect of prothrombinase complex assembly on the heparin-catalyzed rate of FXa inhibition by AT. The second order rate constants (k_2) for inhibition of 2.5 nM FXa by 25 nM AT were determined in the presence of 5 mM $CaCl_2$ and 33 nM heparin (panel A) or 667 nM fondaparinux (panel B) and in the absence or presence of 25 nM FVa, 20 μM PCPS and/or 2 μM prothrombin plus 10 μM hirudin. Bars represent the mean of at least 3 experiments, while the lines above the bars reflect the SD. *Denotes $p<0.05$ compared with FXa alone.

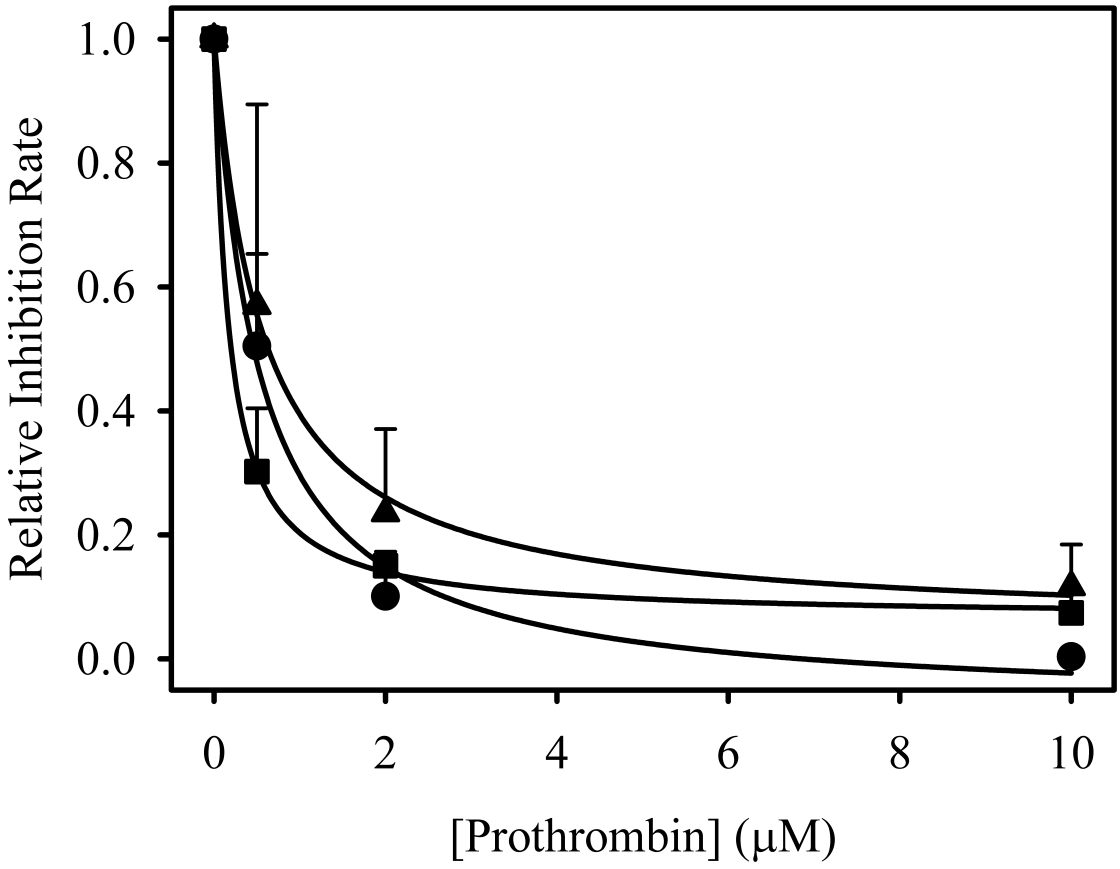
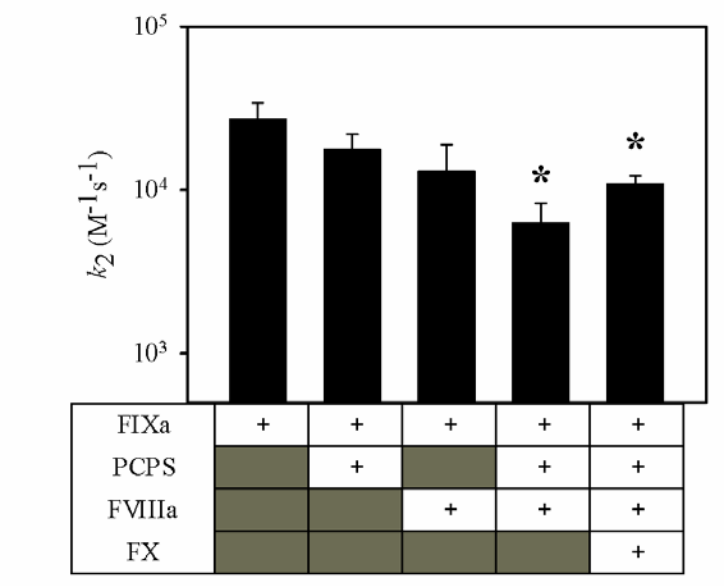


FIGURE 3. Effect of varying prothrombin concentrations on the extent to which FXa incorporated into the prothrombinase complex is protected from inhibition by heparin-, enoxaparin- or fondaparinux-AT. Second order rate constants (k_2) for inhibition of 2.5 nM FXa by 25-1000 nM AT were determined in the presence of 25 nM FVa, 20 μM PCPS, 5 mM $CaCl_2$, 0.5-10 μM prothrombin, 100 μM hirudin, and either 33 nM heparin (circles), 2.6 μM enoxaparin (triangles) or 667 nM fondaparinux (squares). Rates of FXa inhibition in the presence of prothrombin were normalized relative to those obtained in its absence. Symbols represent the mean of 3 experiments, while the bars above the symbols reflect the SD. Lines were fit by non-linear regression analysis to calculate IC_{50} values.

(A)



(B)

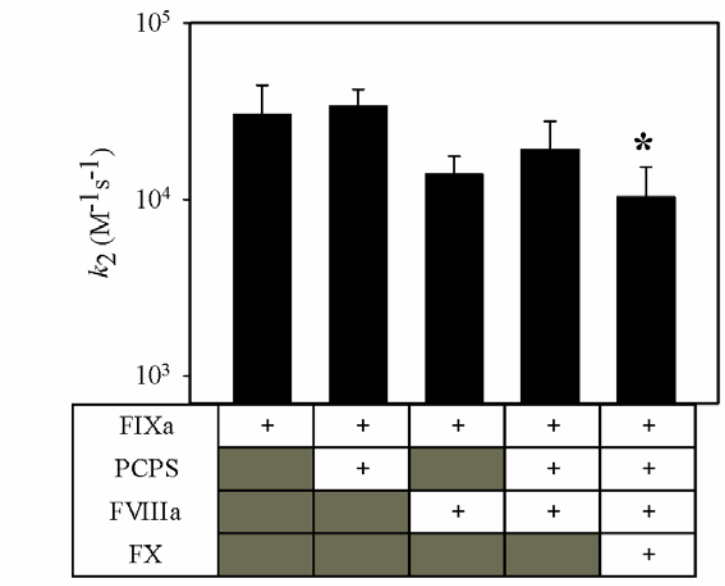


FIGURE 4. Effect of intrinsic tenase complex assembly on the heparin-catalyzed rate of FIXa inhibition by AT. The second order rate constants (k_2) for inhibition of 50 nM FIXa by 0.5-1.0 µM AT were determined in the presence of 5 mM $CaCl_2$ and 40 nM heparin (panel A) or 667 nM fondaparinux (panel B), and in the absence or presence of 500 nM FVIIIa, 50 µM PCPS and/or 10 µM FX plus 30 µM TAP. Bars represent the mean of at least 3 experiments, while the lines above the bars reflect the SD. *Denotes $p<0.05$ compared with FIXa alone.

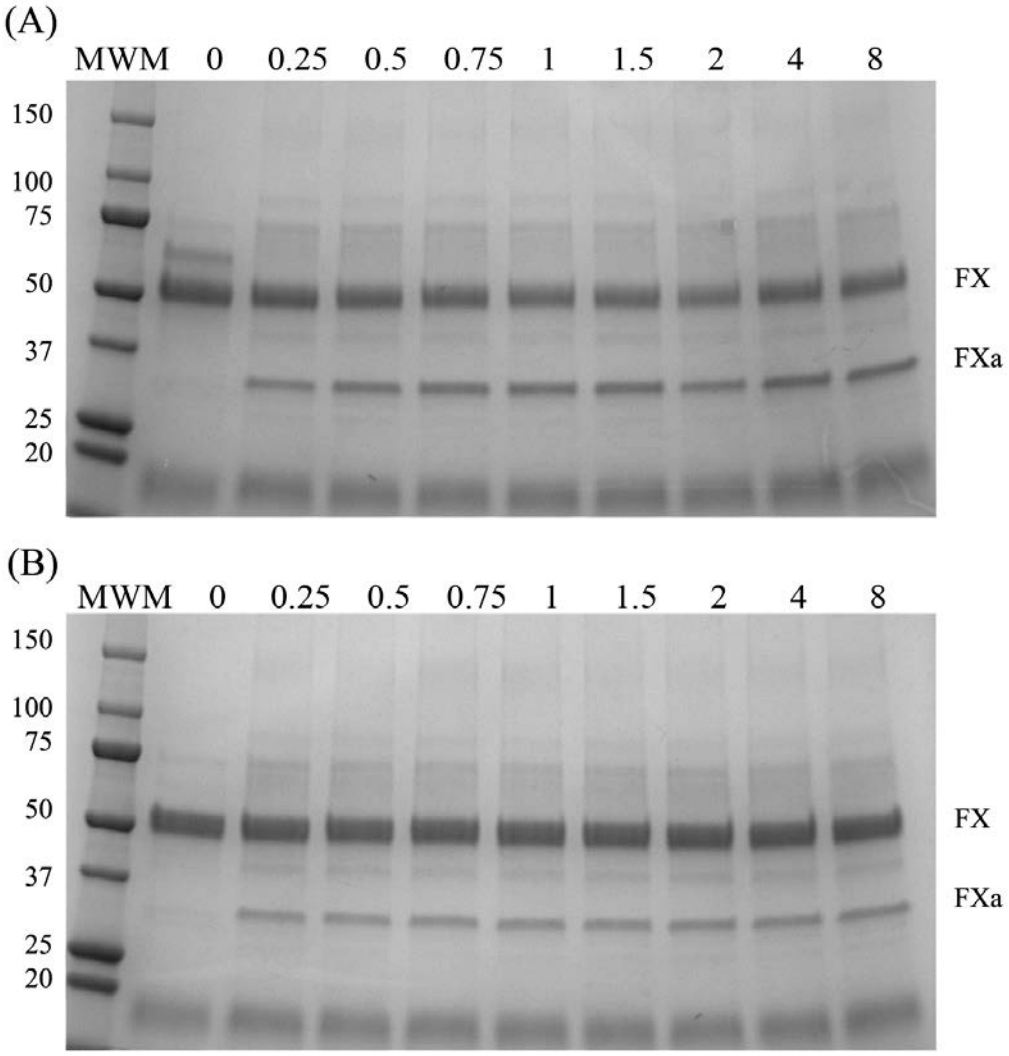
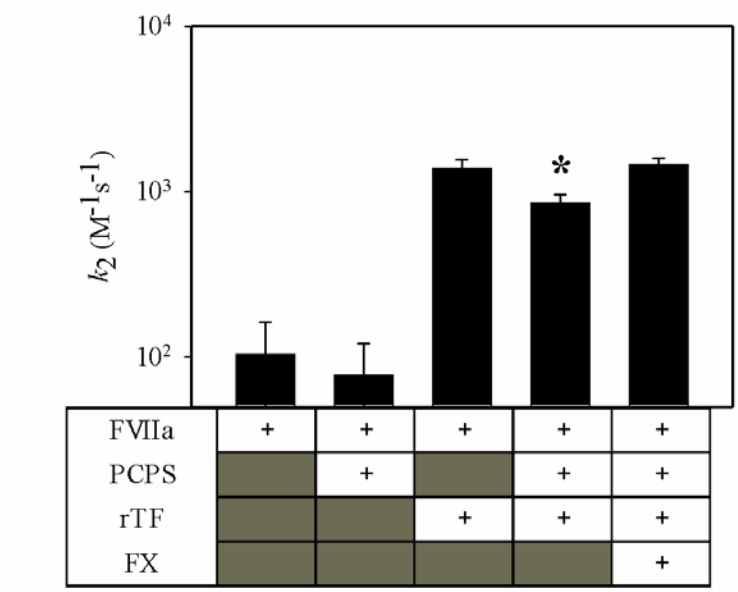


FIGURE 5. SDS-PAGE analysis of FX activation by intrinsic tenase in the absence or presence of AT. FX (3 µM) was activated by 50 nM FIXa, 200 nM FVIIIa, 5 mM $CaCl_2$ and 50 µM PCPS in the presence of 40 nM heparin and 30 µM TAP and in the absence (panel A) or presence (panel B) of 0.5 µM AT. Aliquots removed at the times indicated were subjected to SDS-PAGE analysis under reducing conditions. Molecular weight markers (MWM) are identified on the left, and FX and FXa are identified on the right.

(A)



(B)

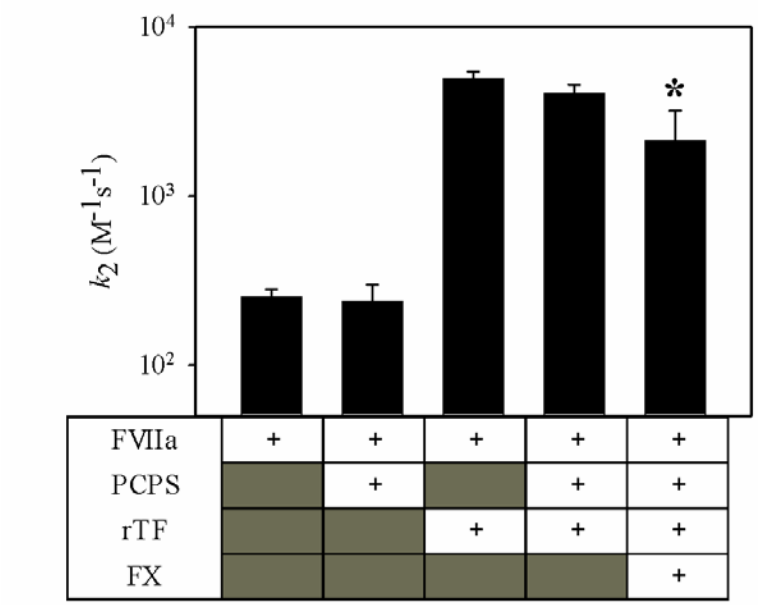
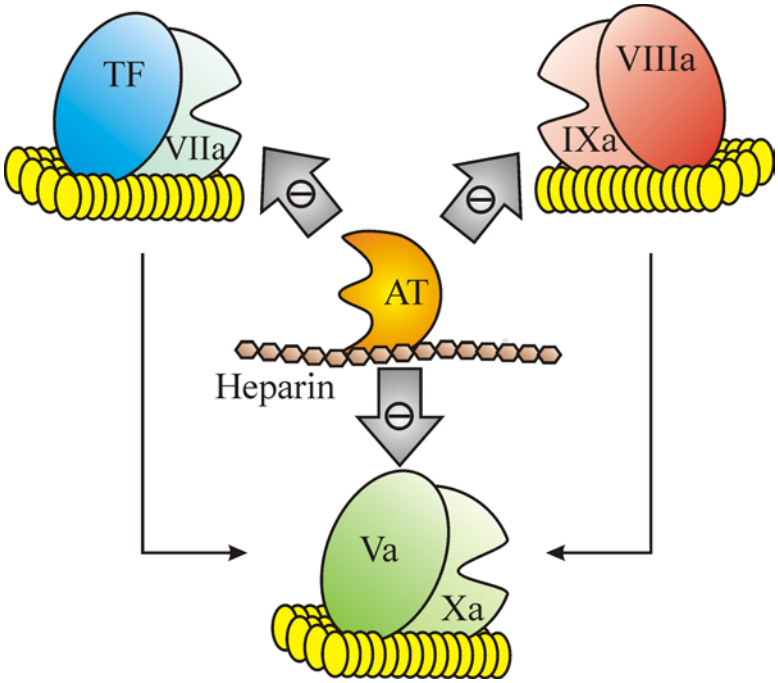


FIGURE 6: Effect of extrinsic tenase complex assembly on the heparin-catalyzed rate of FVIIa inhibition by AT. The second order rate constants (k_2) for inhibition of 25 nM FVIIa by 10 µM AT were determined in the presence of 5 mM $CaCl_2$ and 1.6 µM heparin (panel A) or 66.7 µM fondaparinux (panel B), and in the absence or presence of 500 nM TF, 50 µM PCPS, or relipidated TF vesicles containing 200 nM nominal TF and 66.6 µM PCPS and/or 10 µM FX plus 100 µM TAP. Bars represent the mean of at least 3 experiments, while the lines above the bars reflect the SD. *Denotes $p<0.05$ compared with FVIIa plus TF.



Factors VIIa, IXa, and Xa are differentially protected from inhibition by the heparin-antithrombin complex when incorporated into their respective activation complexes

Calvin H. Yeh, Hashem Alshurafa, James C. Fredenburgh, Alan R. Stafford, Paul Y. Kim, Jeffrey I. Weitz

CHAPTER TWO

Characterization of the role of prothrombin activation on thrombin exosite maturation.

Yeh CH, Stafford AR, Kretz CA, Fredenburgh JC, Weitz JI.

Departments of Medicine and Biochemistry and Biomedical Sciences, McMaster University, and the Thrombosis and Atherosclerosis Research Institute, Hamilton, Canada

INTRODUCTION

Thrombin is the key procoagulant enzyme in hemostasis because of its ability to rapidly cleave fibrinogen and the protease activated receptor PAR-1 on platelets. In addition to these pro-hemostatic functions, thrombin can also cleave a variety of other substrates such as thrombin activatable fibrinolysis inhibitor (TAFI) or protein C (PC) that confer it anti-fibrinolytic and anti-coagulant functions, respectively. How are these multiple contrasting activities regulated? Current data suggest that two anion binding exosites (ABE), ABE I and II, flanking the active site regulate the activity of thrombin.

ABE I primarily controls the various functions of thrombin by binding to substrates such as factors V and VIII, fibrinogen, and the platelet-associated protease-activated receptors (PARs). Thrombin activity may also be modulated by binding to thrombomodulin, which limits the binding of other exosite 1 ligands, but provides new binding sites for substrates such as PC or TAFI. ABE II is mainly used by ligands such as heparin cofactor II and glycoprotein 1bα to localize thrombin for reactions with antithrombin or the PARs, respectively.

In order for thrombin is act as an explosive hemostatic activator, its protease activity must be tightly controlled. One such regulation mechanism is to ensure that the zymogen prothrombin lacks expression of its catalytic triad, and exosite binding capacity. Accordingly, the ABEs of thrombin are not thought to be expressed in prothrombin because exosite-binding ligands bind to the zymogen with very low affinity. Activation of prothrombin allows for the formation of the catalytic triad, as well as unmasking of the ABEs.

Processing of prothrombin to mature α-thrombin by prothrombinase requires cleavage of the single chain molecule at two distinct cleavage sites (Fig 1). Cleavage at Arg^{271} separates the Gla-domain containing fragment 1.2 from the catalytically inactive prethrombin-2 intermediate. The inactive, but pre-formed ABE II is unmasked by cleavage at Arg^{271}, which removes steric hinderance by fragment 2. Cleavage at Arg^{320} allows for the intra-molecular conformational rearrangement in the catalytic domain of thrombin that yields the formation of the catalytic triad and subsequent activation of protease activity of the membrane bound intermediate, meizothrombin. This rearrangement is characteristic of all trypsin-like enzymes and endows their catalytic activity.

Ligands to ABE I such as thrombomodulin (TM) bind to thrombin with a high affinity (K_d = 1.6 ± 2.1 nM), but do not bind to prothrombin (K_d>> 10 µM). The leech anticoagulant Tyr^{63}-sulfated peptide fragment $Hir^{54-65}(SO_3^-)$, also known as hirugen, binds to thrombin ABE I (K_d = 25 ± 2 nM) while exhibiting markedly lower binding affinity for prothrombin (K_d= 2.6 ± 0.6 µM) (Anderson *et al.*, 2000a). Unlike exosite 2, exosite 1 is not sterically hindered, and prothrombin activation alone appears to be able to potentiate the exosite for binding. Therefore, ABE I on prothrombin is thought to be regulated by conformational changes conferred by prothrombin cleavage.

Our laboratory has previously characterized a thrombin exosite 1-binding DNA binding aptamer, HD1, and found that it bound to thrombin with similar high affinity as prothrombin (K_D = 34 ± 4.8 nM and 86 ± 8.4 nM, respectively) (Kretz et al., 2006). These findings suggest that the degree of capacitation of ABE I may be ligand specific since TM and hirugen do not bind prothrombin, a finding that potentially reflects unique conformational changes within subdomains of the ABE I region on thrombin. Characterization of the effects of prothrombin activation on ABE I ligand binding can shed light on the regulation of thrombin activity.

SPECIFIC AIMS AND RATIONALE

Specific aim #1: To explore the functional consequences of differential maturation on ABE I-dependent thrombomodulin binding.

TM is chiefly responsible for endowing thrombin with anti-coagulant and anti-fibrinolytic activity through high affinity binding to exosite 1. TM does not bind to prothrombin, making it useful for probing exosite 1 through functional and direct binding studies on prothrombin, its intermediates, and thrombin. In order to explore this concept, we tested the binding of prothrombin and its intermediates and thrombin to the ABE I binding EGF-like domains 4, 5, 6 on TM (TM456). These studies can give further insight into the exosite dependent regulatory mechanisms of prothrombin activation and subsequent thrombin activity toward its various substrates.

Specific aim #2: To explore the consequences of exosite maturation FVIII binding.

Cleavage of FVIII by thrombin is a major positive feedback signal in hemostasis. Activation of FVIII by thrombin activation is dependent on both exosites 1 and 2 (Myles *et al.*, 2002, Nogami *et al.*,

2005). Elucidation of the development of pro-cofactor binding during thrombin maturation will shed light on the regulation of thrombin mediated positive feedback.

Specific aim # 3: To explore the consequences of exosite maturation on factor Va binding.

Prothrombin binding to factor Va in the prothrombinase complex a critical event in hemostasis leading to the generation of thrombin. The mechanism of interaction of prothrombin with factor Va during activation has not been elucidated, and is potentially important for the development of new anticoagulant targets. Activation of prothrombin by prothrombinase has been shown to be dependent on exosites distant from the active site (Betz and Krishnaswamy, 1998, Bianchini *et al.*, 2005, Krishnaswamy and Betz, 1997) rather than the site of cleavage (Orcutt et al., 2002). Thus, in order for prothrombin to activate, a multi-step cleavage process must occur requiring the zymogen to repeatedly dissociation and re-associate with the enzyme (Bianchini *et al.*, 2005, Orcutt and Krishnaswamy, 2004). To examine the kinetics of these interactions, we generated prothrombin intermediates and measured their binding to factor Va using SPR.

Specific aim # 4: To explore the consequences of exosite maturation on fibrinogen and fibrin binding.

Clotting fibrinogen is the central role of thrombin, while binding to fibrin allows for localization of this action. Binding of the ɣA/ɣA fibrin(ogen) occurs exclusively through interaction of exosite 1.(Fredenburgh et al., 2004) Characterization of fibrinogen binding to prothrombin intermediates during activation provides insight into the regulation of fibrinogen clotting. Fibrin polymers can bind and sequester thrombin, an effect that increases thrombogenicity by decreasing thrombin inhibition by heparin-antithrombin complexes.(Fredenburgh et al., 2008)

MATERIALS

Human prothrombin, factor Xa, α-thrombin, protein C, and plasminogen-free fibrinogen were purchased from Enzyme Research Laboratories (South Bend, IN), while human FV was from Haematologic Technologies Inc. (Essex Junction, VT). γA/γA- and γA/γ′-fibrinogens were separated by fractionation on DEAE-Sepharose (GE Healthcare) as described previously (Fredenburgh *et al.*, 2001, Petrera *et al.*, 2009,

Pospisil *et al.*, 2003, Vu *et al.*, 2011). Chromogenic substrate S2366 was from Chromogenix (Milano, Italy), whereas Chromozyme-thrombin (Chz-Th) was from Hyphen BioMed (Neuvillesur Oise, France). Criterion and Mini-PROTEAN precast 4-15% polyacrylamide gradient gels and Precision Plus Protein Dual Xtra molecular weight standards were obtained from Bio-Rad. Phospholipid vesicles were prepared in a 3:1 ratio of phosphatidylcholine-phosphatidylserine (PCPS) and stored in 10% sucrose at -80°C, as described previously (Barenholz et al., 1977). Hirudin was acquired from Bachem Bioscience, Inc. Thrombomodulin EGF-like domains 4,5,6 (TM456) peptide was a generous gift from Dr. Tim Mather. Recombinant human factor VIII (Kogenate FS; Bayer) was generously provided by Drs. Mark Crowther and Howard Chan. FVIII concentration was based on a specific activity of 2000 U/mg and functionally verified using a FX activation assay. Reactions were performed in clear or black flat-bottom 96-well polystyrene plates (Corning Inc) pre-coated with 1% Tween in HBS.

METHODS

Generation and isolation of prothrombin intermediates

All prothrombin intermediates were prepared using modifications of published techniques (Heldebrant *et al.*, 1973, Kretz *et al.*, 2006). The progress of each reaction was monitored by subjecting time point aliquots to SDS-PAGE analysis on 4-15% acrylamide gels under reducing and non-reducing conditions. Thrombin activity was quantified in all intermediates using Chz-Th hydrolysis. Residual FPR activity was assessed by titrating 5 nM thrombin with each intermediate up to 10 μM and measuring remaining chromogenic activity.

Prethrombin 1 was prepared by incubating prothrombin at 2 μM with 20 nM thrombin in 20mM TRIS, 150 mM NaCl (TBS), pH 7.4 for 20 h at 25°C to maximize Arg^{155} cleavage and minimize cleavage at the second thrombin sensitive site, Arg^{284}. The reaction was terminated with the addition of 500 nM FPR-ck or dansyl-arginyl-(4'-ethyl)piperidine amide (DAPA). The sample was then subjected to anion-exchange chromatography on a 5 mL DEAE cartridge (BioRad).

Prethrombin 2 was prepared by digesting prethrombin 1 (2 μM) with 50 nM FXa at 37°C overnight in 25% sodium citrate in the presence 5 μM DAPA as previously described (Mann, 1976). The

reaction was terminated with 1 μM FPR-ck and 1 μM dansyl-Glu-Gly-Argchloromethyl ketone (dEGR). The sample was dialyzed against 40 mM sodium phosphate buffer pH 7 and then subjected to chromatography on a 1 mL S1 column (BioRad).

Meizothrombin and meizothrombin-des-F1 were generated by treating prothrombin and prethrombin 1, respectively, with ecarin. The reactions were conducted in the presence of 1mM FPR-ck to prevent autocatalysis. 5 mg/mL of prothrombin or 1 mg/mL prethrombin 1 was incubated with 100 μg/mL of ecarin in 20 mM HEPES, 150 mM NaCl, pH 7.4, 0.1% Tween 20 (HBST) containing 25 mM $CaCl_2$ overnight at 37°C. The samples were then subjected to chromatography on a 1 mL Q1 column (BioRad).

Chromatography was performed in a BioRad BioLogic DuoFlow chromatography system. Fractions of 1 - 2 mL were collected and A280 values were used to identify peaks for pooling. Protein concentrations were determined at 280 nm using extinction coefficients: $\varepsilon = 1.64$, $\varepsilon = 1.95$, $\varepsilon = 1.44$, and $\varepsilon = 1.64$ $mL \cdot mg^{-1} \cdot cm^{-1}$ for prethrombin 1, prethrombin 2, meizothrombin, and meizothrombin-des-F1, respectively (Rabiet et al., 1986). All of the proteins were concentrated using an Amicon Centriprep YM-10 (Beverly, MA). The integrity of each of the prothrombin intermediates was assessed by SDS PAGE. Samples of prethrombin 1 and prethrombin 2 were also activated with ecarin and subsequent thrombin activity monitored. Aliquots were stored at -80 °C.

Determination of kinetic parameters of protein C activation by thrombin-TM456

All kinetic experiments were conducted at 25°C in HBS containing 0.01% Tween-80, 0.1% Prionex and 0.1 or 2 mM $CaCl_2$. The kinetic parameters of TM456-mediated thrombin activation of PC were determined by titrating one of TM456 or PC to obtain apparent $K_{d,app}$ and k_{cat}/K_M values, respectively. To initiate PC activation, a 10 μL solution of PC was added to 10 μL of thrombin in HBST containing 0.1 or 2 mM $CaCl_2$ in a 96-well plate. The reaction was stopped at specific time intervals by addition of 20 μL hirudin to 400 nM and EDTA to 8 mM. APC activity was quantified by the addition of 60 μL S2366 to 500 μM and absorbance was measured at 405 nm using a SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, CA). The specific activity of APC for S2366 was 2.08 mOD min^{-1} nM^{-1}. The rate of APC generation was obtained by dividing the concentration by the time interval. To determine the apparent binding affinity of

TM456 to thrombin, increasing concentrations of TM456 (0 – 200 nM) were incubated with 100 nM PC prior to addition of 40 nM thrombin. $K_{d,app}$ values were 16.47 +/- 9.13 nM and 32.64 +/- 3.01 nM for 0.1 or 5 mM $CaCl_2$, respectively. Because PC binds to the thrombin-TM456 complex with low affinity (K_M = 7.1 µM) (Yang and Rezaie, 2003), we were unable to determine K_M as titration of PC produced a linear rate vs substrate response up to 4 µM.

Functional measurement of prothrombin intermediates binding to TM456

Thrombin-TM456 activation of PC was used as a functional measure of exosite 1 maturity of prothrombin intermediates. A 10-µL aliquot containing varying concentrations of prothrombin or intermediate, exosite 1 binding ligands such as Hir54-65 or HD1, or FPR-IIa was mixed with 10 µL thrombin. Final concentrations were 100 nM PC, 40 nM thrombin and 40 nM TM456 in HBST and 0.1 or 2 mM $CaCl_2$. Reactions were stopped at 30 s or 5 min by addition 20 µL of 400 nM hirudin. APC concentration and rate of generation were quantified by S2366 hydrolysis as above. The rate of APC generation was plotted against the competitor concentration and data fit by nonlinear regression using Table Curve (Jandel Scientific, San Rafael, CA) using the equation to obtain the IC50 value:

$$y = A + [(B \cdot S) / (C + S)]$$

where A is the maximal rate of APC generation, B is the minimal rate of APC generation following competition, C is the IC50, and S is the competitor concentration. K_i values were obtained from the IC50 using the equation:

$$IC50 = [1 + (TM / K_d)] K_i$$

Where TM is the TM456concentration, and K_d is the value obtained for TM456-thrombin binding.

Surface plasmon resonance (SPR) – Studies were performed using a Biacore T200 (GE Healthcare). TM456 was biotinylated with sulfo-NHS-LC-biotin. 100 RU was bound to a flow cell of a streptavidin coated biosensor chip in a Biacore T200. Proteins were passed through both the b-TM456 and a control flow cell at 100µl/min for 115 seconds in HBS containing 0.05% Tween-20 and $CaCl_2$ and the dissociation monitored for 300 seconds, followed by 1M NaCl for regeneration. γ_A-fibrinogen, γ'-fibrinogen, factor Va, and ovalbumin were immobilized to separate flow cells of a CM5 sensor chip using

the amine coupling kit from GE Healthcare. Briefly, after injecting the 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride/N-hydroxysuccinimide mixture into the flow cell at a rate of 10 µl/min for 420 s, 50 µg/ml γ_A- or γ′-fibrinogen in 10 mM acetate buffer, pH 4.5, 28 mg/ml FVa in 10 mM acetate buffer, pH 5.5, or 0.1 mg/ml in 10 mM acetate buffer, pH 5.5, was injected at a rate of 5 µl/min until 8000-9000 response units (RU) were immobilized. Flows cells were then washed with 1 M ethanolamine for 420 s, followed by 20 mM HEPES, 150 mM NaCl, pH 7.4 (HBS) containing 0.01% Tween-80 (HBS-Tw) and 5 mM $CaCl_2$. To convert the immobilized fibrinogen to fibrin, flow cells were subjected to successive 60-min injections of up to 1 µM thrombin, each followed by a wash with HBS-Tw containing 0.5 M $CaCl_2$ (Fredenburgh *et al.*, 2008, Petrera *et al.*, 2009, Vu *et al.*, 2013).

To measure the binding to fibrin and factor Va, increasing concentrations of up to 5 µM thrombin, FPR-thrombin, WTEColi-thrombin, S195A-thrombin, γ-S195A-thrombin, or R93E-S195A-thrombin were injected into the flow cells. To measure the effects of dabigatran, argatroban, and DAPA on the binding of thrombin variatns to fibrin and factor Va, 50 – 500 nM thrombins were pre-incubated in a 96 well plate with 0-5 µM inhibitor and subsequently injected into the flow cells. All SPR runs for this sensor chip were performed with HBS-Tw plus 5mM $CaCl_2$ at a flow rate of 25µl/min and an association time of 120s and dissociation time of 600s. For regeneration, a 45s wash with 0.5M $CaCl_2$ was used.

Kd values for one-site binding were determined by steady state analysis of the RU values at equilibrium (Req) for thrombin or FPR-thrombin binding to immobilized ligands using BIAEvaluation software version 3.2, as described.(Petrera et al., 2009) To calculate two-site binding of thrombin to γ′-fibrin and factor Va, Req values were plotted against input protease concentrations and analyzed by nonlinear regression analysis of a two-site binding equation using BIAEvaluation software, as described.(Fredenburgh et al., 1997) EC_{50} values for the effect of dabigatran, argatroban, or DAPA on thrombin binding was determined by steady state analysis of the Req plotted against the concentration of active site inhibitor using BIAEvaluation software as above.

PRELIMINARY RESULTS

Functional measurement of prothrombin intermediates binding to TM456

In order to determine the impact of prothrombin activation cleavages on functional capacity of exosite 1, prothrombin intermediates were prepared and used as competitors for TM456- mediated thrombin activation of PC. The rates of PC activation in the control, to which all experiments were normalized, achieved with 40 nM thrombin, 40 nM TM456, 100 nM PC were 6.84 ±1.25 x 10^{-3} and 1.17 ± 0.13 10^{-3} nM^{-1} s^{-1} for 0.1 and 2 mM $CaCl_2$, respectively (n=10). FPR-thrombin was used as a positive control for disruption of thrombin-TM456 complex via exosite 1. FPR-thrombin inhibited TM456 mediated thrombin cleavage of PC in a dose dependent manner with a K_i of 45.9 ± 24.9 nM in 0.1 mM calcium (Fig 2A). This value is comparable to the K_d of the thrombin-TM456 complex of 16.47 +/- 9.13 nM, which validates that exosite 1 containing species of FPR-thrombin are capable of directly competing with active thrombin. HD1 and hirugen were used as indirect controls for the disruption of exosite 1 binding, and produced results comparable to FPR-thrombin. Since prothrombin does not express an exosite 1 region that is capable of binding TM456, addition of prothrombin up to 10 μM did not affect the rate of APC generation by thrombin-TM456.

The zymogen prethrombin 1, generated by removal of fragment 1 by cleavage at Arg^{155}, inhibited APC generation in a dose dependent manner, with a K_i of 498 ± 188.5 nM in 0.1 mM $CaCl_2$ (Fig. 1A). This result is consistent with previous accounts of exosite 1 formation upon removal of F1, using hirugen as an exosite 1 ligand (Anderson and Bock, 2003). Further removal of F2 from prethrombin 1 to yield prothrombin 2 did not substantially impact TM binding to exosite 1 (K_i 346.1 ± 90.3 nM) in functional assays. Cleavage of prothrombin at Arg^{320} to generate FPR-meizothrombin confers the largest effect on capacitation of exosite 1 for TM456 binding, with a K_i value of 64.2 ± 5.7 nM, which is indistinguishable from FPR-thrombin in this assay. Further processing of meizothrombin at Arg^{155} to form meizothrombin-des-F1 produces little change in the inhibition constant (Fig 1A). Due to the large effect of calcium concentration on the kinetics of PC cleavage by thrombin-TM456, these experiments were repeated with 2 mM $CaCl_2$ with no appreciable differences in inhibition constants (Fig 1B; Table 1).

Direct binding studies of prothrombin intermediates to TM456

The affinity of prothrombin and intermediates for binding to TM456 was determined by surface plasmon resonance (SPR). TM456 was biotinylated and immobilized on a streptavidin sensor chip. Increasing concentrations of each prothrombin intermediate were passed over the surface of the chip for kinetic or stead-state analysis (Fig. 2). From these data, dissociation constants were determined for each prothrombin intermediate (Table 1). Because engagement of the active site of thrombin by FPR-ck is known to modulate exosite 1 activity (Kamath et al., 2010), active thrombin was also examined. FPR-thrombin and thrombin had comparable K_d values of 0.79 ± 0.59 and 1.6 ± 2.1 nM, respectively, suggesting that use of FPR-ck does not affect exosite 1 dependent binding to TM456.

The SPR data and the competition data provide different results concerning the roles of F1 and F2 and cleavage at Arg^{320} on the development of exosite 1. Our SPR data are in line with previous reports that removal of F1 significantly increases the affinity of exosite 1 ligand binding to TM. However, our SPR data also show that F2 may also regulate exosite 1 development as the K_d decreases from 486 ± 34.7 nM in prethrombin 1 to 87.3 ± 28.0 nM for prethrombin 2 (Table 1). An increase of exosite 1 binding capacity with removal of F1 is also seen between FPR-meizothrombin and FPR-meizothrombin-des-F1, as the K_d decreases from 44.4 ± 18.9 nM to 5.9 ± 1.3 nM with removal of F1, with a further 5-fold increase in affinity as F2 is removed in FPR-thrombin (Table 1).

Cleavage of Arg^{320} to yield FPR-meizothrombin was previously thought to be sufficient for capacitation of exosite 1 for binding.(Kroh et al., 2007) Our data show that sequential capacitation of exosite 1 through removal of F1 and F2 is possible following meizothrombin generation. This difference was not seen in our functional data, where prethrombin 1 and prethrombin 2 had similar K_i values (498 ± 188.5 and 346.1 ± 90.3 nM, respectively), as did FPR-meizothrombin and FPR-meizothrombin-des-F1.

The differences in F2 removal may not have been observed with functional studies because of a lack of sensitivity, as the lowest K_d achieved was in the range of 40 nM as compared with ~1 nM with SPR (Table 1). In all previous accounts of exosite 1 capacitation, the lowest K_d achieved was 35 ± 6 nM with direct titration of FPR-thrombin with hirugen.

To verify these results and to examine the functional consequences of sequential exosite capacitation in prothrombin activation the multiple ligands of prothrombin, we repeated SPR experiments using immbolized factors Va and VIII, and fibrin(ogen). These results are summarized in Table 2.

Determination of the effect of active site occupancy on function of exosite 1 in meizothrombin

Previous studies have shown that occupancy of the active site of mature thrombin enhances the expression of thrombin's proteinase-like characteristics, including exosite 1 ligand binding (Croy *et al.*, 2004, Kamath *et al.*, 2010). Because meizothrombin is proposed to exist in an equilibrium of both zymogen-like and proteinase-like forms (Bradford and Krishnaswamy, 2012a), the use of an active site inhibitor such as FPR-ck may affect the degree to which exosite 1 binding is capacitated. Does the use of FPR-ck in our assays cause allosteric changes that lock thrombin or meizothrombin in a protease rather than zymogen-like state, thereby affecting our measurement of TM456 binding at exosite 1? Employing recombinant prothrombin S195A and thrombin S195A (generous gifts from Dr. James Huntington) which are catalytically inactive and therefore obviate the need for PPA-ck, we compared the S195A with the FPR variant of meizothrombin for binding to HD1 and TM (not shown) using SPR. There were no discernable differences in the affinity or binding profile of the variants of meizothrombin binding to exosite 1 ligands. Therefore, despite previous reports of meizothrombin’s zymogen-like character and associated decreased capacitation of exosite 1, our results suggest that meizothrombin has nearly full capacitation of exosite 1 for TM and HD1 binding.

	TM456 Kd (nM)	Inhibition of PC Activation - Ki (nM)	
		100µM calcium	2mM calcium
HD1	ND	34.4 ± 1.5	41.4 ± 6.1
Hirugen sulfate	ND	137.3 ± 68.5	133.8 ± 10.6
FPR-Thrombin	0.79 ± 0.59	45.9 ± 24.9	65.6 ± 15.3
Prothrombin	>> 10 000	NB	NB
Prethrombin 1	486 ± 34.7	498 ± 188.5	411.6 ± 125.2
Prethrombin 2	87.3 ± 28.0	346.1 ± 90.3	733.2 ± 428.2
FPR-Meizothrombin	44.4 ± 18.9	64.2 ± 5.7	176 ± 229.3
FPR-Meizothrombin-des F1	5.9 ± 1.3	52.4 ± 12.8	42.9 ± 9.8
Thrombin	1.6 ± 2.1	ND	ND

Table 1. Summary of dissociation constants and inhibition constants as determined by surface plasmon resonance and APC generation by thrombin-TM456, respectively. NB, no binding.

	Exosite 1				Exosite 2	Bivalent			
	HD1	**TM**	**Fn1**	**Fgn1**	**ckHD22**	**Fn2**	**Fgn2**	**FVa**	**FVIII**
Prothrombin	10756.7 ± 458.8	20823.3 ± 2239.8	NB	NB	NB	NB	NB	Weak	NB
Prethrombin 1	2227.3 ± 119.3	420.3 ± 75.4	NB	NB	NB	NB	NB	11390 ± 6800	NB
Prethrombin 2	187.9 ± 3.0	62.8 ± 32.2	4044 ± 67.0	1802.7 ± 176.4	25.4 ± 0.4	2546 ± 10	1176.3 ± 93.0	1458 ± 69	1211.4 ± 401.6
FPR-Meizothrombin	164.1 ± 17.7	29.9 ± 3.8	4590 ± 120	NB	NB	4903 ± 130	NB	5321 ± 540	NB
FPR-Meizothrombin-dF1	77.8 ± 2.5	6.0 ± 0.8	3505 ± 510	155.2 ± 40.7	123.3 ± 8.0	2011 ± 24	1004.8 ± 121.6	1886 ± 190	629.8 ± 91.2
FPR-α-thrombin	45.8 ± 1.04	7.5 ± 0.2	2238.7 ± 61.6	1830 ± 651.7	16.1 ± 4.8	1161 ± 33	1882 ± 247.5	943.2 ± 82	163.7 ± 102.3
α-thrombin	42.8 ± 1.7	5.7 ± 0.3	3323 ± 29.5	ND	39.1 ± 13.3	1783 ± 65	ND	11735	ND

Table 2. Summary of dissociation constants (KD) of prothrombin intermediates for exosite 1 and 2 and bivalent ligands as determined by surface plasmon resonance

FIGURES

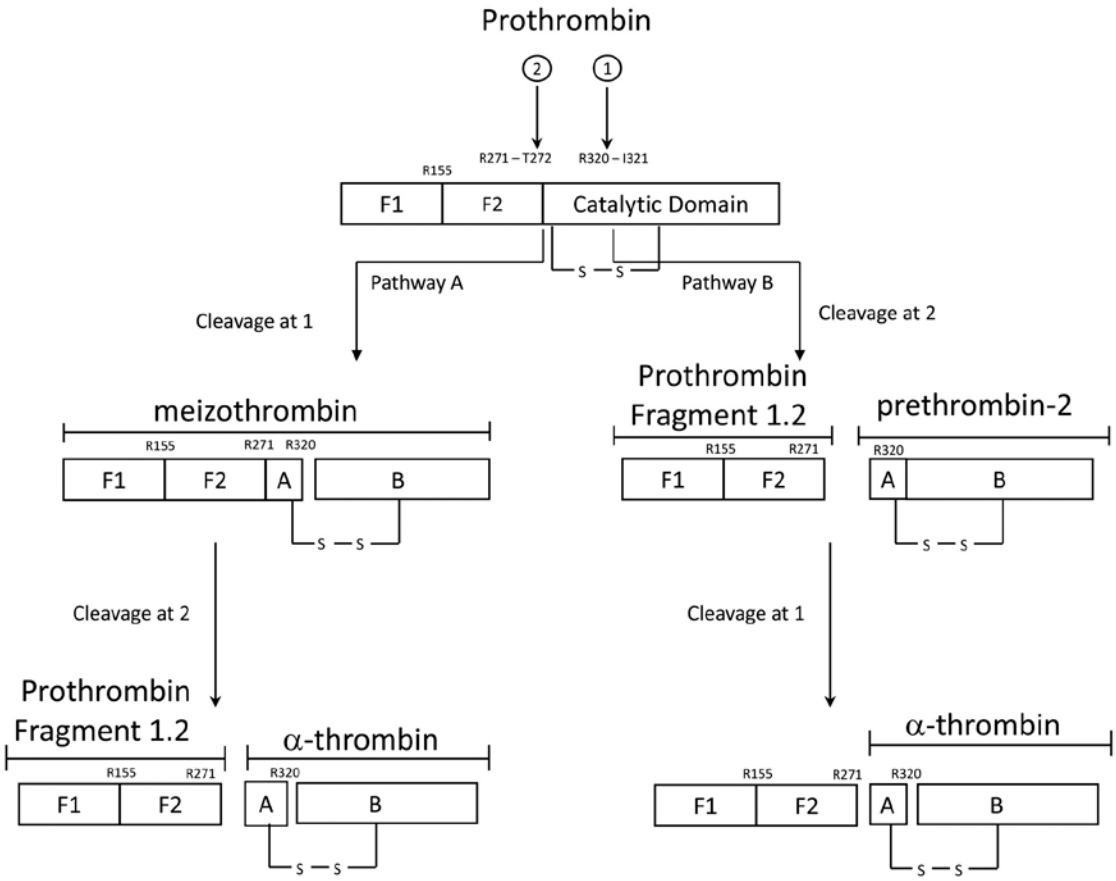


Figure 1. Potential prothrombin pathways by factor Xa. Prothrombin may be cleaved preferentially by factor Xa alone at Arg271 or by factor Xa in the prothrombinase complex on the surface of PCPS vesicles at Arg320.

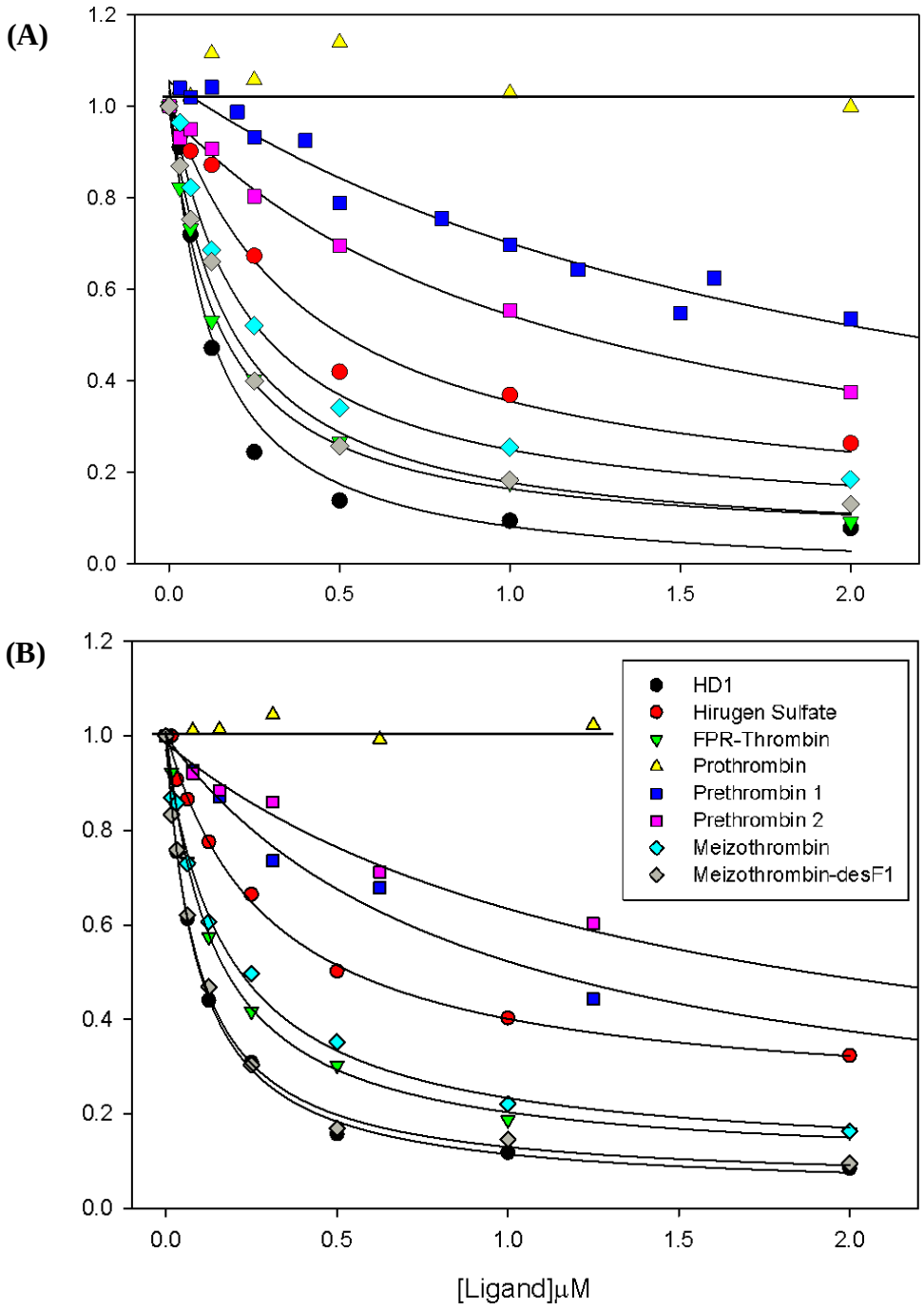


Figure 2. Effect of various prothrombin intermediates and exosite 1 ligands on the rate of APC generation by 40 nM thrombin, 40 nM TM456, 100 nM PC, in 20 mM HEPES 150 mM NaCl + 0.1% Tween with (A) 0.1 or (B) 5 mM $CaCl_2$.

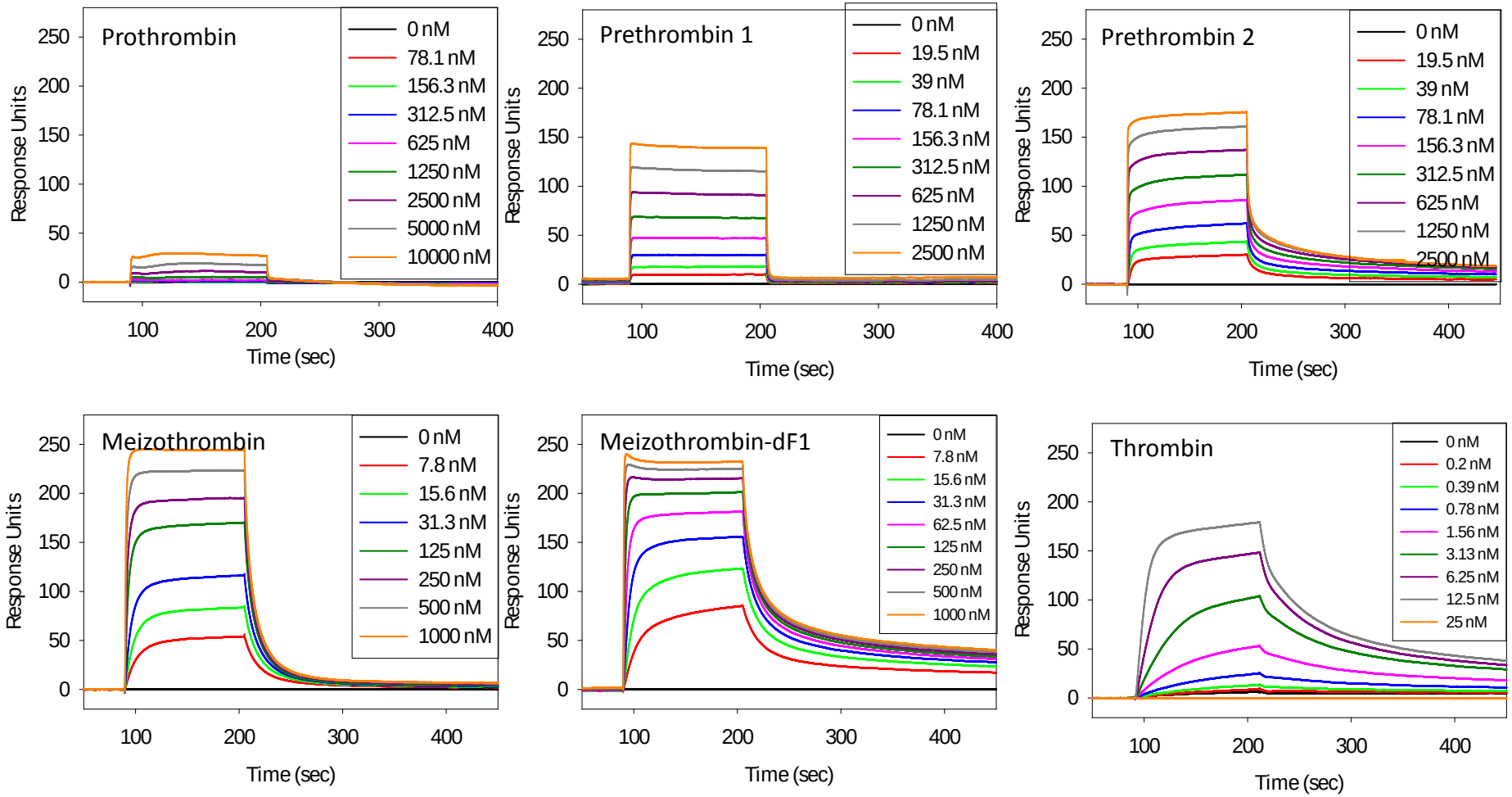


Figure 3. Sensorgram tracings of the binding of prothrombin and its intermediates, and thrombin to TM456 as measured by surface plasmon resonance.

CHAPTER THREE

Rivaroxaban associates with factor Xa faster than apixaban: A potential explanation for their differential effects on global tests of coagulation

Running title: Differential effects of rivaroxaban and apixaban

Calvin H. Yeh[1-3], Brian J. Dale[3,4], Beverly Leslie[1,3], Paul Y. Kim[1,3], James C. Fredenburgh[1,3], Jack Hirsh[1], John W. Eikelboom[1,3], and Jeffrey I. Weitz*[1-3].

[1]Department of Medicine, McMaster University, Hamilton, Canada; [2]Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Canada; and [3]Thrombosis and Atherosclerosis Research Institute, Hamilton, Canada; [4]Sansom Institute for Health Research, University of South Australia, Adelaide, Australia

***Correspondence to Dr. Jeffrey I. Weitz, Thrombosis and Atherosclerosis Research Institute, 237 Barton St. E, Hamilton, Ontario, Canada L8L 2X2. Phone: 905-574-8550, FAX: 905-575-2646, email: weitzj@taari.ca**

REGULAR ARTICLE
Section: THROMBOSIS AND HEMOSTASIS

KEY POINTS

Rivaroxaban inhibits factor Xa faster than apixaban

This difference explains why rivaroxaban prolongs the PT and aPTT more than apixaban

ABSTRACT

As oral factor Xa inhibitors, rivaroxaban and apixaban are both small molecules that reversibly inhibit factor Xa. Surprisingly, compared with rivaroxaban, apixaban has minimal effects on the prothrombin time and activated partial thromboplastin time. To investigate this phenomenon, we used a factor Xa-directed substrate in a buffer system. Although rivaroxaban and apixaban inhibited factor Xa with similar K_i values (1.0 ± 0.6 and 0.9 ± 0.8 nM, respectively), kinetic measurements revealed that the rate of binding to factor Xa inhibition by rivaroxaban was 3.3-fold faster than that of apixaban (75.7 ± 6.5 x 10^5 and 22.9 ± 2.9 x 10^5 M^{-1} sec^{-1}, respectively; p = 0.00002); a difference that persisted in the presence of prothrombinase components, and in the presence of recalcified plasma. Using a discontinuous chromogenic assay to monitor thrombin production by prothrombinase in a purified system, rivaroxaban was 2.6-fold more potent than apixaban (K_i values of 2.5 ± 0.2 and 6.5 ± 1.3 nM, respectively; p = 0.02). Likewise, in thrombin generation assays in plasma, rivaroxaban prolonged the lag time and suppressed peak thrombin to a greater extent than apixaban. Collectively, these findings suggest that rivaroxaban has a greater effect on global tests of coagulation than apixaban because it associates with factor Xa at a faster rate.

INTRODUCTION

Rivaroxaban and apixaban are oral factor Xa inhibitors that were developed as alternatives to warfarin for the prevention and treatment of venous and arterial thrombosis.(Yeh et al., 2012) Both agents are licensed for stroke prevention in patients with atrial fibrillation(Granger *et al.*, 2011, Patel *et al.*, 2011) and for the prevention of venous thrombosis after elective hip or knee replacement surgery.(Quinlan and Eriksson, 2013) In addition, rivaroxaban is approved for treatment of venous thromboembolism and apixaban is under regulatory consideration for this indication.(Yeh *et al.*, 2014)

Rivaroxaban and apixaban are inhibitors with a similar mechanism of action; as small molecules that bind reversibly to the active site of factor Xa, both agents inhibit the enzyme with high affinity as evidenced by inhibition constant (K_i) values of 0.4 ± 0.02 and 0.3 ± 0.11 nM, respectively.(Luettgen *et al.*, 2011, Perzborn *et al.*, 2005) In addition to inhibiting free factor Xa, both agents have also been shown to inhibit factor Xa incorporated in prothrombinase, the complex of factor Xa and factor Va that assembles on platelets and activates prothrombin.(Luettgen *et al.*, 2011, Perzborn *et al.*, 2005) Prothrombinase is the central effector of clotting because assembly of the complex induces structural changes in factor Xa that increase the catalytic efficiency of prothrombin activation by over 100,000-fold.(Mann et al., 1990) Prothrombinase propagates coagulation by rapidly generating thrombin from prothrombin at the site of vessel injury. Thus, the anticoagulant activity of rivaroxaban and apixaban reflects their rapid association with factor Xa in the prothrombinase complex.

Despite their similar affinity for factor Xa, surprisingly, rivaroxaban prolongs the prothrombin time (PT) and activated partial thromboplastin time (aPTT) more than apixaban.(Baglin, 2013, Barrett *et al.*, 2010, Eller *et al.*, 2014, Garcia *et al.*, 2013, Hillarp *et al.*, 2014) The explanation for their different effects on global tests of coagulation is currently unknown, but this divergence is important because it influences the interpretation of the results of coagulation tests in patients taking these drugs. The K_i value is an equilibrium constant determined by the rates of association and dissociation of the inhibitor with its target enzyme. Although rivaroxaban and apixaban inhibit factor Xa with similar K_i values, we hypothesized that the greater effect of rivaroxaban relative to apixaban on global tests of coagulation may reflect differences in the kinetics of factor Xa inhibition, which would influence their inhibitory effects on

prothrombinase-induced thrombin generation. To test this hypothesis, we compared the effects of rivaroxaban and apixaban on (a) the inhibition constants for factor Xa alone and factor Xa incorporated into the prothrombinase complex in buffer and plasma systems, (b) the PT and aPTT, which are global tests of coagulation, and on clotting induced by factor VIIa or Xa in plasma, (c) thrombin generation in plasma, (d) inhibition constants of prothrombin activation by prothrombinase in a purified system, and (e) rates of factor Xa inhibition.

MATERIALS AND METHODS

Materials – Human thrombin, prothrombin and factor Va were purchased from Haematologic Technologies (Essex Junction, VT), while factor Xa and thrombin were from Enzyme Research Laboratories (South Bend, IN). Recombinant factor VIIa (NiaStase) was acquired from Novo Nordisk. The factor Xa-directed chromogenic substrate, S-2765, was from Chromogenix (Milano, Italy), whereas the thrombin-directed substrate, Chromozym-thrombin (Chz-Th), was from Hyphen BioMed (Neuville sur Oise, France). Hirudin was from Bachem Bioscience, Inc. (Philadelphia, PA). Phospholipid vesicles were prepared in a 3:1 ratio of phosphatidylcholine and phosphatidylserine (PCPS) and stored in 10% sucrose at -80°C as described previously.(Barenholz et al., 1977) RecombiPlasTin 2G, which contains recombinant tissue factor at a concentration of 0.3 µg/mL(Yau et al., 2011), was from Instrumentation Laboratory (Bedford, MA). Rivaroxaban and apixaban, obtained from Suzhou Howsine Biological Technology Company (Suzhou, China), exhibited single peaks by HPLC analysis. After dissolving the agents in 100% dimethyl sulfoxide (DMSO) to a concentration of 10 mg/mL, they were stored in aliquots at -80°C. To prepare pooled normal human plasma (PNP), blood was collected from the antecubital veins of 13 healthy volunteers into Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) containing 0.105 M sodium citrate. Platelet-poor plasma was obtained by twice subjecting the blood to centrifugation at 2,500 x g for 15 min. The plasma was then pooled and stored in aliquots at -80°C.

Determination of inhibition constants of factor Xa – Reactions were performed in clear or black flat-bottom 96-well polystyrene plates (Corning Inc, Corning, NY). All inhibition experiments were conducted at 25°C in 20 mM HEPES, 150 mM NaCl, pH 7.4 (HBS) containing 0.01% Tween-80 (HBST),

0.1% Prionex and 5 mM $CaCl_2$. To monitor factor Xa inhibition in buffer, 10 µL aliquots containing rivaroxaban or apixaban in concentrations ranging from 0 – 20 nM were added to wells containing 10 µL of factor Xa at a final concentration of 5 nM, and brought to a final volume of 100 µL with a solution containing S-2765 and $CaCl_2$ in final concentrations of 400 µM and 5 mM, respectively. Stock solutions of rivaroxaban and apixaban were diluted in HBST, such that the final concentration of DMSO was < 0.01% (v/v); a concentration that in control experiments did not influence the hydrolysis of S-2765 by factor Xa. Absorbance was monitored at 405 nm using a SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, CA) and the rates of S-2765 hydrolysis were converted to factor Xa concentrations using a specific activity of 25.73 ± 0.95 mOD min^{-1} nM^{-1}, which was determined in a separate experiment. The residual concentration of factor Xa was then plotted against the anticoagulant concentration, and the data were fit to a rectangular hyperbola equation by nonlinear regression analysis using TableCurve version 4.04 (Jandel Scientific):

$$V = V_0 + (V_{min} \cdot I) / (IC_{50} + [I])$$

where V_0 and V_{min} are the rates of substrate hydrolysis in the absence and presence of saturating concentrations of inhibitor, respectively, IC_{50} is the concentration of inhibitor required to produce half-maximal reduction, and I is the inhibitor concentration. K_i values were then obtained from the IC_{50} value using the Cheng-Prusoff equation:

$$IC_{50} = [1 + ([S] / K_M)] K_i$$

where S is the S-2765 concentration and K_M is the Michaelis-Menten constant of factor Xa for S-2765.

PT and aPTT - Stock solutions of rivaroxaban or apixaban (MW = 435.9 and 459.5 Da, respectively) were diluted in PNP to 500 ng/mL (1,088 and 1,150 µM, respectively) and then serially diluted with PNP to a final concentration of 31.25 ng/mL (72 and 68 nM, respectively). The concentrations range of inhibitors used encompass the peak and trough plasma levels in clinical use.(Frost *et al.*, 2013, Mueck *et al.*, 2011) Final concentrations of rivaroxaban and apixaban were confirmed with the Rotochrom chromogenic anti-Xa assay (Diagnostica Stago, Doncaster, Australia), which was performed using a ST4 coagulometer (Diagnostica Stago) with commercial rivaroxaban or apixaban calibrators (Technoclone, Vienna, Austria). The PT was performed using HemosIL HS PLUS (Instrumentation Laboratory,

Lexington, MA) on an ACL 7000 coagulometer (Instrumentation Laboratory) according to the manufacturer's instructions. The aPTT was performed using Actin FSL reagent (Siemens, Marburg, Germany) on a BCS XP analyzer (Siemens) according to the manufacturer's instructions. All assays were performed three times in duplicate. The PT and aPTT reagents were chosen because of their wide availability and their reported sensitivities to rivaroxaban and apixaban.(Funatsu *et al.*, 2012, Hillarp *et al.*, 2014)

Plasma-based thrombin generation assays –Thrombin generation assays were performed as previously described(Hemker *et al.*, 2006, Kretz *et al.*, 2010, Yau *et al.*, 2011) using final concentrations of 3 pM tissue factor, 4 µM PCPS, 0.5 mM Z-Gly-Gly-Arg-AMC, and 7.5 mM $CaCl_2$, without added corn trypsin inhibitor. The assay was calibrated using Technothrombin TGA thrombin calibrators (Technoclone) according to the manufacturer's instructions. High and low thrombin concentration controls were included in each run. All samples and controls were tested in quadruplicate.

Factor VIIa and factor Xa clot times - Factor VIIa or factor Xa was used to initiate clotting in plasma. After incubating 100 µL aliquots of PNP containing 0 – 1000 nM rivaroxaban or apixaban at 37°C for 15 min, a 20 µL solution containing either 1 µM factor VIIa or 20 nM factor Xa and 100 mM $CaCl_2$ was added. Absorbance was monitored at 405 nm and the clotting time was calculated as the time to half maximal increase in absorbance as determined using the instrument software. Tests were performed in quadruplicate and mean clotting times were plotted *versus* anticoagulant concentrations.

Determination of inhibition constants of prothrombinase-induced thrombin generation – To examine their inhibitory effect on prothrombinase activation of prothrombin, rivaroxaban or apixaban was pre-incubated with prothrombin, then added a solution containing factor Xa, factor Va, PCPS, and calcium. The final activation solution contained 20 µL of 0 – 200 nM inhibitor, 0.5 nM factor Xa, 5.5 nM factor Va, 50 µM PCPS, 500 nM prothrombin, and 5 mM $CaCl_2$. Reactions were stopped after 20 s by addition of 20 µL of 10 mM EDTA, and thrombin activity was determined by adding 60 µL of 500 µM Chz-Th and monitoring its hydrolysis at 405 nm using a plate reader. The rates of Chz-Th hydrolysis were converted to thrombin concentrations using a specific activity of 17.29 ± 0.66 mOD min^{-1} nM^{-1}, which was determined in a separate experiment. Thrombin concentrations were divided by reaction time to obtain the rate of

thrombin generation (nM/s), and divided by the factor Xa concentration to obtain the turnover number (k_{cat}). The turnover number was then plotted against the concentration of inhibitor, and the data were analyzed as described above to obtain the K_i value, using a K_M value of 157.4 nM for 500 nM prothrombin in prothrombinase (data not shown).

Determination of association rate constants for factor Xa inhibition by rivaroxaban and apixaban – The kinetic rate constants for factor Xa inhibition by rivaroxaban and apixaban were determined by chromogenic assay in a Beckman DU7400 spectrophotometer using a DU600 high performance transport assembly to monitor control and inhibition reactions simultaneously. Absorbance at 405 nm was monitored at 3.7 s intervals in 10 x 4 mm disposable cuvettes, with samples mixed continuously using a micro stir bar. Reactions were conducted under pseudo first-order conditions by addition of a 50 µL solution of factor Xa to a cuvette containing 700 µL of inhibitor and S-2765, yielding final concentrations of 5 nM, 50 nM and 1 mM, respectively, in HBST buffer containing 5 mM $CaCl_2$. Absorbance *versus* time data were fit by non-linear regression analysis using TableCurve to an equation for slow-binding enzyme inhibitors, as described previously(Sinha *et al.*, 2003):

$$A405 = v_f\, t + (v_i - v_f)\,(1 - e^{-k1t}) / k_1 + A405_o$$

where A405 is the absorbance at 405 nm at time t, $A405_o$ is the baseline absorbance prior to addition of the enzyme, v_f and v_i are the derived final and initial rates of substrate hydrolysis, respectively, and k_1 is the apparent pseudo-first-order rate constant. The second-order rate constant of inhibition (k_2) was then obtained by dividing k_1 by the inhibitor concentration and by correcting for competitive binding between the inhibitor and the chromogenic substrate.

To determine the rates of factor Xa inhibition by rivaroxaban and apixaban in the presence of the components of the prothrombinase complex or in recalcified plasma, rivaroxaban or apixaban and S-2765 were preincubated in buffer containing 25 nM factor Va, 50 µM PCPS, 2 µM prothrombin and 10 µM hirudin in 5 mM $CaCl_2$. For studies in plasma, factor Xa was pre-mixed with $CaCl_2$ for final concentrations of 5 nM and 25 mM, respectively, then added to plasma containing 10 µM hirudin and either rivaroxaban or apixaban. Inhibition rate constants were obtained as described above.

Statistical analyses – All experiments were performed at least in triplicate and data are expressed as mean ± SD. Inhibition constants (K_i) and inhibition rates (k_2) for rivaroxaban and apixaban were compared by Student t-tests using Microsoft Office Excel 2010. Clot times, PT, aPTT, and thrombin generation data were compared by repeated measures two-way analysis of variance using GraphPad Prism 6 (San Diego, CA). P-values < 0.05 were considered statistically significant.

RESULTS

Effect of rivaroxaban and apixaban on the PT and aPTT – The effects of rivaroxaban and apixaban on the PT and aPTT were directly compared in global clotting assays. Inhibitor concentrations up to 500 ng/ml for rivaroxaban and apixaban cover the peak plasma levels seen in clinical use.(Frost *et al.*, 2013, Mueck *et al.*, 2013) Although both agents prolonged the PT and aPTT in a concentration-dependent fashion (Fig. 2), as previously reported(Barrett *et al.*, 2010, Funatsu *et al.*, 2012), the effect of rivaroxaban on these tests was significantly greater than that of apixaban ($p<0.0001$). Therefore, rivaroxaban is more potent than apixaban regardless of whether coagulation is activated via the extrinsic or intrinsic pathway.

Effect of rivaroxaban and apixaban on factor VIIa and factor Xa clot times – To determine whether the divergent effects on clotting are the result of differential inhibition of factor Xa, we compared the effects of rivaroxaban and apixaban in clotting assays initiated with factor VIIa or factor Xa (Fig. 3). Both agents prolonged the factor VIIa and factor Xa clot times in a concentration-dependent fashion, but at equimolar concentrations, rivaroxaban was 1.5-fold more potent than apixaban; a difference that was statistically significant ($p<0.0001$). Thus, the greater inhibitory effect of rivaroxaban on clotting relative to apixaban appears to be at the level of factor Xa.

Plasma-based thrombin generation assay – The inhibitory effects of rivaroxaban and apixaban in plasma were compared using a calibrated thrombin generation assay. After triggering thrombin generation with dilute tissue factor, we examined the effects of rivaroxaban and apixaban on the lag time, peak thrombin concentration and endogenous thrombin potential (ETP). Although both agents prolonged the lag time, reduced peak thrombin concentration and decreased ETP in a concentration-dependent fashion, the effect of rivaroxaban on these variables was significantly ($p<0.0001$) greater than that of apixaban (Fig. 4).

Therefore, these data show that rivaroxaban inhibits thrombin generation in plasma to a greater extent than apixaban; a finding that suggests that the differences in the effect of the drugs on global clotting assays reflect divergent inhibitory effects on prothrombinase-induced thrombin generation.

Inhibition of prothrombinase-induced thrombin generation by rivaroxaban and apixaban – To directly measure the effect of rivaroxaban and apixaban against the activity of prothrombinase, we measured the rate of prothrombin activation in a purified prothrombinase system.(Mann et al., 1990) Both agents inhibited prothrombinase-induced thrombin generation by over 95% in a concentration-dependent fashion (Fig. 5) with K_i values that were 5- to 10-fold higher than those for inhibition of the chromogenic activity of free factor Xa. Importantly, the K_i value for rivaroxaban was 2.6-fold lower than that for apixaban (2.5 ± 0.2 and 6.5 ± 1.3 nM, respectively; p = 0.02). Therefore, although rivaroxaban and apixaban bind free factor Xa and factor Xa incorporated in prothrombinase with comparable affinities, rivaroxaban is 2.6-fold more potent than apixaban at inhibiting the rate of prothrombin activation by prothrombinase. These results are consistent with the observation that rivaroxaban is more potent than apixaban in global tests of coagulation and in plasma thrombin generation assays.

Inhibition of free factor Xa and factor Xa incorporated into prothrombinase by rivaroxaban and apixaban – Rivaroxaban and apixaban inhibited the chromogenic activity of factor Xa in a concentration-dependent fashion (Fig. 1) with K_i values of 0.5 ± 0.06 and 0.6 ± 0.04 nM, respectively (p=0.25, n=3); values comparable to those previously reported.(Luettgen *et al.*, 2011, Perzborn *et al.*, 2005) At a concentration of 20 nM, both agents inhibited the chromogenic activity of 5 nM factor Xa by over 95%.

To determine whether prothrombinase assembly influences the inhibition of factor Xa, studies were repeated in the presence of factor Va, PCPS, and prothrombin. Hirudin was added to inhibit the generated thrombin, thereby preventing it from hydrolyzing the chromogenic substrate. As observed with factor Xa alone, rivaroxaban and apixaban inhibited over 95% of factor Xa chromogenic activity in the presence of prothrombinase components in the same dose-dependent fashion (not shown) with K_i values of 1.0 ± 0.6 and 0.9 ± 0.8 nM, respectively; values not significantly different from those determined with free factor Xa. Together, these data demonstrate that rivaroxaban and apixaban bind to free factor Xa and factor Xa incorporated into the prothrombinase complex with comparable affinities.

Rate constants for factor Xa inhibition by rivaroxaban or apixaban – To test the hypothesis that rivaroxaban and apixaban have divergent effects on the rate of factor Xa inhibition, we used a chromogenic assay to compare the rate constants for inhibition of factor Xa by rivaroxaban and apixaban.(Luettgen *et al.*, 2011, Perzborn *et al.*, 2005) Although both agents demonstrated progressive inhibition of factor Xa (Fig. 6), rivaroxaban inhibited factor Xa 3.3-fold more rapidly than apixaban, with second-order rate constants of inhibition, k_2 values, of 75.7 ± 6.5 and 22.9 ± 2.9 x 10^5 M^{-1} sec^{-1}, respectively (p = 0.00002). These results suggest that despite the fact that the two drugs display similar affinities for factor Xa, rivaroxaban interacts with factor Xa more rapidly than apixaban; a difference that could explain the more potent inhibitory effect of rivaroxaban on prothrombinase-induced thrombin generation.

To determine whether the difference in inhibition rate constants between rivaroxaban and apixaban persists when factor Xa is incorporated into the prothrombinase complex, we repeated the experiment in the presence of factor Va, PCPS, prothrombin, and hirudin. Again, rivaroxaban inhibited factor Xa in the prothrombinase complex at a 3.5-fold faster rate than apixaban (95.6 ± 9.6 and 27.0 ± 0.7 x 10^5 M^{-1} sec^{-1}, respectively; p = 0.0003); rates similar to those observed with free factor Xa. To determine whether the difference persists in a physiological milieu, the rate of factor Xa inhibition by rivaroxaban or apixaban was measured in recalcified human plasma containing hirudin. As expected, the rates of factor Xa inhibition by rivaroxaban and apixaban in plasma were slower than those in buffer, reflecting the fact that both drugs exhibit avid binding to albumin leaving only ~10% free to inhibit factor Xa.(Scaglione, 2013) However, the rate of factor Xa inhibition by rivaroxaban remained 2.6-fold faster rate than that by apixaban (k_2 values of 25.4 ± 11.4 and 9.5 ± 1.6 x 10^5 M^{-1} sec^{-1}, respectively; p = 0.03). Therefore, although rivaroxaban and apixaban bind and inhibit free factor Xa and factor Xa incorporated into prothrombinase with similar affinities, the rate of association with factor Xa is faster with rivaroxaban than with apixaban.

DISCUSSION

Rivaroxaban and apixaban are administered in fixed doses without routine coagulation monitoring. Nonetheless, assessment of their anticoagulant activity may be useful to detect accumulation or overdose, to ensure offset prior to surgery, to determine their contribution to bleeding, or to assess adherence. Despite

their similar mechanism of action, rivaroxaban has a greater effect on the PT and aPTT than apixaban.(Baglin, 2013, Barrett *et al.*, 2010, Garcia *et al.*, 2013) To understand this phenomenon, we compared the effect of these agents in three different systems. First, we determined the K_i values for free factor Xa and factor Xa incorporated into prothrombin. Second, we compared their effects in plasma on the PT, aPTT, factor VIIa and factor Xa clot times, and thrombin generation. Third, we measured the rates of inhibition of free factor Xa, factor Xa incorporated into prothrombinase and prothrombinase-induced prothrombin activation by rivaroxaban and apixaban.

As previously reported, rivaroxaban and apixaban inhibit free factor Xa and factor Xa incorporated into the prothrombinase complex with similar K_i values, suggesting that the two agents bind factor Xa with comparable affinities.(Luettgen *et al.*, 2011, Perzborn *et al.*, 2005) Consistent with previous results, rivaroxaban prolongs the PT and aPTT more than apixaban. Furthermore, rivaroxaban has a greater effect than apixaban on factor VIIa and factor Xa clot times and thrombin generation; findings that suggest that the two agents have divergent effects on prothrombinase-induced thrombin generation. In support of this concept, rivaroxaban inhibits prothrombinase-induced thrombin generation with a K_i value 2.6-fold lower than that for apixaban. The explanation for this difference comes from the demonstration that the rate of inhibition of free factor Xa or factor Xa incorporated into prothrombinase by rivaroxaban is up to 3.5-fold faster than that by apixaban. Collectively, therefore, these findings suggest that rivaroxaban has a greater effect on global tests of coagulation and thrombin generation than apixaban because rivaroxaban associates with factor Xa at a faster rate. These findings give credence to the concept that in addition to K_i values, rates of association are needed to predict the effect of factor Xa inhibitors on thrombin generation and global tests of coagulation.(Sinha *et al.*, 2003)

Examination of the crystal structure of the complex of factor Xa with rivaroxaban or apixaban may provide insight into their divergent rates of inhibition. Although both agents bind to the active site, they exhibit distinct interactions with substrate-binding subsites S1 and S4 within the active site cleft,(Husten *et al.*, 1987, Scaglione, 2013) which may account for the differences in their association rate constants and their effects on prothrombin activation. Therefore, these results raise the possibility that the interactions of

inhibitors with individual subsites within the active site of factor Xa may be exploited to design anticoagulants with varying properties.

The finding that apixaban inhibits thrombin generation to a lesser extent than rivaroxaban may have important implications. Indirect comparisons suggest that at the doses used clinically, apixaban is associated with less bleeding than rivaroxaban.(Lip *et al.*, 2012, Schneeweiss *et al.*, 2012) It is possible that slower inhibition of thrombin generation by apixaban at sites of vascular injury may attenuate bleeding by allowing the generation of sufficient amounts of thrombin to enable hemostasis. Head-to head trials of apixaban and rivaroxaban would be needed to test this hypothesis.

In conclusion, we show that rivaroxaban is a more potent inhibitor of prothrombinase-induced thrombin generation than apixaban because it inhibits factor Xa more rapidly. This difference provides a plausible explanation for the observation that rivaroxaban has a greater effect on global tests of coagulation than apixaban. The slower rate of factor Xa inhibition by apixaban may endow it with a safety advantage by allowing sufficient thrombin generation to ensure stable hemostatic plug formation; a concept that requires exploration in head-to-head clinical trials.

ACKNOWLEDGEMENTS

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AUTHORSHIP CONTRIBUTIONS

C.H.Y., B.J.D., designed and performed experiments, analyzed and interpreted data, and wrote the manuscript. B.L. performed experiments. J.C.F., J.H., J.W.E., and J.I.W. designed experiments and interpreted data, and wrote the manuscript.

DISCLOSURE OF CONFLICTS OF INTEREST

J.W.E. has received consulting fees and/or honoraria from Bayer, Janssen. Bristol-Myers Squibb, and Pfizer, and J.I.W. has served as a consultant and has received honoraria from Bristol-Myers Squibb, Pfizer, Bayer, and Janssen. None of the other authors declares conflicts of interest.

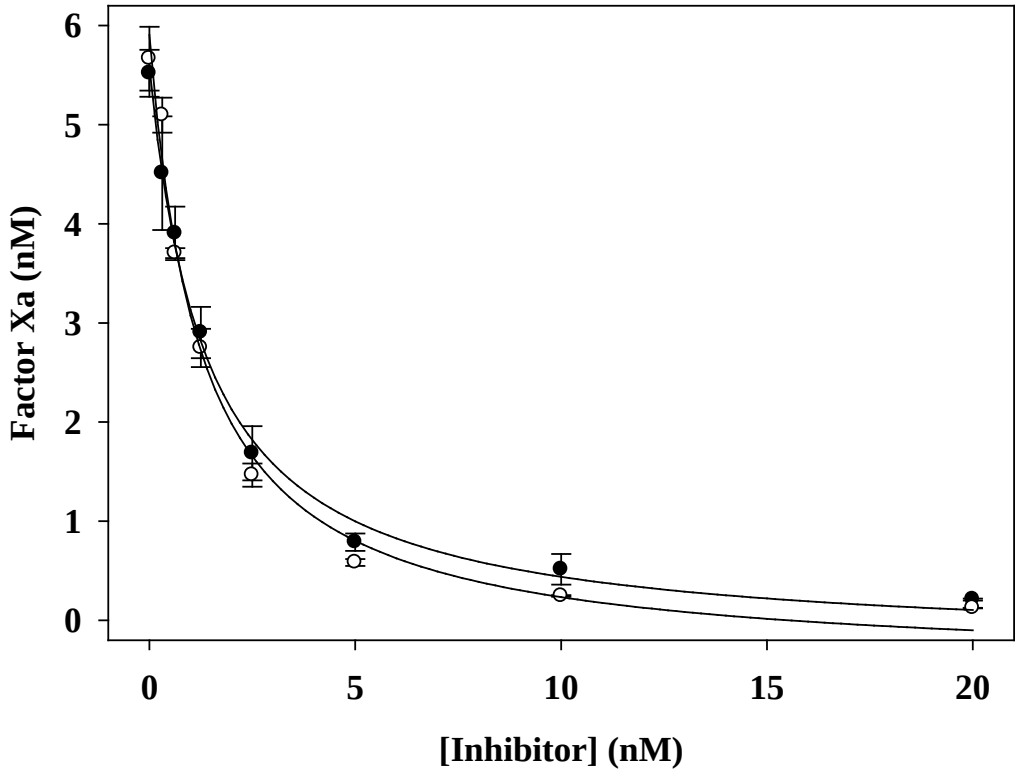

Figure 1. Comparison of the effects of rivaroxaban and apixaban on factor Xa chromogenic activity. Inhibition constants (K_i) were determined by incubating 0.5 nM factor Xa with rivaroxaban (open circles) or apixaban (closed circles) at the indicated concentrations, and residual factor Xa concentration was determined by monitoring hydrolysis of 500 μM S-2765. Symbols reflect the mean ± SD of 3 to 4 experiments. Lines are fit to a rectangular hyperbola equation by nonlinear regression analysis.

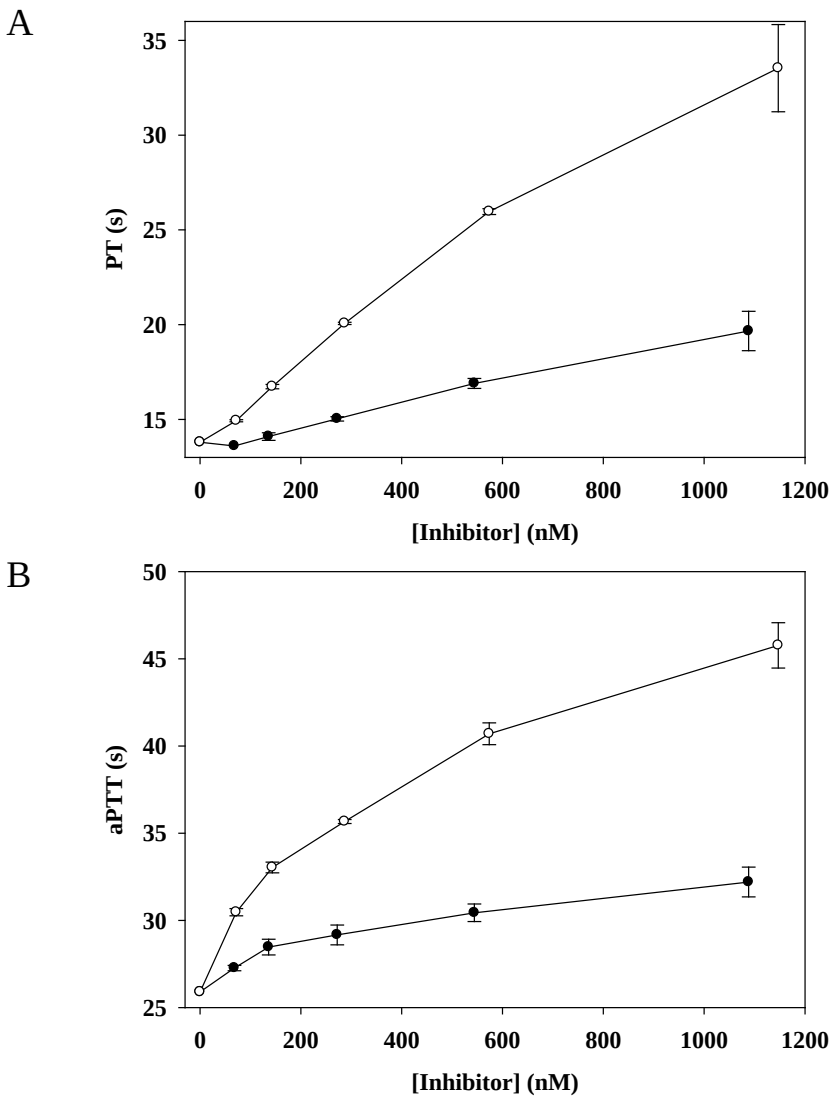

Figure 2. Comparison of the effects of rivaroxaban and apixaban on the prothrombin time (PT) and activated partial thromboplastin time (aPTT). The PT (A) and aPTT (B) were determined in human plasma in the presence of increasing concentrations of rivaroxaban (open circles) or apixaban (closed circles). Symbols reflect the mean ± SD of 4 experiments.

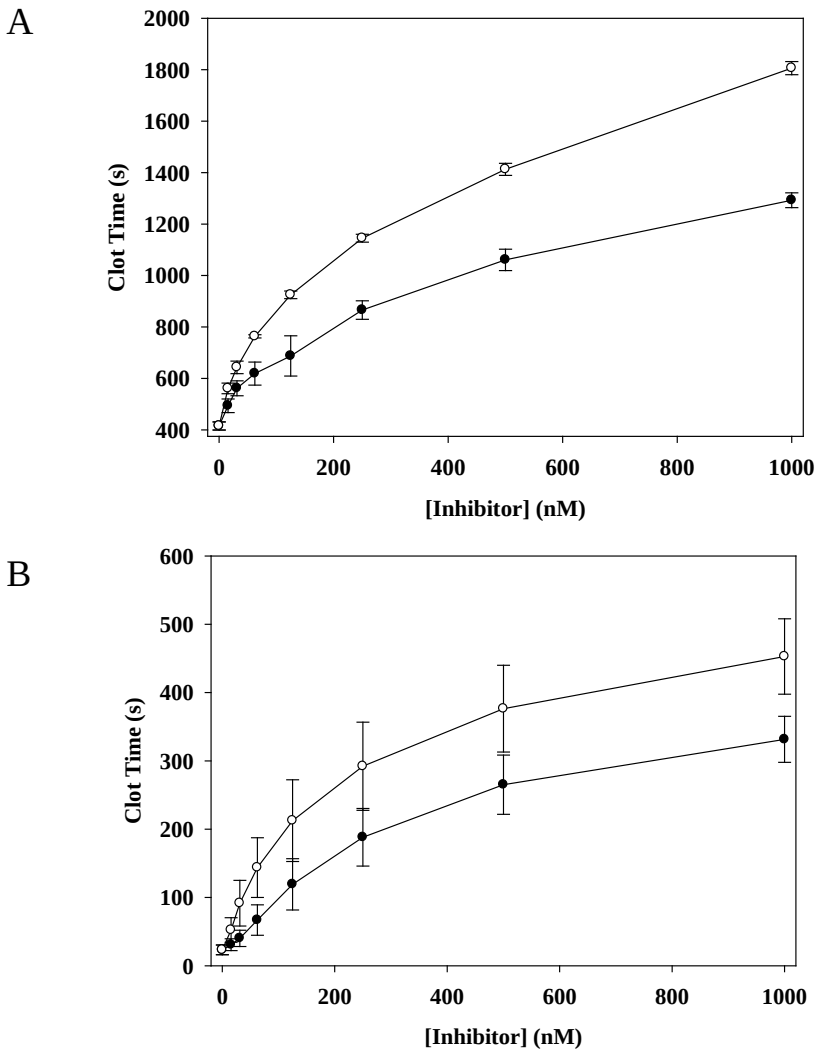

Figure 3. Comparison of the effects of rivaroxaban and apixaban on factor VIIa or factor Xa clot times. Plasma was clotted by addition of (A) 200 nM factor VIIa and 20 mM $CaCl_2$, or (B) 4 nM factor Xa and 15 mM $CaCl_2$ in the presence increasing concentrations of rivaroxaban (open circles) or apixaban (closed circles), and absorbance at 405 nm was monitored. Clot time was determined as the time to half maximal increase in absorbance. Symbols reflect the mean ± SD of 3 to 4 experiments.

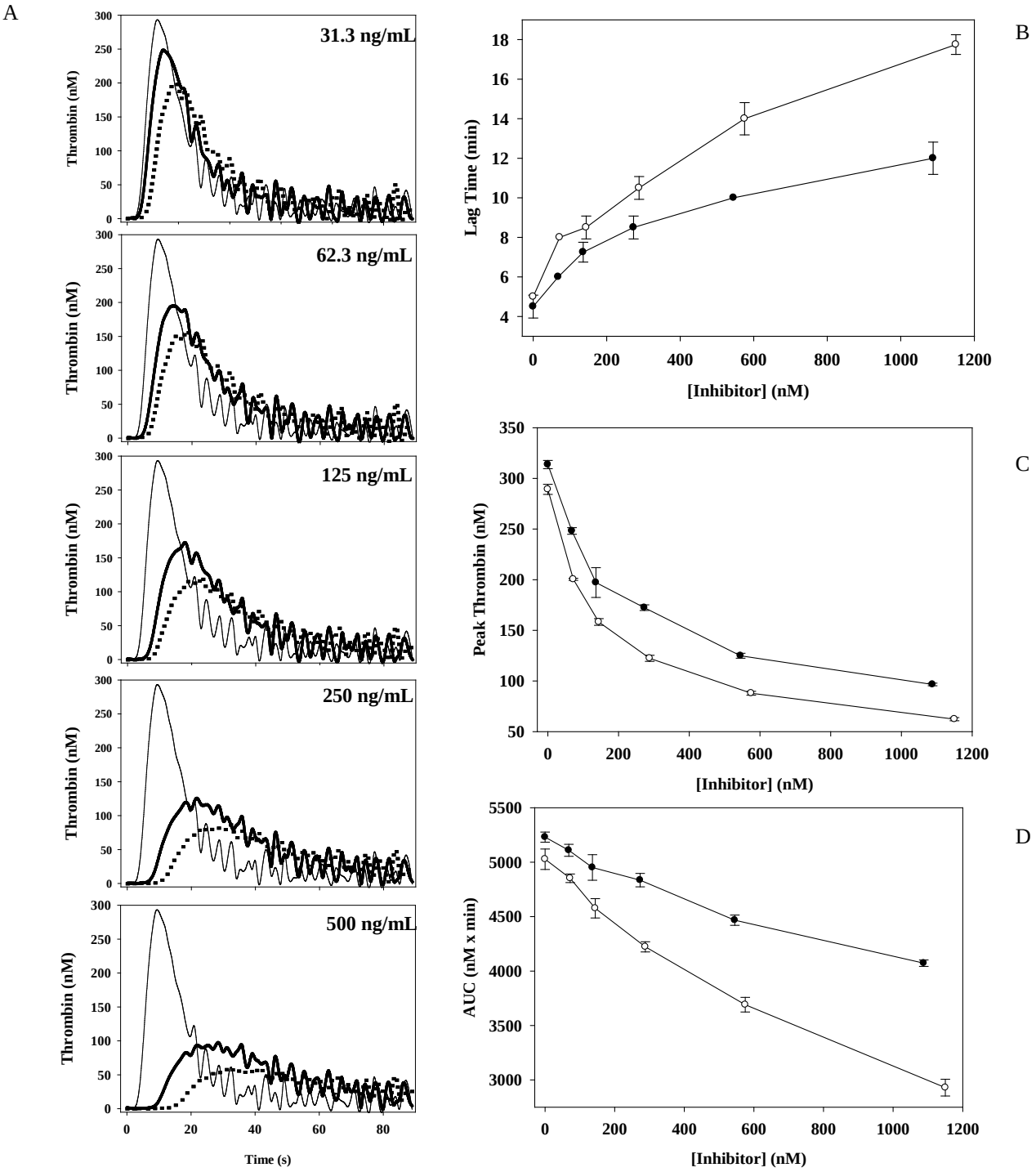

Figure 4. Comparison of the effects of rivaroxaban and apixaban on thrombin generation in plasma. Thrombin generation in plasma containing 4 μM PCPS was triggered by addition of 3 pM tissue factor and 7.5 mM $CaCl_2$ and quantified by monitoring the hydrolysis of 0.5 mM Z-Gly-Gly-Arg-AMC in the absence (thin lines) or presence of rivaroxaban (solid lines) or apixaban (dotted lines) at the indicated concentrations. Thrombin generation profiles are shown for indicated concentrations of inhibitor in (A), and parameters for lag time (B), peak thrombin (C), and AUC (D) are plotted *versus* the concentrations of rivaroxaban (open circles) or apixaban (closed circles). Data reflect the mean ± SD of 3 experiments.

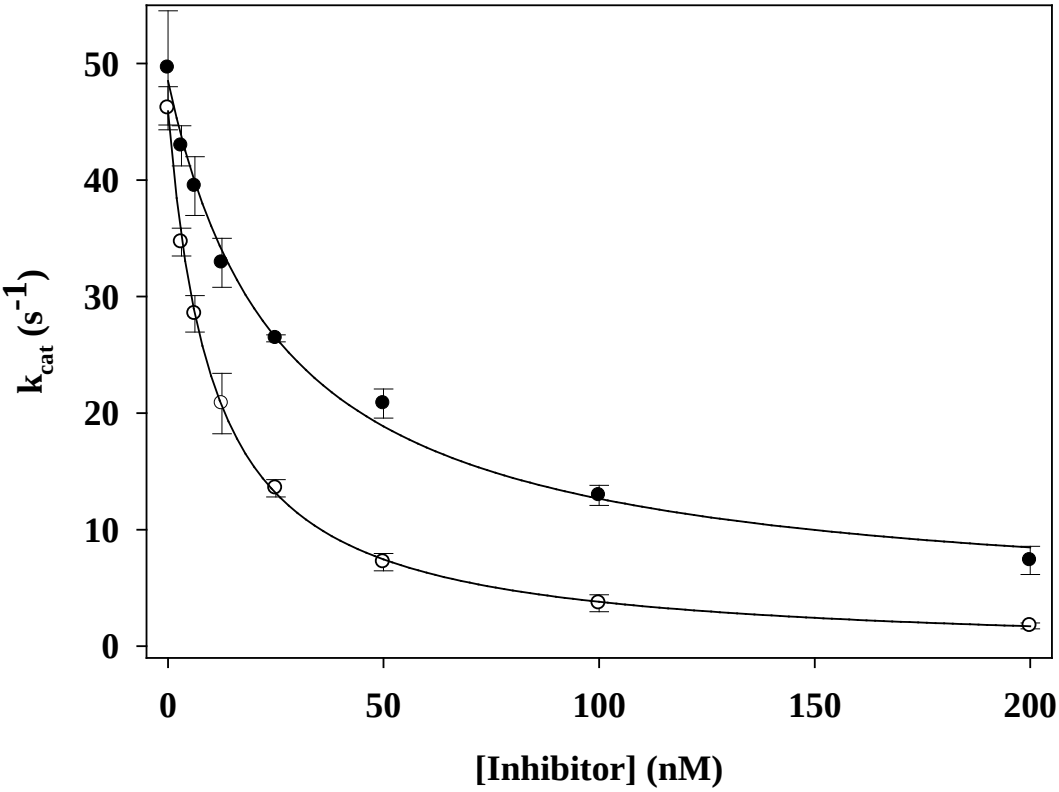

Figure 5. Comparison of the effects of rivaroxaban and apixaban on prothrombin activation by prothrombinase. Inhibition constants (K_i) were determined by incubating increasing concentrations of rivaroxaban (open circles) or apixaban (closed circles) with 0.5 nM factor Xa, 5.5 nM factor Va, 50 µM PCPS, 500 nM prothrombin and 5 mM $CaCl_2$. After 20 s, reactions were stopped by addition of EDTA to 10 mM and generated thrombin was quantified by chromogenic assay with Chz-Th. Rates of prothrombin activation are plotted versus inhibitor concentration. Symbols reflect the mean ± SD of 3 to 4 experiments. Lines are fit to a rectangular hyperbola equation by nonlinear regression analysis.

Figure 6

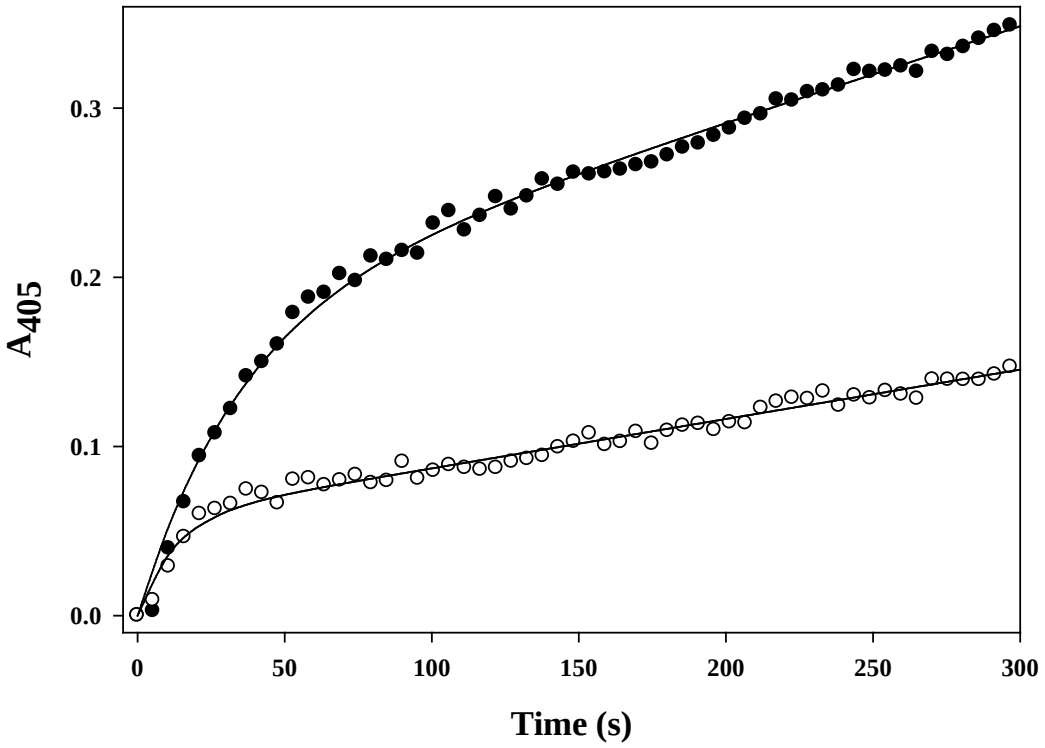

Figure 6. Kinetics of inhibition of factor Xa chromogenic activity by rivaroxaban or apixaban. Rivaroxaban (open circles) or apixaban (closed circles), at a concentration of 50 nM, was incubated with 5 nM factor X and 5 mM $CaCl_2$, and residual factor Xa activity was quantified by monitoring the hydrolysis of 1 mM S-2765 every 3 s for 5 min. Absorbance values were corrected for background signal prior to addition of factor Xa. A representative plot is illustrated. Lines were fit by non-linear regression analysis to an equation for slow-binding enzyme inhibitors as previously described.(Sinha *et al.*, 2003)

CHAPTER FOUR

Engagement of the active site of thrombin by dabigatran or argatroban modulates its exosite-mediated interactions with fibrin, factor Va, and glycoprotein Ibα

Calvin H. Yeh, Alan R. Stafford, Beverly A. Leslie, James C. Fredenburgh, Jeffrey I. Weitz*

Departments of Medicine and of Biochemistry and Biomedical Sciences
McMaster University and the Thrombosis and Atherosclerosis Research Institute, Hamilton, Canada

*Correspondence to Dr. Jeffrey I. Weitz, Thrombosis and Atherosclerosis Research Institute, 237 Barton St. E, Hamilton, Ontario, Canada L8L 2X2. Phone: 905-574-8550, FAX: 905-575-2646, email: weitzj@taari.ca

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Conflict of Interest.
The authors declare no competing financial interest.

Abbreviations.

b	biotin
Chz-Th	Chromozym Thrombin
COOH	carboxy-terminal
DAPA	dansylarginine N-(3-ethyl-1,5-pentanediyl)amide (DAPA)
EC_{50}	effective concentration for 50% change
Va	activated factor V
Fg	fibrinogen
FPR	Phe-Pro-Arg
Gp	glycoprotein
GpIb	GpIb
HBS	Hepes buffered saline
NH_2	amino-terminal
PAR	protease activated receptor
RA-thrombin	exosite 2 thrombin variant with Arg residues 93, 97 and 101 replaced with Ala
Req	response units at equilibrium
RU	response units
SPR	surface plasmon resonance
SD	standard deviation
Tw	Tween 80

ABSTRACT

Thrombin is a highly plastic molecule whose activity and specificity are regulated by exosites 1 and 2, positively-charged domains that flank the active site. Exosite binding by substrates and cofactors regulates thrombin activity by localizing thrombin, guiding substrates, and by inducing allosteric changes at the active site. Although inter-exosite and exosite-to-active-site allostery have been demonstrated, the impact of active site ligation on exosite function has not been examined. To address this gap, we used surface plasmon resonance to determine the effects of dabigatran, argatroban, and dansylarginine N-(3-ethyl-1,5-pentanediyl)amide (DAPA), active site-directed inhibitors, on thrombin binding to immobilized □A/ A-fibrin or glycoprotein Ib A/ peptide via exosite 1 fibrin or factor Va, which is mediated by both exosites. Whereas dabigatran attenuated binding, argatroban and DAPA increased thrombin binding to and A -fibrin and to factor Va. The results with immobilized fibrin were confirmed by examining the binding of radiolabeled thrombin to fibrin clots. Thus, dabigatran modestly accelerated the dissociation of thrombin from A/ A-fibrin clots, whereas argatroban and DAPA attenuated dissociation. Dabigatran had no effect on thrombin binding to glycoprotein Ib peptide, whereas argatroban and DAPA promoted binding. These findings not only highlight the bidirectional nature of thrombin allostery, but also suggest that individual active site-directed thrombin inhibitors uniquely modulate exosite function, thereby identifying potential novel mechanisms of action.

Thrombin, the final effector in blood coagulation, catalyzes the conversion of fibrinogen to fibrin and activates factor XIII, which then crosslinks the fibrin to form a stable clot. In addition, by activating factors V, VIII, and XI, protein C, and thrombin activable fibrinolysis inhibitor, thrombin influences procoagulant, anticoagulant and anti-fibrinolytic pathways. Because of its multiple roles, regulation of thrombin activity is critical for hemostasis.(Bock *et al.*, 2007, Huntington, 2012, Lane *et al.*, 2005)

The substrate specificity of thrombin is dependent on exosites 1 and 2, positively-charged domains that flank the active site.(Bock *et al.*, 2007, Krishnaswamy, 2013) These exosites modulate the reactivity of thrombin by providing initial binding sites for substrates, inhibitors or cofactors, sterically hindering other interactions, and by allosterically modifying the active site. Exosite 1 is more versatile because it (a) serves as a docking site for substrates such as fibrinogen, factor V, factor VIII, and protease-activated receptors (PAR) on platelets, (b) redirects thrombin activity by binding thrombomodulin, and (c) mediates thrombin inhibition by binding the COOH-terminus of hirudin and the NH_2-terminus of heparin cofactor II. In contrast, exosite 2 has a more limited role because it mainly serves to tether or localize thrombin. For example, exosite 2 contributes to thrombin binding to glycoprotein (Gp) Ib □, thereby prom activation via protease activated receptor 1.(De Candia *et al.*, 2001, Zarpellon *et al.*, 2011) In addition, by binding heparin, exosite 2 accelerates the inhibition of thrombin by the heparin-antithrombin complex. Exosite 2 also binds the -chain of fibrinogen, thereby mediating a bivalent, high affinity interaction with the variant -fibrinogen.(Wolfenstein-Todel and Mosesson, 1981) In contrast, because the -chain lacks a thrombin binding site, thrombin binds the bulk -fibrinogen with lower affinity solely via exosite 1.(Meh *et al.*, 1996, Pospisil *et al.*, 2003) Both exosites of thrombin also are involved in the interaction of thrombin with factor V, and activated factor V (factor Va) retains affinity for thrombin.(Dharmawardana *et al.*, 1999, Esmon and Lollar, 1996, Segers *et al.*, 2007) Therefore, the exosites are important regulators of thrombin activity.

Numerous studies have shown that thrombin is subject to allosteric modulation. For example, binding of Na^+ to a conserved site on thrombin increases the catalytic activity of thrombin by altering the conformation of the active site.(Gohara and Di Cera, 2011, Pozzi *et al.*, 2011) Likewise, ligand binding to exosites 1 or 2 can induce allosteric changes at the active site and/or the reciprocal exosite. Thus, binding of

the hirudin peptide,(Fredenburgh *et al.*, 1997, Lechtenberg *et al.*, 2010, Verhamme *et al.*, 2002) platelet PAR-1 peptide,(Gandhi *et al.*, 2008, Lechtenberg *et al.*, 2010) and thrombomodulin(Adams *et al.*, 2009, Gasper *et al.*, 2012) to exosite 1 or GpIb (Li *et al.*, 2001) and prothrombin fragment 2 to exosite 2 (Fredenburgh *et al.*, 1997) elicits conformational changes at the active site. Likewise, binding of prothrombin fragment 2, γ′-peptide (an analog of the thrombin-binding domain on the chain of fibrinogen), or HD22 (an exosite 2-binding aptamer) attenuates exosite 1-mediated thrombin interactions, thereby providing evidence of inter-exosite allostery.(Kamath *et al.*, 2010, Malovichko *et al.*, 2013, Petrera *et al.*, 2009, Sabo *et al.*, 2006, Treuheit *et al.*, 2011) Therefore, abundant structural and functional evidence supports the existence of allosteric connections between the two exosites and between the exosites and the active site. However, it is uncertain whether the exosite to active site connection is bidirectional such that ligand binding to the active site of thrombin induces reciprocal allosteric changes at the exosites. To address this gap, we used surface plasmon resonance (SPR) to determine the effects of dabigatran, argatroban, and dansylarginine N-(3-ethyl-1,5-pentanediyl)amide (DAPA), active site-directed small molecules that inhibit thrombin with Ki values of 4.5 nM,(Wienen *et al.*, 2007) 39 nM,(Fitzgerald and Murphy, 1996) and 31 nM,(Nesheim *et al.*, 1979a) respectively, on thrombin binding to immobilized γ_A-fibrin, γ′′-fibrin, factor Va, or GPIbα peptide. In addition, the effects of these inhibitors on the binding of radiolabeled thrombin to fibrin clots and its subsequent dissociation were examined.

MATERIALS

Reagents – Human thrombin and plasminogen-free fibrinogen were from Enzyme Research Laboratories (South Bend, IN). Human factors Va and XIII and DAPA were from Haematologic Technologies Inc. (Essex Junction, VT). Recombinant thrombin with Arg residues 93, 97, and 101 changed to Ala (RA-thrombin) was a generous gift from Dr. C. Esmon (Oklahoma Medical Research Foundation). Prionex was from Pentapharm (Basel, Switzerland). γ_A- and γ′-fibrinogen were isolated and characterized as previously described.(Fredenburgh *et al.*, 2008, Pospisil *et al.*, 2003, Schaefer *et al.*, 2006) Batroxobin from the venom of *Bathrops atrox moojeni* was from Pentapharm (Basel, Switzerland). D-Phe-Pro-Arg (FPR) chloromethyl ketone was from Calbiochem. Chromozym-Thrombin (Chz-Th) was from Hyphen BioMed (Neuville sur

Oise, France). Dabigatran was generously provided by Dr. J. van Ryn (Boehringer-Ingelheim, Biberach, Germany), whereas argatroban was a gift from Dr. D. Stump (Genentech, South San Francisco, CA). Recombinant hirudin was from Dade-Behring (Marburg, Germany). Chloramine T and sodium metabisulfite were from Sigma-Aldrich and Bolton-Hunter reagent was from Pierce Biotechnology (Rockford, IL). The synthetic peptide corresponding to the thrombin exosite 2-specific binding site at residues 269-286 on GpIb [illegible]-Glu-Asp-Thr-Asp-Leu-Tyr(PO3)-Asp-Tyr(PO3)-Tyr(PO3)-Pro-Glu-Glu-Asp-Thr-Glu-Gly; GpIb [illegible]-286pp) was synthesized with three phosphorylated Tyr residues by Mimotopes (Minneapolis, MN).(Lechtenberg *et al.*, 2014, Sabo and Maurer, 2009)

Radiolabeled thrombin - To 10 µl of 0.2 M sodium borate, pH 8.0, and 10 µl (1 mCi) of $Na^{125}I$ (McMaster University Nuclear Reactor, Hamilton, ON) was added 5 µl of 1.5 mM Bolton-Hunter reagent in DMSO. The reaction was initiated by adding 10 µl of 5 mg/ml chloramine T in 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS). After incubation for 1 min at 23°C, the reaction was stopped by addition of 10 µl of 12 mg/ml sodium metabisulfite in PBS. To the reaction mixture was added 150 µg of thrombin and after incubation for 1 hr at 23°C, the reaction was terminated by addition of 100 µl of 0.2 M glycine in 0.2 M sodium borate, pH 8.0. Labeled thrombin was isolated on a PD-10 column (GE Healthcare, Baie d'Urfe, PQ), which was equilibrated and eluted with 20 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TBS) containing 0.01% Tween-80 (TBS-Tw). The specific activity of the labeled thrombin was 3.8×10^8 cpm/mg.

Active site-blocked thrombin – Thrombin (3–3.5 mg/ml) was incubated with 2-fold molar excess of FPR chloromethyl ketone at 23°C. After 30 min, absence of residual activity of 2 µM FPR-thrombin was determined at 405 nm with 200 µM Chz-Th in a Spectramax plate reader (Molecular Devices, Sunnyvale CA). The sample was diluted to 15 ml with TBS and concentrated using an Amicon Ultra 4 ml – 3000 MW centrifugal cartridge (Millipore); a procedure that was repeated 3 times prior to determining the thrombin concentration by measuring absorbance at 280 nm.(Fredenburgh *et al.*, 1997)

METHODS

SPR – Studies were performed using a Biacore T200 (GE Healthcare). α-fibrinogen, γ' fibrinogen, and factor Va were immobilized to separate flow cells of a CM5 sensor chip using the amine coupling kit from GE Healthcare.(Vu *et al.*, 2013) Briefly, after injecting the 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride/N-hydroxysuccinimide mixture into the flow cell at a rate of 10 µl/min for 420 s, 50 µg/ml of α-or γ'-fibrinogen or 28 µg/ml factor Va in 10 mM acetate buffer, pH 4.5, was injected at a rate of 5 µl/min until 8000-9000 response units (RU) were immobilized. Flows cells were then washed with 1 M ethanolamine for 420 s, followed by 20 mM HEPES, 150 mM NaCl, pH 7.4 (HBS) containing 0.01% Tween-80 (HBS-Tw) and 5 mM $CaCl_2$. To convert the immobilized fibrinogen to fibrin, flow cells were subjected to successive 60-min injections of up to 1 µM thrombin, each followed by a wash with HBS-Tw containing 0.5 M $CaCl_2$ to remove thrombin.(Fredenburgh *et al.*, 2008, Petrera *et al.*, 2009, Vu *et al.*, 2013)

To measure binding to immobilized α-fibrin, γ'-fibrin or factor Va, increasing concentrations of up to 5 µM thrombin or FPR-thrombin were injected into the flow cells. To measure the effects of dabigatran, argatroban, and DAPA on thrombin binding to fibrin or factor Va, 250 – 500 nM thrombin or FPR-thrombin was incubated with 0-5 µM inhibitor and injected into the flow cells. All SPR experiments were performed in HBS-Tw containing 5 mM $CaCl_2$ at a flow rate of 25 µl/min. Association times were 120 s and dissociation was monitored by washing the flow cells with HBS-Tw for 600 s. Between runs, flow cells were regenerated with a 45 s wash with HBS-Tw containing 0.5 M $CaCl_2$.

The Kd values for one-site binding of thrombin to γ_A-fibrin were determined from plots of RU values at equilibrium (Req) *versus* thrombin or FPR-thrombin concentration using Biacore T200 Evaluation software v 1.0. Nonspecific binding was accounted for by subtracting the control RU values obtained from the unmodified flow cell. To calculate two-site binding of thrombin to γ'-fibrin or factor Va, plots were subjected to kinetic analysis using a two-site binding model. EC_{50} values for the effect of dabigatran, argatroban, or DAPA on thrombin binding were determined by plotting Req values against the concentration of active site inhibitor and using non-linear regression to fit these to a rectangular hyperbola.

To examine the effect of the active site inhibitors on exosite 2-mediated binding, 1 mg of GpIb-(269)-pp in HBS was biotinylated at its NH_2-terminus by incubation with a 10-fold molar excess

of Hook-Sulfo-NHS-LC biotin (G-Biosciences, St. Louis, MO) for 1 h at 23°C. After separation from unincorporated reagent by chromatography on Sephadex G15, fractions containing biotinylated peptide were identified by monitoring absorbance at 204 nm. Streptavidin (Sigma) was attached to flow cells of a CM5 chip to about 12000 RU and biotin (b)-GpIb -2366pp was then adsorbed to about 150 RU above the streptavidin background. Aliquots of thrombin or RA-thrombin up to 2 µM in HBS-Tw were injected into the flow cell at 25 µl/min for 80 s, followed by HBS-Tw for 200 s. Between runs, flow cells were regenerated with 1 M NaCl for 30 s. To measure the effect of dabigatran, argatroban, or DAPA on this interaction, 0-2 µM thrombin was injected in the presence of 2.5 µM active site inhibitor at 25 µl/min for 60 s. Binding affinities were calculated as described above.

Effect of dabigatran, argatroban and DAPA on thrombin binding to fibrin clots – Binding of ^{125}I-thrombin to α/γ_A- or α/γ'-fibrin clots in the absence or presence of active site inhibitors was assessed by measuring the amount of ^{125}I-thrombin in the supernatants of compacted clots prepared with α/γ_A- or α/γ'-fibrinogen.(Fredenburgh *et al.*, 2008, Petrera *et al.*, 2009, Vu *et al.*, 2013a) To a series of 1.5-ml micro-centrifuge tubes containing 0-1 µM dabigatran or argatroban, 0-4 µM DAPA, or 0-0.1 µM hirudin were added 10 nM ^{125}I-thrombin, 3 µM α_A/γ_A-fibrinogen or 1 µM α_A/γ'-fibrinogen, 2 mM $CaCl_2$ and 1 unit/mL batroxobin in TBS-Tw in a total volume of 100 µl. Batroxobin was used to induce clotting because its activity is unaffected by the inhibitors (not shown). After incubation for 1 h at 23°C, clots were pelleted by centrifugation at 14,000 × g for 4 min and 50-µl aliquots of supernatant were removed and counted for radioactivity using a gamma counter (Wizard^2 2470, Perkin-Elmer) to determine the concentration of unbound ^{125}I-thrombin. Bound ^{125}I-thrombin was calculated by expressing this value relative to the total ^{125}I-thrombin in supernatants of tubes without fibrinogen. EC_{50} values for the effect of the inhibitors were calculated by fitting plots of bound ^{125}I-thrombin versus the inhibitor concentration by non-linear regression analysis to an equation for a rectangular hyperbola. $K_{i\ obs}$ values were then determined using the Cheng-Prusoff equation.

$$EC_{50} = (1 + ([Fg]/ K_d))\ K_{i\ obs}$$

where [Fg] is the fibrinogen concentration and K_d is the dissociation constant of ^{125}I-thrombin for fibrinogen as determined in a separate experiment.

Dissociation of ^{125}I-thrombin from fibrin clots. Dissociation of ^{125}I-thrombin from preformed fibrin clots was performed as described.(Fredenburgh *et al.*, 2008) Briefly, clots were formed around plastic inoculation loops by clotting a 130 µl solution containing 3 µM α/γ_A-fibrinogen and 30 nM factor XIII in TBS-Tw containing 2 mM $CaCl_2$ with 10 nM ^{125}I-thrombin. After incubation for 1 h, clots were immersed in 50 ml plastic tubes containing 10 ml of 100 nM dabigatran, argatroban or DAPA, or 2 M NaCl in TBS-Tw at 23°C. At defined intervals, aliquots were removed, counted for radioactivity, and returned to the tubes. Plots of residual radioactivity versus time were analyzed using a 2-phase exponential decay equation.(Fredenburgh *et al.*, 2008)

Statistical analysis – All experiments were performed at least three times. Results are presented as the mean ± standard deviation (SD). Paired data were compared using Student t-tests and p values less than 0.05 were considered statistically significant.

RESULTS

SPR analysis of the interaction of thrombin with immobilized fibrin or factor Va – Increasing concentrations of thrombin or FPR-thrombin were injected into flow cells containing immobilized α/γ_A-fibrin, α/γ'-fibrin, or factor Va.(Fredenburgh *et al.*, 2008, Petrera *et al.*, 2009) Because sensorgram profiles rapidly reached equilibrium, Req values were determined and plotted versus the analyte concentration to determine Kd (Fig. 1). As expected, more thrombin and FPR-thrombin bound to α/γ'-fibrin than to α/γ_A-fibrin. Binding to γ_A-fibrin was via a single lower affinity site, whereas binding to α/γ'-fibrin occurred through higher and lower affinity interactions, demonstrating involvement of both exosites (Table 1).(Fredenburgh *et al.*, 2008, Vu *et al.*, 2013) More thrombin and FPR-thrombin also bound to factor Va than to α/γ_A-fibrin. The higher affinities for factor Va than for α/γ_A-fibrin are consistent with interaction mediated by

both exosites.(Dharmawardana *et al.*, 1999, Esmon and Lollar, 1996, Segers *et al.*, 2007) The affinities of FPR-thrombin for fibrin and factor Va were ~50% higher than those of active thrombin ($p < 0.001$), demonstrating that presence of a covalent adduct in the active site promotes thrombin binding to these ligands. These results suggest that immobilized fibrin and factor Va are suitable targets to examine the effects of active site ligands on exosite binding.

Effects of dabigatran, argatroban or DAPA on thrombin binding to immobilized fibrin or factor Va– To assess the effect of active site inhibitors on the exosite-dependent binding of thrombin to immobilized α_A/γ_A-fibrin, α_A/γ'-fibrin, or factor Va, samples containing 250-500 nM thrombin or FPR-thrombin pre-incubated with increasing concentrations of dabigatran, argatroban, or DAPA were injected. Dabigatran reduced thrombin bound to α_A/γ_A-fibrin, α_A/γ'-fibrin, and factor Va in a concentration-dependent manner (Fig. 2) with EC_{50} values of 184.6 ± 4.3 nM, 182.4 ± 15.0 nM, and 204.2 ± 17.0 nM, respectively. At saturation, dabigatran reduced thrombin binding to α_A/γ_A-fibrin, α_A/γ'-fibrin, and factor Va by 47.6 ± 0.4%, 28.4 ± 1.6%, and 37.9 ± 3.2%, respectively (Table 2). As a control, experiments using FPR-thrombin in place of thrombin demonstrated no effect of dabigatran on binding, confirming that dabigatran binds specifically to the active site (not shown). These results provide evidence for active site-mediated perturbation of exosite function by dabigatran.

Experiments were then repeated with argatroban and DAPA to determine whether attenuation of thrombin binding to fibrin or factor Va was unique to dabigatran. In contrast to the results with dabigatran, argatroban and DAPA increased thrombin binding to α_A/γ_A-fibrin, α_A/γ'-fibrin, and factor Va in a concentration-dependent manner with EC_{50} values for argatroban of 62.4 ± 4.8 nM, 59.4 ± 5.1 nM, and 23.4 ± 8.8 nM, respectively, and for DAPA of 514.1 ± 24.0 nM, 515.9 ± 31.0 nM, and 565.1 ± 200 nM, respectively (Fig. 2). Although argatroban and DAPA are reported to bind thrombin with similar affinities, the EC_{50} values for argatroban were more than 10-fold lower than those for DAPA. As observed with dabigatran, neither argatroban nor DAPA had any effect on FPR-thrombin binding to α_A/γ_A-fibrin, α_A/γ'-fibrin, or factor Va, thereby highlighting the importance of access to the active site of thrombin for their

activity (not shown). Therefore, reversible engagement of the active site by small molecules allosterically modulates the exosite-mediated interactions of thrombin with fibrin and factor Va.

Effects of dabigatran, argatroban and DAPA on thrombin binding to GpIb – The effects of the active site inhibitors on the exosite 2-mediated interaction of thrombin with GpIb, a peptide to which it binds exclusively via exosite 2,(Lechtenberg *et al.*, 2014) was investigated using SPR. Thrombin bound to the immobilized peptide with a Kd of 306 ± 11 nM (Fig. 3A; Table 1); an affinity intermediate between that reported for a similar peptide and for glycocalicin.(De Cristofaro *et al.*, 2000, Lechtenberg *et al.*, 2014) In contrast, R93A-thrombin, an exosite 2 variant, did not bind (not shown), consistent with the reported specificity of this interaction for exosite 2. Thrombin binding to the GpIb 269 286ppp peptide was then quantified in the presence of increasing concentrations of inhibitors. Whereas dabigatran had no effect on thrombin binding, argatroban and DAPA increased the amount of thrombin bound by 17% and 25%, respectively (Fig. 3B; Table 2). Binding to GpIb was then quantified in the presence of 2.5 μM dabigatran, argatroban or DAPA, Whereas thrombin bound with similar affinity in the presence of dabigatran (306 ± 10 nM; not shown) as in its absence, the affinity of thrombin for GpIb 286ppp significantly ($p < 0.001$) increased by 25-35% in the presence of argatroban or DAPA (Kd values of 229 ± 7 nM and 197 ± 5 nM, respectively). These findings demonstrate that occupation of the active site by some, but not all, small molecules increases the affinity of ligands for exosite 2, similar to the observation for exosite 1-dependent interactions.

Effects of dabigatran, argatroban and DAPA on thrombin binding to fibrin clots – Although the affinities of thrombin and FPR-thrombin for α/γ_A- and α/γ'-fibrin determined by SPR are comparable with those obtained with fibrin clots,(Fredenburgh *et al.*, 2008) it was important to confirm that the allosteric response observed with SPR also occurred with three-dimensional fibrin clots. ^{125}I-thrombin bound α/γ_A- and α/γ'-fibrin clots with K_d values of 3.6 ± 0.3 μM and 1.2 ± 0.2 μM (not shown), respectively; values comparable with those obtained using SPR and with those published previously.(Vu *et al.*, 2013) Up to

70% of the ^{125}I-thrombin bound to fibrin, demonstrating that labeling had little effect on the capacity of thrombin to bind to fibrin.

Dabigatran reduced ^{125}I-thrombin binding to γ_A/γ_A- and γ_A/γ'-fibrin clots in a concentration-dependent manner with K_i values of 3.8 ± 1.5 nM and 26.0 ± 4.0 nM, respectively (Figure 3). As observed using SPR, dabigatran produced a 2-fold greater reduction in thrombin binding to γ_A/γ_A-fibrin than to γ_A/γ'-fibrin ($p < 0.05$); a difference that likely reflects the higher affinity of thrombin for γ_A/γ'-fibrin. To verify the role of exosite 1 in binding, the effect of hirudin also was examined. As expected, hirudin fully inhibited ^{125}I-thrombin binding to γ_A/γ_A- and γ_A/γ'-fibrin. In agreement with the SPR observations, argatroban and DAPA enhanced the binding of ^{125}I-thrombin to γ_A/γ_A- and γ_A/γ'-fibrin clots to a similar extent (~15-25%) (Figure 4, Table 3). Collectively, therefore, the data obtained with fibrin clots support the SPR results and suggest that active site engagement induces allosteric changes in the thrombin exosites, with the direction and magnitude determined by the ligand.

Effects of dabigatran, argatroban and DAPA on ^{125}I-thrombin dissociation from fibrin clots – To determine whether the alteration in the affinity of thrombin for fibrin in the presence of active site inhibitors influences the amount of thrombin associated with fibrin, dissociation from fibrin clots was examined.(Fredenburgh *et al.*, 2008) Clots formed from γ_A/γ_A-fibrinogen in the presence of ^{125}I-thrombin were incubated in buffer in the absence or presence of active site inhibitors and the amount of ^{125}I-thrombin released from the clot over time was quantified. Biphasic analysis was used to evaluate the fast non-adsorbent and slow bound phases of dissociation of thrombin from fibrin. In the control, the bound fraction dissociated with a half-life of 20.5 ± 3.1 h, whereas in the presence of 2 M NaCl the rate of dissociation was significantly faster ($p = 0.02$) at 14.2 ± 1.2 h (Fig. 5). The half-life values in the presence of 2.5 μM dabigatran, argatroban, and DAPA were 17.7 ± 2.3 h, 25.5 ± 2.9 h, and 23.7 ± 2.9 h, respectively. Although these values were not significantly different from the control, there was a trend for more rapid dissociation in the presence of dabigatran, and the half-lives in the presence of argatroban and DAPA were significantly longer than that in the presence of dabigatran ($p = 0.01$ and 0.02, respectively). These data are consistent

with the effects of the inhibitors on the affinity of thrombin for fibrin, and confirm the differences in response between dabigatran and argatroban or DAPA.

DISCUSSION

This purpose of this study was to determine whether occupation of the active site of thrombin by small molecules induces allosteric changes at its exosites. We chose fibrin, factor Va and GpIbα269-286ppp as targets for two reasons. First, their use enabled studies with unmodified, active thrombin. Second, the mode of interaction of thrombin with these ligands has been well characterized; thus, binding of thrombin to γ_A-fibrin and GpIbα269-286ppp is solely dependent on exosite 1 and exosite 2, respectively, whereas thrombin binding to γ'-fibrin and factor Va requires both exosites. Dabigatran and argatroban were chosen because they are highly specific for thrombin and their interactions with the active site of thrombin have been extensively investigated and because both agents are in clinical use. DAPA was chosen to complement the results with argatroban because both incorporate an Arg group that is essential for specificity.(Brandstetter *et al.*, 1992, Mathews and Tulinsky, 1995)

Dabigatran saturably reduces thrombin binding to γ_A-fibrin and factor Va by 28-48%, but has no effect on the interaction of thrombin with GP1bα269-286ppp. Surprisingly, argatroban and DAPA increase thrombin binding to γ_A-fibrin, factor Va and GpIbα269-286ppp by 13-47%. The observations with fibrin were confirmed using three-dimensional fibrin clots and were not dependent on the affinity of the inhibitors for thrombin because dabigatran, argatroban and DAPA inhibit thrombin with similar nanomolar K_i values. DAPA and argatroban also slow the dissociation of thrombin from fibrin clots in a manner consistent with their enhancement of the affinity of thrombin for fibrin. Thus, these studies demonstrate the bidirectional nature of the allosteric network in thrombin, and provide evidence that responses are ligand specific.

Investigation into the allosteric regulation of thrombin has shown that binding of a variety of effectors to the exosites modulates the active site. This phenomenon has been attributed to a complex allosteric network within thrombin that is subject to modulation through surface residues.(Gandhi *et al.*,

2008, Lechtenberg *et al.*, 2010) Within the active site, residues in the S_{1-4} subsites and the 60- and autolysis-loops contribute to this allosteric network.(Adams *et al.*, 2009, Croy *et al.*, 2004, Gandhi *et al.*, 2008, Ng *et al.*, 2009) These regions within the active site make unique contact with individual substrates and are perturbed by exosite ligands, thereby rendering them central to allosteric regulation. It is interesting to compare the interaction of dabigatran, argatroban and DAPA with the active site of thrombin because dabigatran induces effects that differ from those of argatroban and DAPA. Although dabigatran and argatroban engage the S1, S2, and S4 subsites in the active site, they do so in distinct ways.(Bode *et al.*, 1992, Brandstetter *et al.*, 1992, Hauel *et al.*, 2002, Mathews and Tulinsky, 1995) Thus, the differences in exosite response elicited by the various inhibitors likely reflect unique points of contact within the allosteric network. In an analogous fashion, different exosite 1 ligands evoke unique changes in thrombin activity,(Fredenburgh *et al.*, 1997, Liu *et al.*, 1991, Ng *et al.*, 2009, Petrera *et al.*, 2009, Ye *et al.*, 1991) suggesting that the exosites are composed of distinct subdomains.(Abdel Aziz *et al.*, 2012, Bock *et al.*, 2007, Bode, 2006, Kretz *et al.*, 2006, Page *et al.*, 2005) Consistent with the results observed here, argatroban has previously been shown to increase the affinity of hirudin peptide for exosite 1 on thrombin.(Parry *et al.*, 1993) These observations were not limited to exosite 1 because argatroban and DAPA also increased the affinity of thrombin for GpIb □ peptide; an in 2.(Lechtenberg *et al.*, 2014) The structural explanations for how different small molecules can bind to active site of thrombin and evoke distinct responses at the exosites remain to be determined.

The observation that binding of dabigatran, argatroban or DAPA to the active site of thrombin induces changes at the exosites is consistent with the hypothesis that changes transmitted over the allosteric network are bidirectional. Most studies have focused on the effect of exosite ligands on active site function. However, calorimetry and fluorescence studies demonstrate that structural changes that result from ligand binding at the active site or exosite 1 can run in both directions.(De Cristofaro *et al.*, 1995, Parry *et al.*, 1993, Treuheit *et al.*, 2011) This property suggests that covalent occupation of the active site by FPR should alter exosite function; a concept supported by some studies,(Bock *et al.*, 1997, Croy *et al.*, 2004, Fredenburgh *et al.*, 2001, Li *et al.*, 2010) but not by others.(Figueiredo *et al.*, 2012, Kroh *et al.*, 2007, Pozzi *et al.*, 2013a, Treuheit *et al.*, 2011) Although extensive investigations of thrombin structure have not

provided a unifying model of thrombin allostery, they demonstrate that thrombin is a highly dynamic molecule with numerous conformations and networks of internal communication.

The capacity of dabigatran to attenuate thrombin binding to fibrin and factor Va reveals a unique mechanism by which it might function as an anticoagulant. Thus, in addition to inhibiting the catalytic activity of thrombin, dabigatran may displace thrombin from fibrin or platelets. This would reduce the protection from inhibition afforded by such binding, and would permit access to natural inhibitors such as antithrombin.(Fredenburgh *et al.*, 2008, Mosesson, 2007b) Because argatroban promotes thrombin binding to fibrin, it is likely that not all direct thrombin inhibitors benefit from this secondary effect. This raises the possibility that in addition to simple target potency, future efforts at structure-based drug design should consider the secondary effects of inhibitors; effects that may be altered by refining contacts within the active site.

In summary, our results suggest that active site inhibitors modulate thrombin function mediated by the exosites. This reverse direction of allosteric signaling provides greater insight into the dynamic nature of thrombin. Furthermore, different active site-directed agents evoke unique responses, demonstrating that their inhibitory function can be refined. Thus, exploitation of its allosteric network endows thrombin with additional mechanism of regulation without compromising its repertoire of substrates. This demonstrates that the versatility of thrombin is a consequence of the intricate connections between the active site and the exosites.

Table 1. Dissociation constants (K_d) for the binding of thrombin to fibrin, factor Va and GpIbα269-286ppp. The binding of thrombin or FPR-thrombin to immobilized α_A/γ_A-fibrin, α_A/γ'-fibrin, factor Va or GpIbα269-286ppp was quantified using SPR. K_d values for thrombin binding to α_A/γ_A-fibrin and GpIbα269-286ppp were determined by steady state analysis, whereas bivalent binding of thrombin to α_A/γ'-fibrin and factor Va was determined by kinetic analysis for a two-site model using BIAevaluation software. K_d values are shown as mean ± S.D. for three separate experiments.

	Thrombin		FPR-thrombin	
	Kd1	**Kd2**	**Kd1**	**Kd2**
Target	**(nM)**			
α_A/γ_A-fibrin	3887 ± 36	-	2110 ± 140	-
α_A/γ'-fibrin	2631 ± 18	211.6 ± 0.9	1434.2 ± 56.8	110.4 ± 14.0
Factor Va	1471 ± 73	219.8 ± 189.4	831.2 ± 13.7	130.4 ± 17.7
GpIbα269-286ppp	306 ± 11	-	211 ± 1	-

Table 2. Effect of dabigatran, argatroban, or DAPA on thrombin binding to α_A/γ_A-fibrin, α_A/γ'-fibrin, factor Va, or GpIbα269-286ppp. The effect of 0 – 5000 nM dabigatran, argatroban, or DAPA on the binding of thrombin to immobilized α_A/γ_A-fibrin, α_A/γ'-fibrin, factor Va or GpIbα269-286ppp was quantified using SPR. EC_{50} values were determined by plotting the Req values against the inhibitor concentration and fitting by nonlinear regression analysis using BIAevaluation software. Change is the calculated difference between thrombin binding in the absence of inhibitors and that determined at saturating inhibitor concentration. Values are shown as mean ± S.D. for three separate experiments.

	Dabigatran		Argatroban		DAPA	
Target	**EC50 (nM)**	**Change (%)**	**EC50 (nM)**	**Change (%)**	**EC50 (nM)**	**Change (%)**
α_A/α_A-fibrin	184.6 ± 4.3	- 47.6 ± 0.4	62.4 ± 4.8	+ 47.2 ± 1.0	514.1 ± 24.0	+ 25.1 ± 0.5
α_A/γ'-fibrin	182.4 ± 15.0	- 28.4 ± 1.6	59.4 ± 5.1	+ 44.5 ± 2.3	515.9 ± 31.0	+ 27.7 ± 1.5
Factor Va	204.2 ± 17.0	- 37.9 ± 3.2	23.4 ± 8.8	+ 15.1 ± 3.9	565.1 ± 200	+ 13.2 ± 3.2
GpIb 269-286ppp	--	0	161.0 ± 2.6	+17.3 ± 0.6	1288.3 ± 32.3	+25.4 ± 0.0

Table 3. Effect of dabigatran, argatroban, DAPA or hirudin on ^{125}I-thrombin binding to fibrin clots. Binding of ^{125}I-thrombin to α_A/γ_A- or α_A/γ'-fibrin clots in the absence or presence of dabigatran, argatroban, DAPA, or hirudin was determined by measuring the amount of ^{125}I-thrombin in the supernatants of compacted clots prepared by incubating α_A/γ_A- or α_A/γ'-fibrinogen with a catalytic amount of batroxobin. EC_{50} values were determined by nonlinear regression analysis and converted to K_i values by correction for K_d of ^{125}I-thrombin for fibrin. Change denotes the maximal change in the percentage of bound ^{125}I-thrombin compared with that in the absence of inhibitor.

Ligand	Dabigatran		Argatroban		DAPA		Hirudin	
	K_i (nM)	**Change (%)**	**K_i (nM)**	**Change (%)**	**K_i (nM)**	**Change (%)**	**K_i (nM)**	**Change (%)**
α_A/α_A-fibrin	3.8 ± 1.5	-21.4 ± 3.3	2.4 ± 1.9	+23.5 ± 0.5	2.1 ± 0.9	+17.0 ± 2.7	3.4 ± 0.3	-100
α_A/γ'-fibrin	26.0 ± 4.0	-9.4 ± 1.4	7.0 ± 5.6	+26.1 ± 0.4	19.6 ± 19.1	+20.1 ± 3.6	3.4 ± 0.1	-100

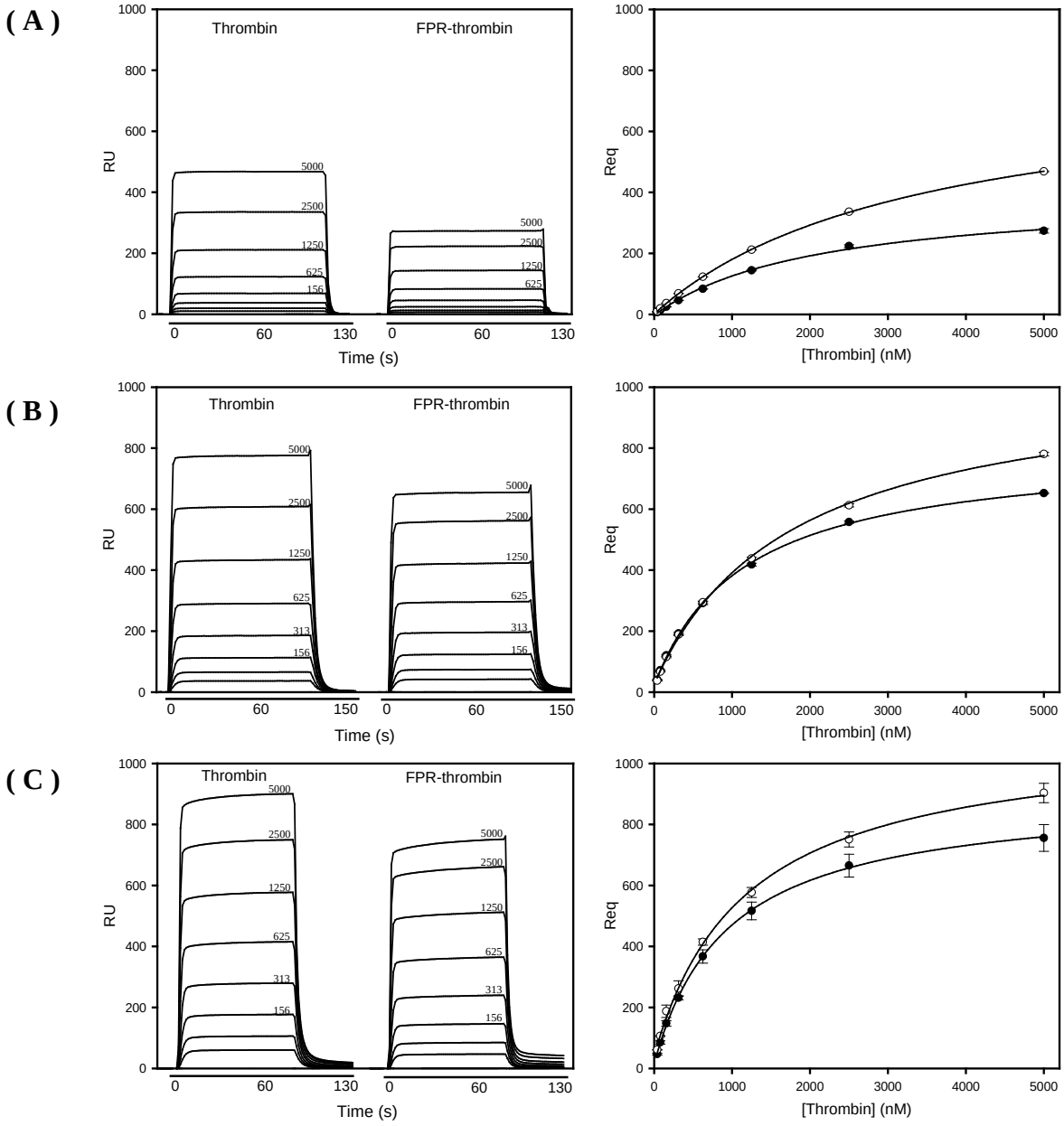


Figure 1. Binding of thrombin or FPR-thrombin to immobilized α_A/γ_A-fibrin, α_A/γ'-fibrin, or factor Va. Increasing concentrations of thrombin or FPR-thrombin up to 5000 nM were injected into individual flow cells of a CM5 biosensor chip containing immobilized (A) α_A/γ_A-fibrin, (B) α_A/γ'-fibrin, or (C) factor Va. Samples were injected for 120 s at 25 µl/min, followed by a 45 s wash to monitor dissociation. *Left panels*; sensorgram tracings of representative runs for thrombin and FPR-thrombin are shown in succession. Analyte concentrations are 39, 78, 156, 313, 625, 1250, 2500, 5000 nM, or as indicated. *Right panels*; from each sensorgram, the amount of thrombin (open circles) or FPR-thrombin (closed circles) bound at equilibrium after background correction (Req) was determined and plotted against the thrombin concentration. Data points represent the mean ± S.D. of 3 experiments, and the *lines* represent nonlinear regression analysis.

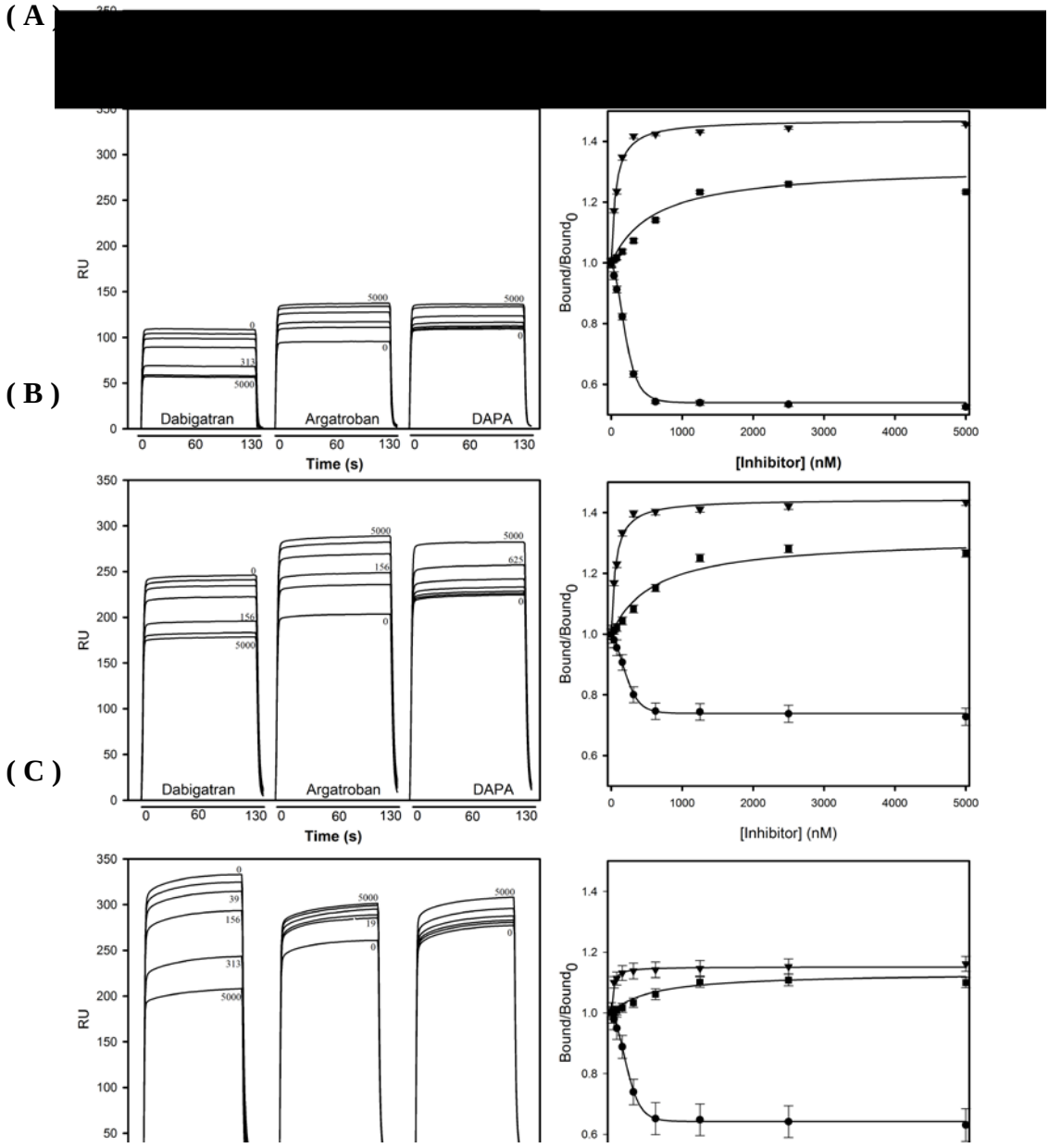


Figure 2. Effect of dabigatran, argatroban, or DAPA on thrombin binding to immobilized α_A/γ_A-fibrin, α_A/γ'-fibrin, or factor Va. Increasing concentrations of dabigatran (circles), argatroban (triangles), or DAPA (squares) up to 5 µM were pre-incubated with thrombin prior to injection into individual flow cells containing immobilized (A) α_A/γ_A-fibrin, (B) α_A/γ'-fibrin, or (C) factor Va. Sample injection was followed by a 45 s wash with buffer to monitor dissociation. *Left panels*; representative sensorgrams of the effects of increasing concentrations of dabigatran, argatroban or DAPA on thrombin binding are shown in succession. *Right panels*; the ratios of Req values of thrombin bound in the absence ($Bound_o$) and presence (Bound) of dabigatran, argatroban or DAPA are plotted against the input inhibitor concentrations. Data points represent the mean ± S.D. of 3 experiments, and the *lines* represent nonlinear regression analysis.

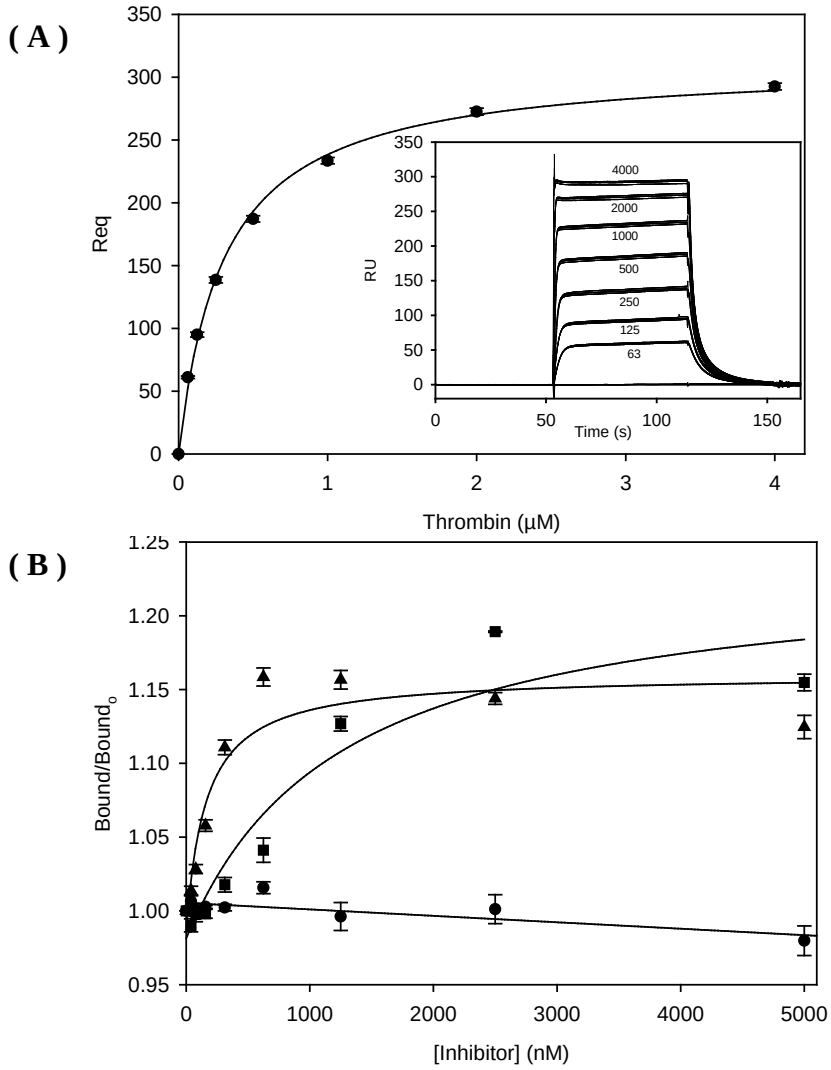


Figure 3. Binding of thrombin to immobilized GpIb [illegible]pp. *A.* Increasing concentrations of thrombin up 4 μM were injected at a rate of 25 μl/min into flow cells of a streptavidin-coated CM5 biosensor chip containing adsorbed biotinylated GpIb [illegible]pp. Response units (RU) are plotted versus time for the indicated thrombin concentrations (in nM) in triplicate (inset). RU values at equilibrium (Req) were determined and plotted versus the input thrombin concentration. The data were analyzed by nonlinear regression (line) to a rectangular hyperbola to determine Kd. Symbols represent mean ±SD for from 3 runs. *B.* Thrombin (250 nM) was injected into flow cells containing immobilized biotinylated GpIb [illegible]pp in the presence of 0-5000 nM dabigatran (circles), argatroban (triangles), or DAPA (squares). Req values were determined, converted to a fraction of that measured in the absence of inhibitor (Bound/Bound$_0$), and plotted versus the inhibitor concentration. Data points indicate mean ± SD for 3 determinations, and the *lines* represent nonlinear regression analyses.

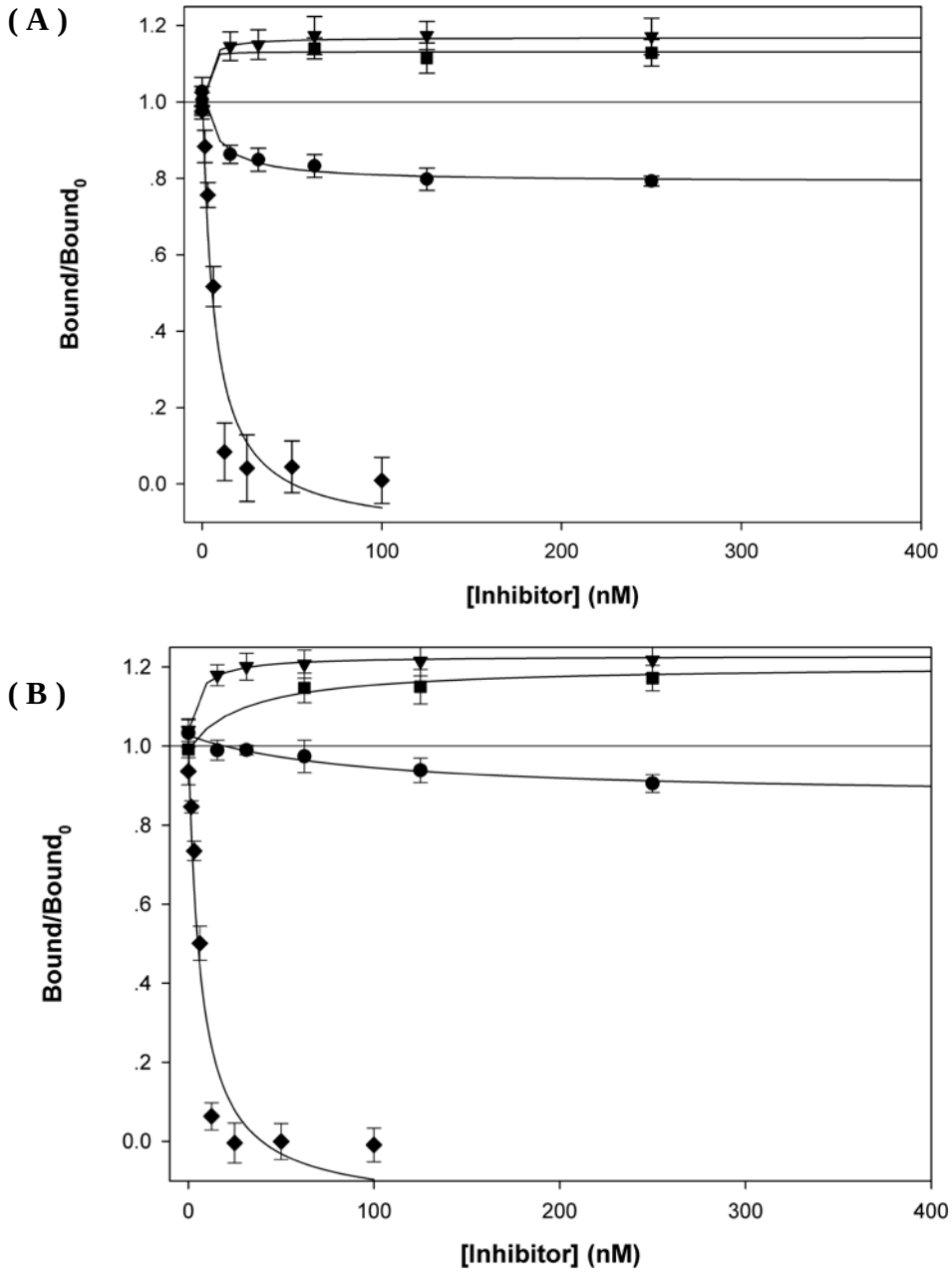


Figure 4. Effect of dabigatran, argatroban, DAPA, or hirudin on the binding of ^{125}I-thrombin to fibrin clots. Increasing concentrations of dabigatran (circles), argatroban (triangles), DAPA (triangles), or hirudin (diamonds) were added to samples containing 10 nM ^{125}I-thrombin, 2 mM $CaCl_2$, 1 U/mL batroxobin and (A) 3 μM □$_A$/γ_A-fibrinogen, or (B) 1 μM □$_A$/γ'-fibrinogen. After incubation for 1 h, clots were pelleted and aliquots of supernatant were counted for radioactivity. The ratios of ^{125}I-thrombin bound in the absence or presence of inhibitor are plotted against the inhibitor concentrations. Data points represent the mean ± S.D. of 2-3 experiments, each performed in duplicate, and the *lines* represent nonlinear regression analysis.

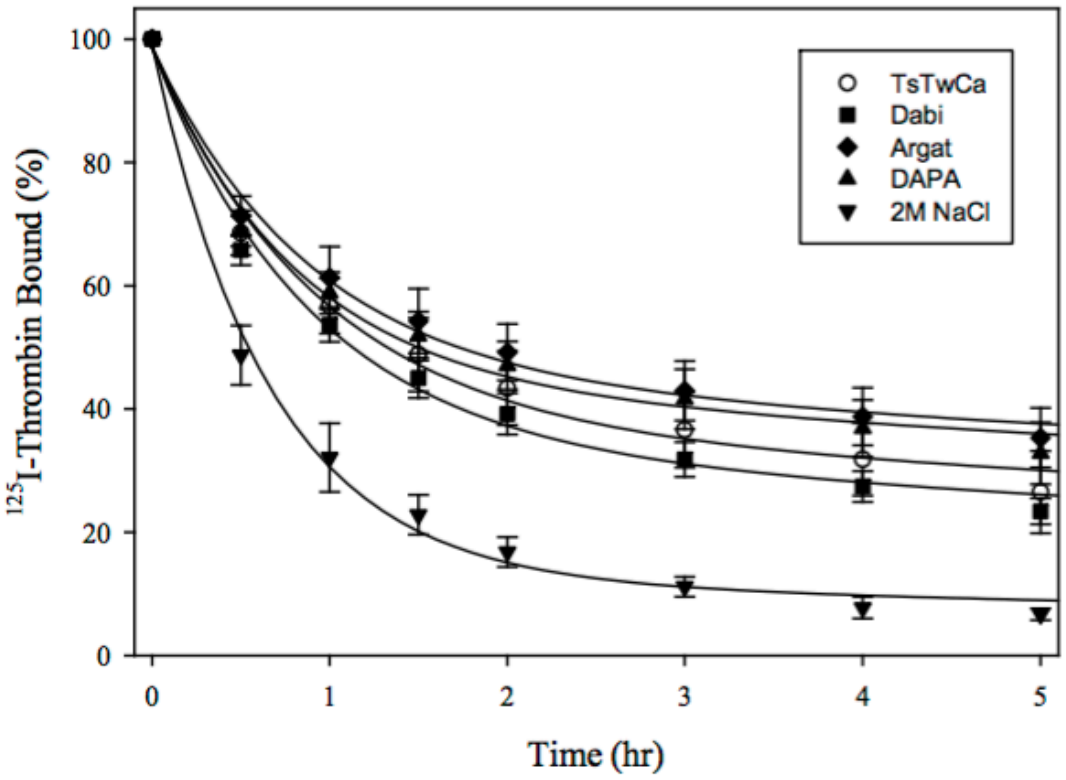
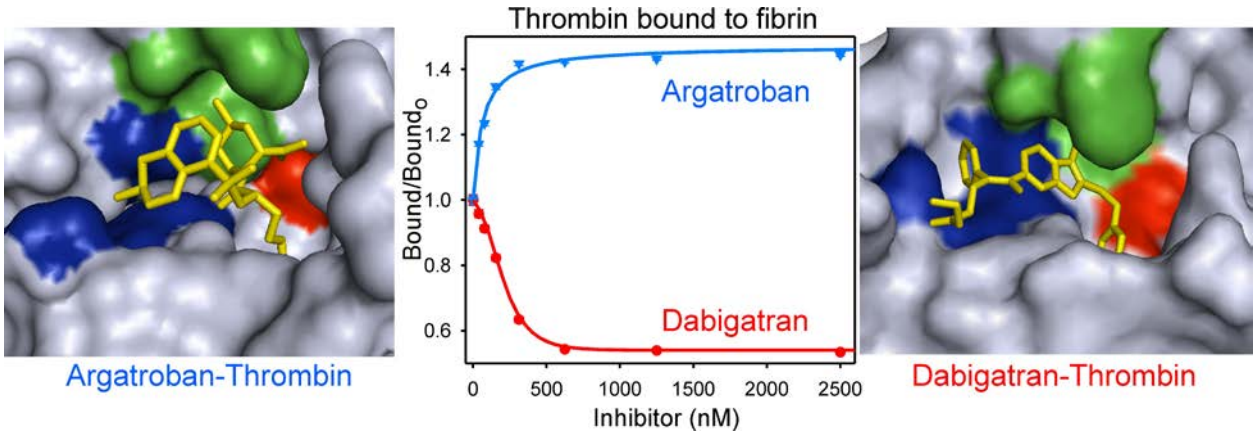


Figure 5. Effect of dabigatran, argatroban, DAPA, or hirudin on the dissociation of ^{125}I-thrombin from fibrin clots. Clots were formed around plastic loops by incubating 3μM α/α-fibrinogen and 30 nM factor XIII with 10 nM ^{125}I-thrombin in the presence of 2 mM $CaCl_2$ for 1 h. Clots were immersed in 10 ml buffer containing 100 nM dabigatran, argatroban, or DAPA, or 2 M NaCl. At intervals, aliquots were removed and counted for radioactivity. Residual counts were normalized with respect to time zero and plotted versus time. Data were analyzed by two phase exponential decay (lines) to determine the half-lives of dissociation. Symbols represent the mean ± SD of 4 experiments.

Engagement of the active site of thrombin by dabigatran or argatroban modulates its exosite-mediated interactions with fibrin, factor Va, and glycoprotein Ibα.
Calvin H. Yeh, Alan R. Stafford, Beverly A. Leslie, James C. Fredenburgh, Jeffrey I. Weitz



CHAPTER FIVE

Mutation of the active site serine to alanine enhances the allosteric capacity of thrombin

Calvin H. Yeh, Alan R. Stafford, Beverly A. Leslie, James C. Fredenburgh, Jeffrey I. Weitz

The Thrombosis and Atherosclerosis Research Institute
Departments of Medicine and of Biochemistry and Biomedical Sciences
McMaster University, Hamilton, Canada

*Correspondence to Dr. Jeffrey I. Weitz, Thrombosis and Atherosclerosis Research Institute, 237 Barton St. E, Hamilton, Ontario, Canada L8L 2X2. Phone: 905-574-8550, FAX: 905-575-2646, email: weitzj@taari.ca

INTRODUCTION

Thrombin is the final effector enzyme in coagulation and exhibits multiple roles through interaction with a large array of target molecules. By cleaving factors (F) V, VIII, XI, XIII, fibrinogen, protein C, and the thrombin activatable fibrinolysis inhibitor, thrombin plays critical roles as the procoagulant, anticoagulant, and antifibrinolytic effector. A major regulatory mechanism for these functions is an extensive allosteric network within the molecule that may be modulated by ligand binding distinct surface contacts on thrombin. Thus, thrombin specificity is selected through binding to target ligands at its active site and the two flanking anion binding domains, exosites 1 and 2.

Ligand engagement governs thrombin activity by (a) localizing the molecule to the site of action, (b) sterically hindering other interactions, and (c) allosterically modulating the adjacent binding sites for ligand recognition. Early studies focused on the electrostatic and steric characteristics of the active site for determining the specificity of thrombin, such as interactions of the substrate with side chains of the S1-S4 residues, and structural observations that the 60s and 149s (γ) loops sterically hinder access to the active site (Di Cera and Cantwell, 2001, Huntington, 2005). Binding of ligands to exosite 1 is responsible for (a) the interaction of thrombin with multiple substrates, such as fibrinogen, FV, FVIII, and the protease-activated receptors (PARs) on platelets, (b) interacting with cofactors such as thrombomodulin (TM) and (c) catalyzing thrombin inhibition by hirudin and heparin cofactor II. Exosite 2 mainly localizes thrombin to other effectors through tethering to heparin, the γ′-chain of fibrin(ogen), and the platelet glycoprotein-1ba.

Allosteric communication between these binding sites has emerged as an important regulatory mechanism for determining thrombin specificity and activity. Thus, ligation of the exosites or the conserved sodium-binding site modulates catalytic activity by altering the conformation of the active site (Gohara and Di Cera, 2011, Kroh *et al.*, 2007, Pozzi *et al.*, 2011). Binding of exosite directed ligands may increase or decrease the cleavage of chromogenic substrates (Ayala and Di Cera, 1994, Liu *et al.*, 1991, Petrera *et al.*, 2009). Ligation of exosite 1 by the hirudin peptide (Fredenburgh *et al.*, 1997, Lechtenberg *et al.*, 2010, Verhamme *et al.*, 2002), platelet PAR-1 peptide (Gandhi et al., 2008) and thrombomodulin (Adams *et al.*, 2009, Gasper *et al.*, 2012, Parry *et al.*, 1993) to exosite 1 cause conformational and functional changes at the active site. Likewise, exosite 2 engagement by prothrombin fragment 2 (Kamath et al., 2010), the

fibrinogen γ'-chain peptide (Siebenlist et al., 2005), and the platelet glycoprotein Ib (Li et al., 2001) affect thrombin activity.

In order to fully explain the unique functional consequences of each ligand, recent work has supported the notion of a network of energetically linked residues within the core of thrombin that governs the global allosteric capacity of the molecule (Gasper *et al.*, 2012, Lechtenberg *et al.*, 2012). This allosteric network can be modulated via surface contacts with exosite or active site binding ligands (Fuglestad *et al.*, 2013, Gasper *et al.*, 2012). These studies explain why site directed mutagenesis of the exosites affects thrombin activity (Qureshi et al., 2009), and places the paradox of ligand-specific reciprocal (Fredenburgh *et al.*, 1997, Lechtenberg *et al.*, 2010, Malovichko *et al.*, 2013, Petrera *et al.*, 2009, Verhamme *et al.*, 2002) or cooperative (Pospisil et al., 2003) intra-exosite and active-site-to-exosite allostery into context. The allosteric capacity of thrombin however, has not been a universally supported phenomena in structural and functional studies (Huntington, 2012, Verhamme *et al.*, 2002).

Many of the structure function relationships have been made by measuring the catalytically inactivated Ser195 to alanine thrombin mutant (S195A-α-thrombin) on the basis of its similarity to thrombin in crystal structures (Huntington and Esmon, 2003). Because mutation of surface residues may cause allosteric effects at other binding sites (Qureshi et al., 2009), the widespread use of S195A-α-thrombin to make inferences on the structural and functional consequences of binding interactions on thrombin may be problematic (Baglin *et al.*, 2002, Gandhi *et al.*, 2010, Izaguirre *et al.*, 2007, Lechtenberg *et al.*, 2010, Li *et al.*, 2004). Many of these studies employ allosteric connections between exosite 1 and the Na+-binding site in S195A-α-thrombin, which has been reported to have altered binding for exosite ligands (Dekker *et al.*, 1999, Krem and Di Cera, 2003, Stone and Hermans, 1995, Stone and Le Bonniec, 1997). Finally, S195A-thrombin variants are also being investigated for clinical applications as an antidote for thrombin directed anticoagulants, such as dabigatran (Sheffield et al., 2014). Thus, the combination of ligand-specific responses and the use of S195A-α-thrombin may reconcile the lack of consensus in measuring thrombin-mediated interactions.

To investigate whether the mutation of the catalytic triad has functional consequences for thrombin allostery, we measured the allosteric capacity of α-thrombin with Ser195 mutated to alanine (S195A-α-

thrombin). Using the physiological ligands fibrin(ogen), and factors Va and VIII, we examined the capacity of S195A-α-thrombin for inter-exosite and active-site-to-exosite allosteric communication compared to α-thrombin.

MATERIALS

Reagents – Human α-thrombin and plasminogen-free fibrinogen were from Enzyme Research Laboratories (South Bend, IN). γA/γA- and γA/γ′-fibrinogens were separated by fractionation on DEAE-Sepharose (GE Healthcare) as described previously (Fredenburgh *et al.*, 2001, Petrera *et al.*, 2009, Pospisil *et al.*, 2003, Vu *et al.*, 2011). Batroxobin from the venom of B. atrox moojeni was from Pentapharm (Basel, Switzerland). D-Phe-Pro-Arg-chloromethyl ketone (FPR) was from Calbiochem. Dabigatran was generously provided by Dr. J. Van Ryn (Boehringer-Ingelheim, Biberach, Germany), whereas argatroban was a gift from Dr. D. Stump (Genentech, South San Francisco, CA). Recombinant full-length hirudin was from Dade-Behring (Marburg, Germany). The *E. coli* derived thrombin variants, S195A-α-thrombin, R93E-S195A-α-thrombin, limited-trypsin digested γ-S195A-α-thrombin, and the wild-type (WTEColi-α-thrombin) were purchased from Cambridge Protein Works Ltd.

Radiolabeled thrombin variants - To 10 µl of 0.2M sodium borate, pH 8.0, and 10 µl (1 mCi) of $Na^{125}I$ (McMaster University nuclear reactor, Hamilton, ON) was added 5 µl of 1.5 mM Bolton-Hunter reagent in DMSO. The reaction was initiated by adding 10 µl of 5 mg/ml chloramine T in 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS). After incubation for 1 min at 23°C, the reaction was stopped by addition of 10 µl of 12 mg/ml sodium metabisulfate in PBS. To the reaction mixture was added 150 µg of thrombin and after incubation for 1 hr at 23°C, the reaction was terminated by addition of 100 µl of 0.2 M glycine in 0.2 M sodium borate, pH 8.0. Labeled thrombin was isolated on a PD-10 column (GE Healthcare, Baie d'Urfe, PQ), which was equilibrated and eluted with 20 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TBS) containing 0.01% Tween-80 (TBS-Tw). The specific activity of the labeled thrombin was 3.8×10^8 cpm/mg.

METHODS

Active site blocked thrombin – Thrombin (3 – 3.5 mg/ml) was incubated with 2-fold molar excess of FPRck at 23°C. After 30 min, residual activity of 2 µM FPR-thrombin was determined at 405 nm with 200 µM ChzTh in a plate reader. The sample was diluted to 15 ml with TBS, then washed three times with 3 ml TBS and concentrated using an Amicon Ultra 4 ml – 3000 MW centrifugal cartridge (Millipore). The concentration of the final volume was determined by measuring absorbance at 280 nm (Fredenburgh et al., 1997).

Surface plasmon resonance (SPR) – Studies were performed using a Biacore T200 (GE Healthcare). γ_A-fibrinogen, γ′-fibrinogen, factor Va, and ovalbumin were immobilized to separate flow cells of a CM5 sensor chip using the amine coupling kit from GE Healthcare. Briefly, after injecting the 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride/N-hydroxysuccinimide mixture into the flow cell at a rate of 10 µl/min for 420 s, 50 µg/ml γ_A- or γ′-fibrinogen in 10 mM acetate buffer, pH 4.5, 28 mg/ml FVa in 10 mM acetate buffer, pH 5.5, or 0.1 mg/ml in 10 mM acetate buffer, pH 5.5, was injected at a rate of 5 µl/min until 8000-9000 response units (RU) were immobilized. Flows cells were then washed with 1 M ethanolamine for 420 s, followed by 20 mM HEPES, 150 mM NaCl, pH 7.4 (HBS) containing 0.01% Tween-80 (HBS-Tw) and 5 mM $CaCl_2$. To convert the immobilized fibrinogen to fibrin, flow cells were subjected to successive 60-min injections of up to 1 µM thrombin, each followed by a wash with HBS-Tw containing 0.5 M $CaCl_2$ (Fredenburgh *et al.*, 2008, Petrera *et al.*, 2009, Vu *et al.*, 2013).

To measure the binding to fibrin and factor Va, increasing concentrations of up to 5 µM thrombin, FPR-thrombin, WTEColi-thrombin, S195A-thrombin, γ-S195A-thrombin, or R93E-S195A-thrombin were injected into the flow cells. To measure the effects of dabigatran, argatroban, and DAPA on the binding of thrombin variatns to fibrin and factor Va, 50 – 500 nM thrombins were pre-incubated in a 96 well plate with 0-5 µM inhibitor and subsequently injected into the flow cells. All SPR runs for this sensor chip were performed with HBS-Tw plus 5mM $CaCl_2$ at a flow rate of 25µl/min and an association time of 120s and dissociation time of 600s. For regeneration, a 45s wash with 0.5M $CaCl_2$ was used.

Kd values for one-site binding were determined by steady state analysis of the RU values at equilibrium (Req) for thrombin or FPR-thrombin binding to immobilized ligands using BIAEvaluation

software version 3.2, as described.(Petrera et al., 2009) To calculate two-site binding of thrombin to γ′-fibrin and factor Va, Req values were plotted against input protease concentrations and analyzed by nonlinear regression analysis of a two-site binding equation using BIAEvaluation software, as described.(Fredenburgh et al., 1997) EC_{50} values for the effect of dabigatran, argatroban, or DAPA on thrombin binding was determined by steady state analysis of the Req plotted against the concentration of active site inhibitor using BIAEvaluation software as above.

Effect of dabigatran, argatroban and DAPA on thrombin binding to fibrin clots – Binding of ^{125}I-thrombin to γA/γA- or γA/γ′-fibrin clots in the presence of active site inhibitors was assessed by measuring the amount of ^{125}I-thrombin in the supernatants of compacted clots prepared with γ_A- or γ′-fibrinogen and a catalytic amount of batroxobin (Dewilde *et al.*, 2013, Fredenburgh *et al.*, 2008, Petrera *et al.*, 2009). To a series of 1.5-ml micro-centrifuge tubes containing 0-1 μM dabigatran or argatroban, 0-4 μM DAPA, or 0-0.1 μM hirudin was added 10 nM ^{125}I-thrombin, 3 μM γA-fibrinogen or 1 μM γ′-fibrinogen, 2 mM $CaCl_2$ and 1 U/mL batroxobin in TBS-Tw to a total volume of 100 μl. Batroxobin was used to induce clotting because its activity is unaffected by dabigatran, argatroban, or DAPA. After incubation for 1 hr at 23'C, clots were pelleted by centrifugation at 14,000 × g for 4 min and 50 μl aliquots of supernatant were removed and counted for radioactivity using a gamma counter (Wizard^2 2470, Perkin-Elmer) to determine the concentration of unbound thrombin. Bound thrombin was calculated be expressing this value relative to the total thrombin in supernatants of tubes without fibrinogen. Bound thrombin was plotted versus the inhibitor concentration and $K_{i\ obs}$ values were then determined by non-linear regression analysis using Table Curve.

Statistical analysis – Results are presented as the mean ± S.D. All experiments were performed at least three times

RESULTS

Effect of active site mutation on thrombin binding to physiological ligands – To examine the effect of Ser195 to ala mutation on the binding capacity of thrombin, we compared binding of native thrombin with S195A-thrombin to physiological ligands using surface plasmon resonance. Factor Va, γA/γA- and γA/γ′-fibrin, were immobilized using direct amine coupling to separate flow cells of a CM5 sensor chip as previously described, and increasing concentrations of α-thrombin, FPR-α-thrombin or S195A-α-thrombin injected to the flow cells in series. Following a dissociation step, the flow cells were regenerated with 0.5M CaCl2 to maintain factor Va integrity. As expected, the SPR sensorgram profile of α-thrombin and FPR-α-thrombin binding to immobilized factor Va, γA/γA- and γA/γ′-fibrin revealed concentration-dependent and saturable responses, which were fit to a steady state analysis based on a one-site binding model (Figure 1). Because the binding of thrombin to factor Va and γA/γ′-fibrin are expected to be bivalent with contribution from both exosites, the KD values for α-thrombin and FPR-α-thrombin were also determined for binding to bivalent ligands using a global fit to a two-site binding model (Table 1). Thus, consistent with previous observations, analysis of α-thrombin binding to γA/γ′-fibrin reveals KD values of 1434.2 ± 56.8 and 110.4 ± 14.0, the latter reflecting high affinity binding to the γ′-sequence. Only 30% of the high affinity binding was observed, likely due to molecular orientation of the immobilized fibrin. This bivalent mechanism accounts for the observed 1.9-fold increase in steady state thrombin affinity for fibrin containing the γ′-sequence (Table 1). Occupation of the active site with chloromethyl-ketone did not affect the mode of thrombin binding based on global analysis of the SPR sensorgram profiles (Figure 1), although the affinity was slightly higher with FPR-α-thrombin compared with active α-thrombin as evidenced by steady state analysis (Table 1). Overall, thrombin bound to factor Va and the two fibrin isoforms with KD values comparable to previously reported values (Dharmawardana *et al.*, 1999, Fredenburgh *et al.*, 2008, Vu *et al.*, 2013).

Surprisingly, mutation of the α-thrombin catalytic Ser195 to alanine markedly altered the binding mechanism to accommodate higher affinity binding to physiological ligands when compared to α-thrombin and FPR-α-thrombin (Figure 1). Whereas α-thrombin fit a one-site model, the binding of S195A-α-thrombin was globally fit with a kinetic analysis of two-site binding, and analysis of the steady state

affinities revealed 1.8-fold, 3.6-fold, and 2.6-fold higher affinity than α-thrombin for factor Va, γA/γA- and γA/γ′-fibrin, respectively (Table 1). Higher affinity binding was also observed when comparing the binding of S195A-α-thrombin and FPR-α-thrombin to factor VIII and the fibrinogen isoforms (Figure 1). Only catalytically inactive thrombins were used for this comparison to prevent cleavage of the immobilized ligands. S195A-α-thrombin bound to factor VIII, γA/γA- and γA/γ′-fibrinogen with a two-site kinetic mechanism, with steady state affinities that were 13.9-fold, 4.8-fold, and 6.8-fold higher than FPR-α-thrombin, respectively (Table 1). Interestingly, the S195A mutation attenuates the importance of exosite 2 binding for cooperative high affinity binding to γA- and γ′-fibrinogen, as evidenced by the comparable KD values (Table 1).

Effect of active site mutation on binding to γA/γA- and γA/γ′-fibrin clots - To confirm the SPR findings, the binding of S195A-thrombin to γA- and γ′-fibrin clots was also assessed as previously described (Dewilde *et al.*, 2013, Fredenburgh *et al.*, 2008, Petrera *et al.*, 2009). In this assay α-thrombin bound to γA/γA- and γA/γ′-fibrin clots with KD values of 3558.22 ± 215.70 and 1231.2 ± 194.3 nM, respectively. ^{125}I-S195A-α-thrombin bound to γA/γA- and γA/γ′-fibrin clots with greater than 10-fold greater affinity than α-thrombin, with KD values of 299.6 ± 131.9 and 132.6 ± 42.6 nM, respectively. Interestingly, there was also increased reactivity of S195A-α-thrombin with Bolton-Hunter reagent.

Effect of N-linked glycosylation on E.Coli derived thrombin binding to fibrin - To control for the lack of N-linked glycosylation in S195A-α-thrombin derived from *E.Coli*, the binding of wild type α-thrombin derived from *E.Coli* (WTEColi-α-thrombin) was also measured. WTEColi-α-thrombin bound to factor Va, γA/γA- and γA/γ′-fibrin with higher steady state affinity than α-thrombin, respectively (Table 1, Figure 1). Although WTEColi-α-thrombin exhibits greater steady state affinity than α-thrombin and FPR-α-thrombin for physiological ligands, it does not bind with as high affinity as S195A-α-thrombin nor does WTEColi-α-thrombin exhibit an altered kinetic binding profile in SPR analysis (Figure 1). Thus, the association and dissociation rates of WTEColi-thrombin reach equilibrium quickly in SPR, following with thrombin's

characteristic fast-on, fast-off profile. These observations therefore suggest that the N-linked glycoslation of thrombin may sterically hinder binding to physiological ligands, but is not linked to the capacity of thrombin to participate in a cooperative binding mechanism. Indeed, WTEColi-thrombin has comparable plasma clot times and chromogenic activity with ChzTh (not shown). Together, these results suggest that S195A-thrombin has a high capacity for exosite-dependent cooperative binding, effects that are limited by the active site Ser195 in the native thrombin molecule.

Cooperative binding S195A-thrombin to physiological ligands requires both exosites – To determine whether both exosites are required for cooperative binding of S195A-α-thrombin to its physiological ligands, we used the limited trypsin-digested γ-variant and R93E mutation of S195A-α-thrombin with disrupted exosites 1 and 2, respectively. Disruption of either exosite abrogated the high affinity binding capacity of S195A-α-thrombin for all physiological ligands, as evidenced by significant impairment in the maximal Req value and steady state KD value achieved by the R93E- or γ-variants of S195A-α-thrombin (Figure 1, Table 1). Furthermore, disruption of either exosite reveals a one-site binding profile similar to those observed in α-thrombin or FPR-α-thrombin (Figure 1). A fraction of the γ-S195A-α-thrombin bound to γA/γA-fibrin(ogen) through exosite 1 due to incomplete trypsin-digestion as determined by western blot (not shown). Thus, these results confirm that the increased binding capacity of S195A-α-thrombin is the result of a cooperative binding mechanism that is dependent on both thrombin exosites. Therefore, Ser195 plays a role in limiting cooperative inter-exosite dynamics of thrombin for engagement of physiological ligands.

Effect of active site mutation on inter-exosite allostery – Reciprocal allosteric connections between the exosites of thrombin have been well described (Petrera et al., 2009). On the basis of the observed cooperativity between exosites 1 and 2 for high affinity S195A-thrombin binding to fibrin(ogen) and factors Va and VIII, we compared the capacity of α-thrombin and S195A-α-thrombin to display reciprocal inter-exosite competitive allostery. HD22, a high affinity exosite 2 binding DNA aptamer (KD = 0.35 nM), was

used to disrupt binding of the thrombin variants to immobilized γA/γA-fibrin. Increasing concentrations of HD22 was incubated with thrombin and injected into the flow cells in series. Consistent with previous observations, HD22 binding at exosite 2 decreased α-thrombin and FPR-α-thrombin binding to γA/γA-fibrin at exosite 1 by 90% with IC50 values of 837.5 ± 24.0 and 830 ± 24.0 nM, respectively (Figure 2). Mutation of Ser195 did not vastly change the maximal response achieved by HD22, but significantly decreased the observed IC50 value 11-fold, down to 73.8 ± 1.4 nM (Table 2). To control for potential steric effects of N-linked glycosylated residues on HD22 binding, inter-exosite reciprocal allostery was also tested with WTEcoli-α-thrombin. The wild type was found to exhibit an intermediate IC50 value of 606.7 ± 16 nM. These results demonstrate that disruption of the catalytic triad and S1 pocket enhances the inter-exosite allosteric potential of thrombin. Therefore, the Ser195 residue may be an important surface residue for regulating allostery in native thrombin by limiting the sensitivity of the inter-exosite reciprocal binding effect.

Effect of active site mutation on allosteric linkage to the binding capacity of the thrombin exosites – To investigate the role of Ser195 in regulating active site to exosite allosteric capacity, we compared the effect of dabigatran, argatroban, and DAPA on α-thrombin or S195A-α-thrombin binding to factor Va, γA/γA- and γA/γ′-fibrin using SPR. As previously described, engagement of the active site of α-thrombin with small inhibitors may capacitate or disrupt exosite-dependent binding to physiological ligands. Because inter-exosite allostery is regulated by the active site Ser195 residue, we explored whether ligand-specific active-site-to-exosite allostery is also modulated by Ser195 mutation. Consistent with our previous observations, subtle differences in the regions of contact in the active site cleft between dabigatran, argatroban, and dansylarginine N-(3-ethyl-1,5-pentanediyl)amide (DAPA) lead to vastly different capacities to either potentiate or disrupt exosite-dependent thrombin binding (Figure 3). Because all three small molecule inhibitors interact with the active site cleft and S1 pocket, mutation of Ser195 had predictably significant consequences for allostery. Thus, whereas dabigatran disrupted up to 30-50% binding of α-thrombin with an IC50 of 180-200 nM, the degree of allosteric disruption by dabigatran was magnified in

S195A-α-thrombin to 60-90% depending on the immbolized ligand, with a decrease in the IC50 to ~40 nM (Table 3). Furthermore, while argatroban and DAPA potentiates α-thrombin exosites for ligand binding, the allosteric effect of these inhibitors is completely reversed in S195A-α-thrombin (Figure 3, Table 3). Thus, argatroban and DAPA become potent allosteric inhibitors of S195A-α-thrombin binding to factor Va, γA/γA- and γA/γ′-fibrin.

To control for the potential effects of the lack of N-linked glycosylation of the 60s-loop given its proximity to the active site, the effects of the inhibitors were also measured with WTEColi-α-thrombin. In a nearly identical fashion to α-thrombin, binding of the unglycosylated wild-type was potentiated by dabigatran and disrupted by argatroban and DAPA (Figure 3, Table 3). Therefore, although the steric effects of glycosylation affects the affinity of thrombin to its exosite ligands, there is no effect on the binding mechanism or active-site-to-exosite allostery. These observations were confirmed with the binding of the radiolabelled thrombin variants to γA/γA- and γA/γ′-fibrin clots (Table 4). Together, the results demonstrate that the Ser195 residue is intimately linked to the allosteric core of thrombin and a critical determinant of the ligand-specificity of thrombin allostery.

DISCUSSION

Mounting evidence suggests that thrombin's myriad of effector roles are regulated through an intricate allosteric network within the molecule that links the exosites and the active site. There has been a lack of consensus between xray crystallographic and solution phase functional data on the allosteric capabilities of thrombin. Thus, inter-exosite and active-site linked allosteric effects appear to be ligand specific, conclusions which have relied on the catalytically inert S195A-thrombin. We demonstrate that the capacity of thrombin for allostery is significantly modified by the mutation. First, substitution of Ser195 to alanine unveils higher binding affinity for fibrin(ogen), factor Va, and FVIII compared to native thrombin. This effect is dependent on cooperativity between both exosites, as high affinity binding is abrogated in the exosite-perturbed R93E- or γ-variants of S195A-thrombin. Further evidence of increased inter-exosite communication is suggested by the increased ability of the exosite 2-directed aptamer to disrupt exosite 1-

mediated yA-fibrin binding in S195A-thrombin compared to thrombin. Finally, the allosteric consequence of active site ligation by small molecules is substantially modified, consistent with the observation that specific residues of contact are responsible allosteric phenomena through connections with the allosteric core. Together, these findings demonstrate that the S195A mutation enhances the allosteric capacity of thrombin.

These findings imply that the catalytic triad Ser195 limits the allosteric capacity of thrombin and may thus be a mechanism for regulating thrombin allostery and ligand-specificity. Since formation of the S1 pocket and the oxyanion hole in the active site cleft is the critical determinant for enzymatic activity (Huntington and Esmon, 2003), disruption of this region may affect ligand-specific surface contacts that are important for allosteric communication (Gasper *et al.*, 2012, Huntington, 2012). Although the active site cleft and S1 pocket are not disordered by S195A mutation in xray crystallographic studies (Huntington and Esmon, 2003), detailed measurement of the energetic consequences of thrombin ligation support functional observations of allostery (Fuglestad *et al.*, 2013, Kamath *et al.*, 2010). This global network allows for a generalized understanding of how mutations to surface residues may cause specific long-range allosteric consequences such as those in the present study and in previous reports (Qureshi et al., 2009). More work is needed to elucidate the specific surface contacts of the allosteric core and the critical residues governing allosteric capacity.

The function of thrombin may be placed on a spectrum of zymogen- to proteinase-like states(Kamath et al., 2010). The transition from an enzymatically inactive zymogen-like state that does not bear functional exosites is driven by activation cleavage. Subsequent molecular rearrangement of the molecule upon cleavage confers specificity by limiting the allosteric interactions and ensemble of conformational states of thrombin (Krishnaswamy, 2013, Lechtenberg *et al.*, 2010, Lechtenberg *et al.*, 2012). Thus, prethrombin 2, which has not been cleaved and cannot undergo rearrangement, exhibits far greater flexibility than thrombin(Kamath *et al.*, 2010, Kamath and Krishnaswamy, 2008). Engagement of the active site by substrate-like ligands such as DAPA force thrombin to adopt a proteinase-like conformation that limits the degree to which inter-exosite allostic interactions with fragment 2 may be expressed (Kamath et al., 2010). Although these studies did not find S195A-thrombin to be vastly

energetically different than proteinase-locked thrombin, our data suggests that the functional capabilities of the allosteric network are greatly modified. S195A-thrombin is a more functionally ambiguous molecule than thrombin. Therefore, our findings are consistent with the view of allostery as a mechanism for modulating thrombin specificity, a capability that is subject to the activation state of the molecule.

Previous studies have described the re-zymogenization of thrombin as a secondary mechanism of thrombin inhibition (Fredenburgh *et al.*, 2001, Li *et al.*, 2010). Thus, inhibition of thrombin by antithrombin disrupted exosite-mediated ligand binding by an allosteric mechanism. Although dabigatran appears to re-zymogenize thrombin by disrupting exosite-mediated binding, DAPA and another inhibitor direct inhibitor, argatroban, may lock thrombin into a proteinase-like state that enhances exosite mediated ligand binding. Our results show that these ligand-dependent effects cannot be observed with the catalytically inert thrombin. The S195A mutation likely modulates the surface contact of the active site ligands, an effect that also re-orders the allosteric network that governs reciprocal or cooperative responses between the binding sites.

In conclusion, our data are consistent with the view of thrombin as a globally dynamic molecule possessing an extensive allosteric network connecting each of its ligand binding domains. Mutation of surface residues predictably modulates the allosteric capacities of the network, an effect observed by the modified consequences of interaction with exosite and active site ligands. Due to the drastically different functional effects S195A mutation has on the thrombin allosteric network, conclusions on the nature of thrombin interactions drawn from mechanistic and structural studies on the S195A-mutant may require more review.

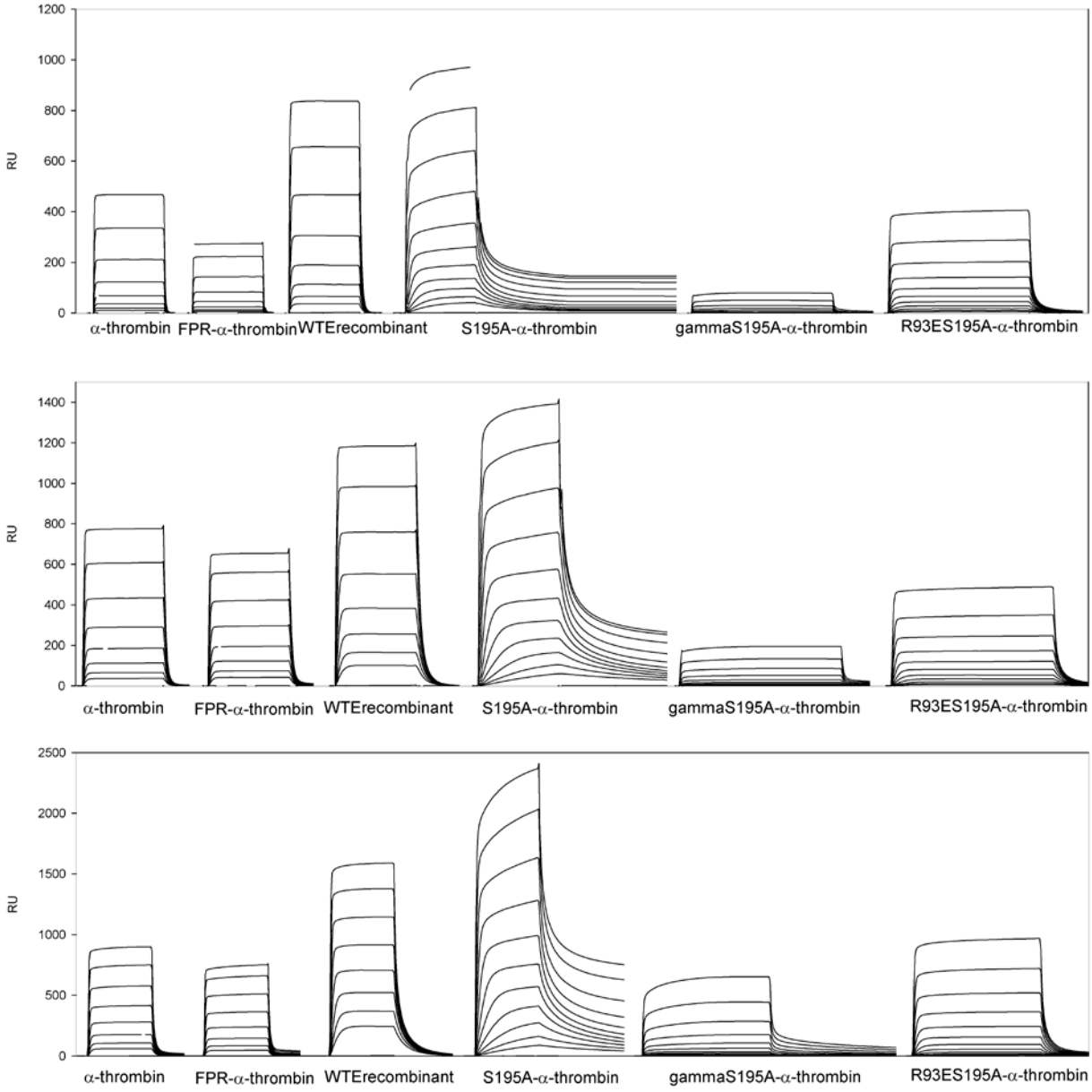


Figure 1A. Steady state analysis and SPR sensorgrams of thrombins to immobilized (top panel) γA-fibrin, (middle panel) γ′-fibrin, or (bottom panel) factor Va. Increasing concentrations of thrombin variants (*x-axis*) up to 5 μM were injected into individual flow cells containing immobilized γ_A-, γ′-fibrin, or factor Va. Samples were injected for 120 s at 25 μl/min, followed by a 45 s wash to monitor dissociation. Analyte concentrations are 39, 78, 156, 313, 625, 1250, 2500, 5000 nM. Representative sensorgrams of the effects of increasing concentrations of thrombin binding are shown in succession.

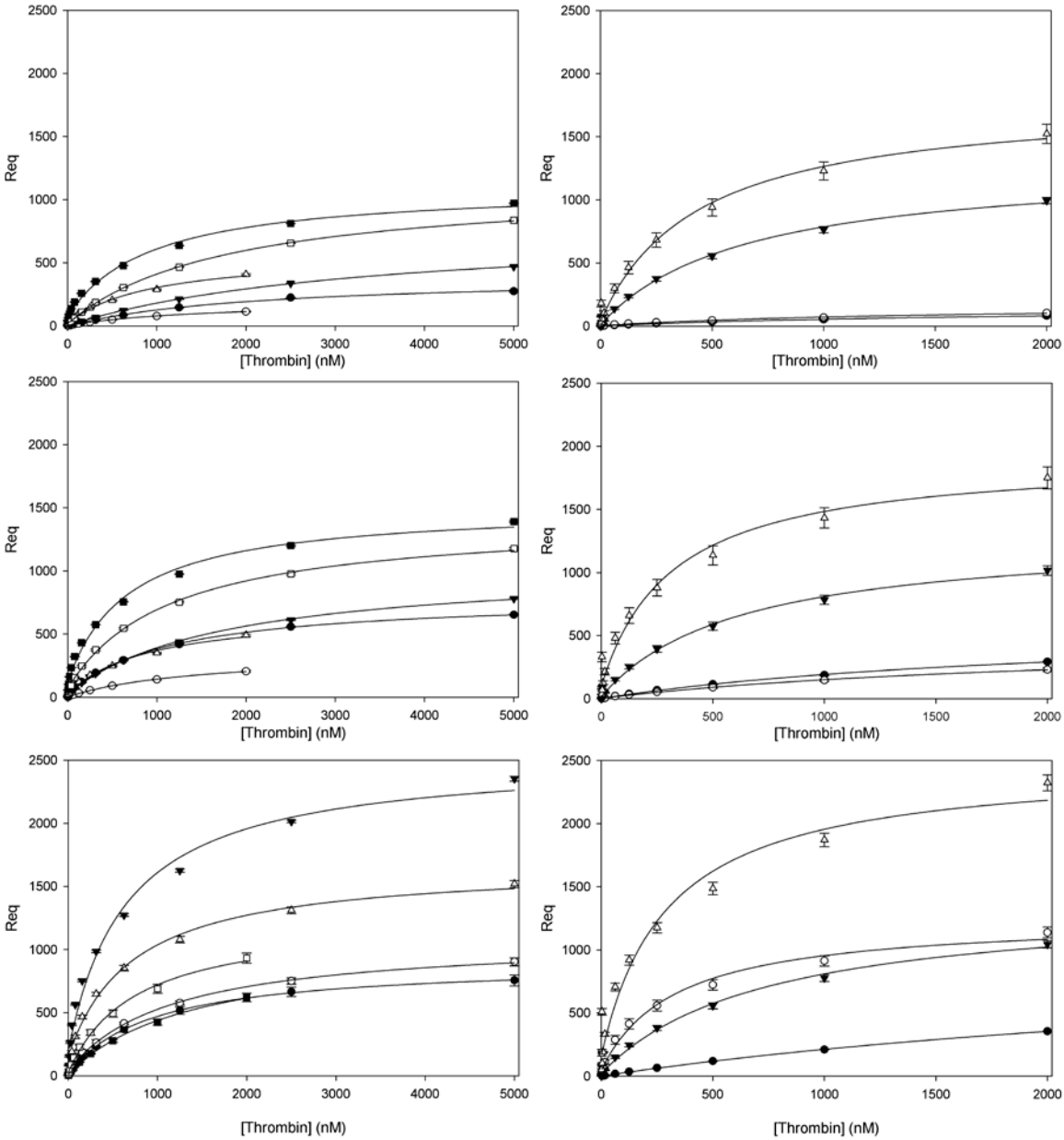


Figure 1B. Sensorgrams and steady state analysis of native thrombin and mutants binding to immobilized (top panels) fibrin and (middle panels) fibrinogen, and (bottom panels) factor Va or factor VIII. Increasing concentrations of thrombin up to 5000 nM were injected into individual flow cells of a CM5 biosensor chip containing immobilized (top left) γ_A-fibrin, (top right) γ′-fibrin, (middle left) γA-fibrinogen, (middle right) γ′-fibrinogen, (bottom left) factor Va, and (bottom right) factor VIII. α-thrombin (closed triangles), FPR-α-thrombin (closed circles), S195A-thrombin (closed squares), WTEColi-thrombin (open squares), gammaS195A-thrombin (open circles), or R93E-S195A-thrombin (open triangles) were injected for 120 s at 25 µl/min, followed by a 45 s wash to monitor dissociation. Analyte concentrations are 39, 78, 156, 313, 625, 1250, 2500, 5000 nM. The amount of thrombin bound at equilibrium after background correction (Req) was determined and plotted against the thrombin concentration. Data points represent the mean ± S.D. of 3 experiments, and the *lines* represent nonlinear regression analysis.

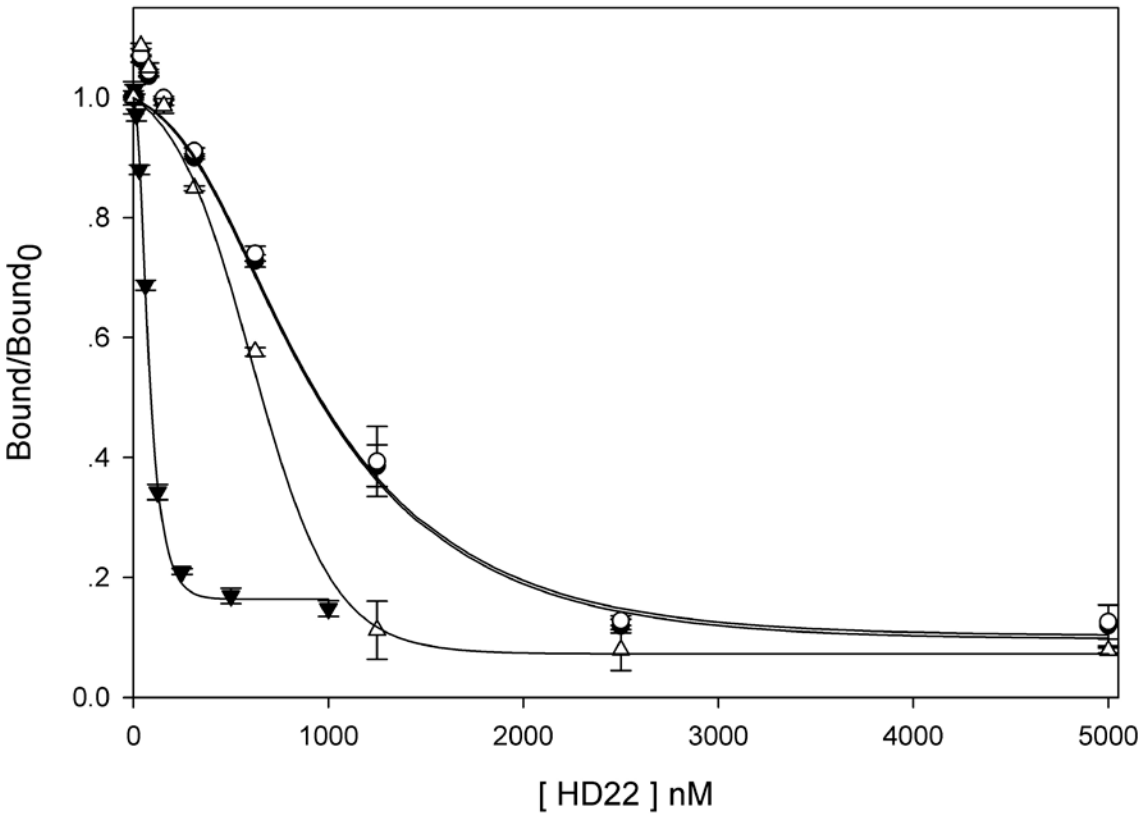


Figure 2. Effect of HD22 on α-thrombin (open circles), FPR-α-thrombin (closed circles), WTEColi-α-thrombin (open triangles), or S195A-α-thrombin (closed triangles) binding to immobilized γA-fibrin. Increasing concentrations of thrombin up to 5000 nM were injected into individual flow cells of a CM5 biosensor chip containing immobilized **γ**A-fibrin. Samples were injected for 120 s at 25 µl/min, followed by a 45 s wash to monitor dissociation. Analyte concentrations are 39, 78, 156, 313, 625, 1250, 2500, 5000 nM. The ratios of Req values of thrombin bound in the absence ($Bound_o$) and presence (Bound) of dabigatran, argatroban or DAPA are plotted against the input inhibitor concentrations. Data points represent the mean ± S.D. of 3 experiments, and the *lines* represent nonlinear regression analysis.

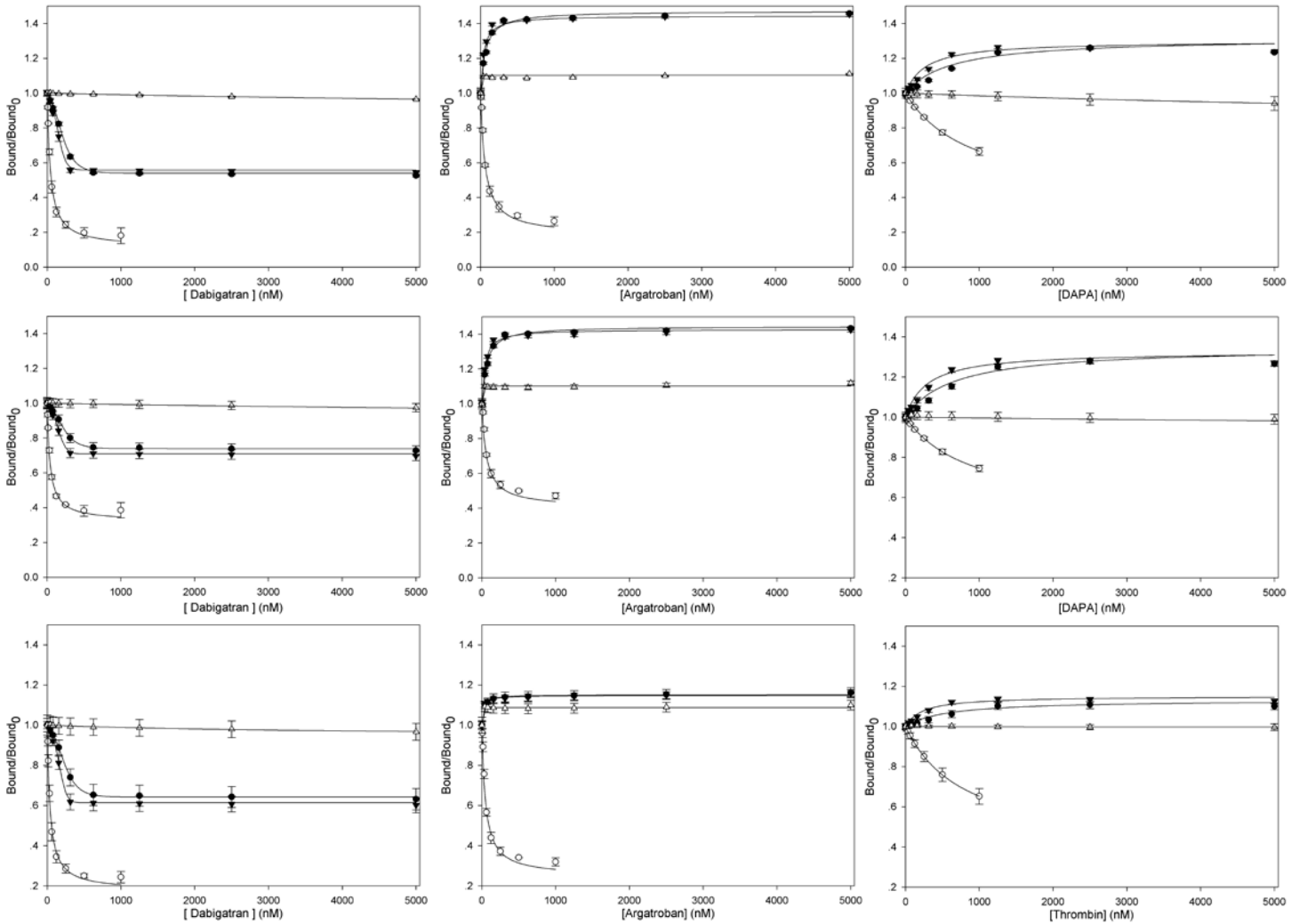


Figure 3. Effect of active site inhibitors on thrombin exosite mediated binding to physiological ligands. Increasing concentrations of dabigatran (left column), argatroban (middle column), DAPA (right column) up to 5 μM were pre-incubated with S195A-α-thrombin (open circles), α-thrombin (closed circles), WTE-α-thrombin (closed squares), or FPR-α-thrombin (open triangles) prior to injection into individual flow cells containing immobilized (top row panels) γ_A-fibrin, (middle row panels) γ′-fibrin, or (bottom row panels) factor Va. Sample injection was followed by a 45 s wash with buffer to monitor dissociation. The ratios of Req values of thrombin bound in the absence ($Bound_o$) and presence (Bound) of dabigatran, argatroban or DAPA are plotted against the input inhibitor concentrations. Data points represent the mean ± S.D. of 3 experiments, and the *lines* represent nonlinear regression analysis.

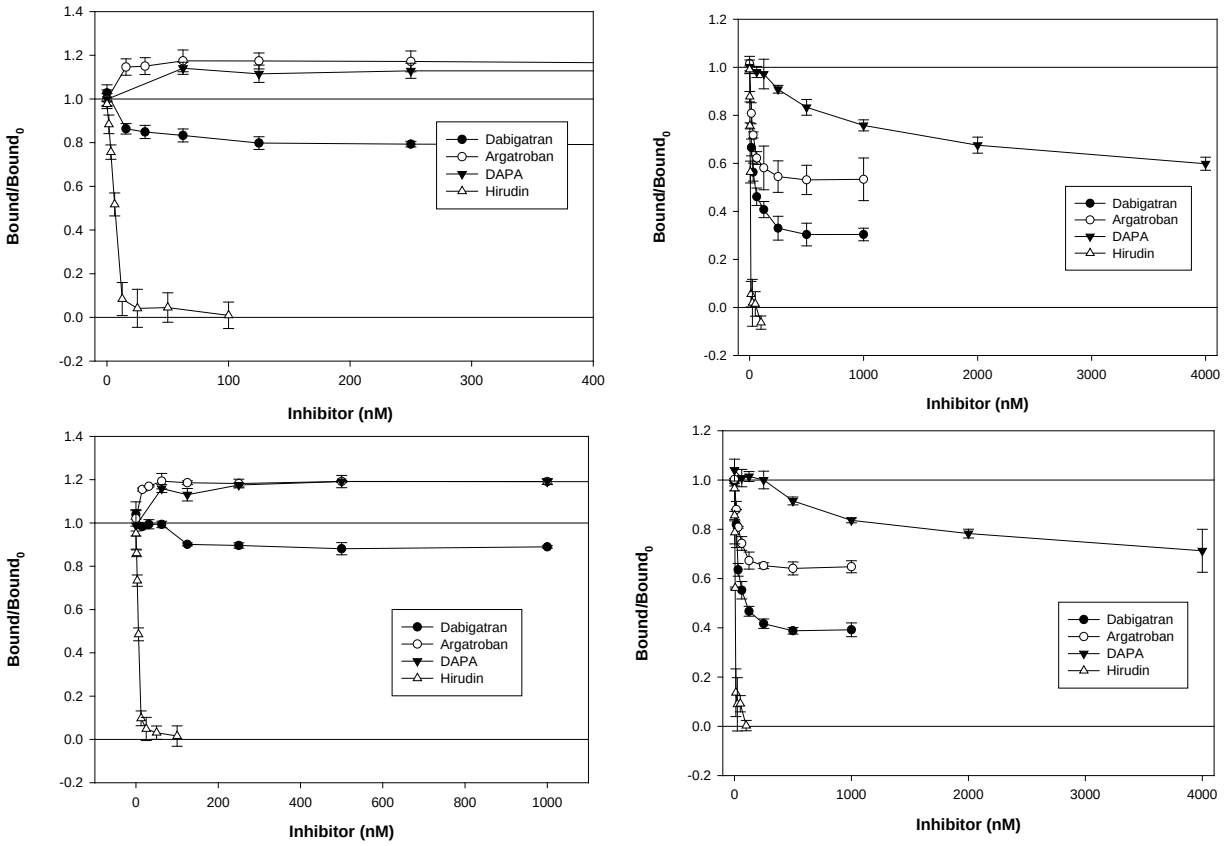


Figure 4. Effect of direct thrombin inhibitors on I125- α-thrombin and I125-S195- α-thrombin binding to γA/γA- and γA/γ′-fibrin clots. Binding of wild-type (*left panels*) or S195A thrombin (*right panels*) to γA/γA- (*top row panels*) and γA/γ′- (*bottom row panels*) fibrin clots.

Table 1. Binding parameters for thrombins to immobilized physiological ligands as determined by surface plasmon resonance. Steady state and fit to global parameters.

		Steady State KD (nM)	Rmax (RU)	KD1 (nM)	RMax (RU)	KD2 (nM)	RMax (RU)
α-thrombin	**Factor Va**	1173 ± 85	1053 ± 21	1471 ± 72.8	1106.1 ± 192.9	219.8 ± 189.4	92.0 ± 57.99
	γA-fibrin	3387 ± 36	784.7 ± 3.8	3323 ± 29.5	781.2 ± 4.0	-	-
	γ′-fibrin	1783.0 ± 65	1015.8 ± 12	2631 ± 17.7	988.7 ± 2.97	211.6 ± 0.89	138.1 ± 6.68
	Factor VIII	-	-	-	-	-	-
	γA-fibrinogen	-	-	-	-	-	-
	γ′-fibrinogen	-	-	-	-	-	-
FPR-α-Thrombin	**Factor Va**	943.2 ± 82	884.2 ± 19	831.2 ± 13.7	837.9 ± 56.6	130.4 ± 17.7	41.5 ± 0.9
	Fn1	2110 ± 140	400.6 ± 9.4	2238.7 ± 61.6	407.1 ± 13.9	-	-
	Fn2	1161 ± 33	777.1 ± 6.0	1434.2 ± 56.8	744.4 ± 0.4	110.4 ± 14.0	76.7 ± 3.6
	Factor VIII	4079 ± 110	1074.8 ± 21	3748.0 ± 106.9	1055.3 ± 5.1	-	-
	Fgn1	1962 ± 470	158.3 ± 21	1830.0 ± 651.7	165.4 ± 2.01	-	-
	Fgn2	2158 ± 120	600.3 ± 19	2349.7 ± 600.6	405.4 ± 246.3	415.8 ± 275.7	13.9 ± 2.8
WTEColi-α-Thrombin	**Factor Va**	723.9 ± 61	1519.3 ± 29	1554.9 ± 107.5	1277.7 ± 7.8	77.8 ± 7.3	574.9 ± 53.0
	Fn1	1824 ± 38	1112.9 ± 7.8	1645.7 ± 16.3	1096.7 ± 3.5	-	-
	Fn2	1159 ± 54	1345.4 ± 17	1892.3 ± 46.9	1228.3 ± 13.1	143.9 ± 17.7	289.6 ± 8.0
	Factor VIII	-	-	-	-	-	-
	Fgn1	-	-	-	-	-	-
	Fgn2	-	-	-	-	-	-
S195A-α-thrombin	**Factor Va**	651.3 ± 66	2331.1 ± 63	69.3 ± 3.1	524.4 ± 4.9	26.3 ± 3.1	196.6 ± 6.4
	Fn1	939.3 ± 71	1032.7 ± 23	939.3 ± 71.0	1032.7 ± 23.0	-	-
	Fn2	679.5 ± 58	1393.8 ± 32	275.6 ± 17.0	1035.3 ± 17.9	48.2 ± 8.6	289.7 ± 10.8
	Factor VIII	294.1 ± 38	2321.8 ± 84	76.4 ± 18.0	1561.7 ± 93.9	9.8 ± 7.8	690.0 ± 107.1

	Fgn1	438.4 ± 38	1762.7 ± 48	138.2 ± 23.7	1719.7 ± 49.7	-	-
	Fgn2	315.7 ± 37	1821.6 ± 60	129.5 ± 17.7	1343.7 ± 38.9	40.3 ± 10.4	454.4 ± 15.7
R93E-S195A-α-thrombin	**Factor Va**	747.0 ± 66	1214.9 ± 41	747.1 ± 66	1214.9 ± 41	-	-
	Fn1	957.2 ± 91	558.6 ± 23	957.2 ± 91	558.6 ± 23	-	-
	Fn2	904.5 ± 85	660.5 ± 26	904.5 ± 85	660.5 ± 26	-	-
	Factor VIII	736.3 ± 51	1371 ± 36	389.9 ± 164.8	1317.0 ± 17.0	-	-
	Fgn1	653.9 ± 34	1280.3 ± 24	553.2 ± 29.3	1250.0 ± 17.0	-	-
	Fgn2	619.1 ± 42	1283.3 ± 31	513.8 ± 34.3	1249.7 ± 27.5	-	-
g-S195A-α-thrombin	**Factor Va**	1415 ± 120	1029.1 ± 42	1415 ± 120	1029 ± 42	-	-
	Fn1	1560 ± 73	197.2 ± 4.7	1215 ± 81.7	184 ± 7.7	-	-
	Fn2	1471 ± 83	349.0 ± 9.9	1471 ± 83.0	349 ± 9.9	-	-
	Factor VIII	331.2 36	1207.2 ± 37	225.3 ± 26.5	1165.3 ± 19.1	-	-
	Fgn1	1464 ± 160	169.7 ± 9.4	698.6 ± 72.8	143.6 ± 7.6	-	-
	Fgn2	2241 ± 260	479.3 ± 32	1535.3 ± 95.0	428.3 ± 16.4	-	-

Table 2. Effect of HD22 on thrombin variants binding to γA/γ′-fibrin, as determined by surface plasmon resonance.

		HD1		HD22	
	Ligand	**EC50 (nM)**	**Change %**	**EC50 (nM)**	**Change %**
α-thrombin	**Factor Va**	460.6 ± 7.7	96.9 ± 1.7	1031 ± 33	92.3 ± 4.4
	γA-fibrin	372.0 ± 7.5	99.0 ± 0.8	837.5 ± 24.0	90.0 ± 1.3
	γ′-fibrin	417.6 ± 7.3	98.2 ± 1.3	930.4 ± 28.0	94.9 ± 2.8
	Factor VIII	-	-	-	-
	γA-fibrinogen	-	-	-	-
	γ′-fibrinogen	-	-	-	-
FPR-α-thrombin	**Factor Va**	443.8 ± 8.7	97.2 ± 1.6	1001 ± 33	93.0 ± 3.7
	γA-fibrin	363.2 ± 7.9	97.4 ± 0.6	830.4 ± 24	90.6 ± 0.9
	γ′-fibrin	429.8 ± 8.4	97.7 ± 1.4	972.5 ± 29	96.9 ± 2.9
	Factor VIII				
	γA-fibrinogen				
	γ′-fibrinogen				
WTEcoli-α-thrombin	**Factor Va**	331.1 ± 6.1	93.7 ± 3.5	736.0 ± 16	95.1 ± 5.1
	γA-fibrin	242.5 ± 4.4	97.5 ± 1.0	606.7 ± 16	93.1 ± 2.1
	γ′-fibrin	268.2 ± 5.0	96.5 ± 1.8	639.6 ± 16	95.3 ± 3.3
	Factor VIII	-	-	-	-
	γA-fibrinogen	-	-	-	-
	γ′-fibrinogen	-	-	-	-
S195A-α-thrombin	**Factor Va**	117.1 ± 2.8	78.9 ± 3.4	75.0 ± 1.3	86.4 ± 3.1
	γA-fibrin	107.9 ± 2.9	81.6 ± 1.1	73.8 ± 1.4	84.0 ± 0.8
	γ′-fibrin	113.3 ± 2.5	78.5 ± 1.3	76.9 ± 1.4	85.7 ± 1.3
	Factor VIII				
	γA-fibrinogen				
	γ′-fibrinogen				

Table 3. Effect of active site inhibitors on thrombin variants binding to immobilized physiological ligands as determined by surface plasmon resonance.

	Ligand	Dabigatran		Argatroban		DAPA	
		EC50 (nM)	Change %	EC50 (nM)	Change %	EC50 (nM)	Change %
α-thrombin	**Factor Va**	204.2 ± 17	- 37.9 ± 3.2	23.4 ± 8.8	+ 15.1 ± 3.9	565.1 ± 200	+ 13.2 ± 3.2
	γA-fibrin	184.6 ± 4.3	- 47.6 ± 0.4	62.4 ± 4.8	+ 47.2 ± 1.0	514.1 ± 24.0	+ 25.1 ± 0.5
	γ′-fibrin	182.4 ± 15	- 28.4 ± 1.6	59.4 ± 5.1	+ 44.5 ± 2.3	515.9 ± 31.0	+ 27.7 ± 1.5
	Factor VIII	-	-	-	-	-	-
	γA-fibrinogen	-	-	-	-	-	-
	γ′-fibrinogen	-	-	-	-	-	-
WTEcoli-α-thrombin	**Factor Va**	154.4 ± 6.5	40.3 ± 4.0	21.7 ± 5.9	+ 15.4 ± 5.7	239.3 ± 21.0	+ 13.1 ± 2.1
	γA-fibrin	138.8 ± 3.4	46.0 ± 0.8	39.8 ± 3.5	+ 44.5 ± 2.0	264.3 ± 16.0	+ 25.0 ± 0.9
	γ′-fibrin	137.5 ± 7.3	31.1 ± 2.0	39.6 ± 3.9	+ 42.7 ± 3.8	269.5 ± 18.0	+ 27.7 ± 1.9
	Factor VIII	-	-	-	-	-	-
	γA-fibrinogen	-	-	-	-	-	-
	γ′-fibrinogen	-	-	-	-	-	-
S195A-α-thrombin	**Factor Va**	38.0 ± 3.4	- 82.5 ± 11	48.6 ± 5.1	- 75.3 ± 11	- 791.7 ± 140.0	- 62.3 ± 29
	γA-fibrin	43.1 ± 3.2	- 88.7 ± 2.8	61.2 ± 6.0	- 81.6 ± 3.0	- 899.7 ± 95.0	- 63.3 ± 5.2
	γ′-fibrin	39.5 ± 4.2	- 64.5 ± 4.2	62.8 ± 6.9	- 59.7 ± 3.9	- 898.8 ± 81.0	- 48.1 ± 5.4
	Factor VIII	37.2 ± 1.2	85.8 ± 0.3				
	γA-fibrinogen	23.8 ± 1.2	- 88.4 ± 0.2				
	γ′-fibrinogen	29.5 ± 1.0	- 77.2 ± 0.2				

Table 4. Effect of direct thrombin inhibitiors on I125- α-thrombin and I125-S195- α-thrombin binding to γA/γA- and γA/γ′-fibrin clots.

	Dabigatran			
	Thrombin		S195A-Thrombin	
Clot	**IC50 (nM)**	**Max Displ %**	**IC50 (nM)**	**Max Displ %**
γA	7.0 ± 2.8	21.4 ± 3.3	20.2 ± 2.2	69.6 ± 2.6
γ′	285.7 ± 343.9	9.4 ± 1.4	23.5 ± 3.4	60.9 ± 3.9
	Argatroban			
	Thrombin		S195A-Thrombin	
Clot	**IC50 (nM)**	**Max Displ %**	**IC50 (nM)**	**Max Displ %**
γA	4.5 ± 3.4	- 23.5 ± 0.5	22.2 ± 4.7	46.7 ± 8.9
γ′	12.9 ± 10.2	- 26.1 ± 0.4	28.8 ± 5.4	38.8 ± 4.4
	DAPA			
	Thrombin		S195A-Thrombin	
Clot	**IC50 (nM)**	**Max Displ %**	**IC50 (nM)**	**Max Displ %**
γA	3.8 ± 1.6	- 17.0 ± 2.7	1103.7 ± 40.1	40.2 ± 2.7
γ′	35.9 ± 35.1	- 20.1 ± 3.6	1576.5 ± 631.4	30.0 ± 4.6
	Hirudin			
	Thrombin		S195A-Thrombin	
Clot	**IC50 (nM)**	**Max Displ %**	**IC50 (nM)**	**Max Displ %**
γA	6.3 ± 0.6	100	7.2 ± 0.8	100
γ′	6.2 ± 0.2	100	6.3 0.05	100

GENERAL DISCUSSION

Thrombotic diseases such as myocardial infarction, thromboembolic stroke in patients with atrial fibrillation, and venous thromboembolism account for significant morbidity and mortality worldwide. Classic antithrombotic treatment and prevention of these diseases include anticoagulant drugs such as heparin and heparin-derived glycosaminoglycans, as well as vitamin K antagonists like warfarin. Despite their use for over 60 years, inherent biological and pharmacologic weaknesses have spurred the development of the direct oral anticoagulant drugs (DOACs). The studies in this dissertation examine the biochemical mechanisms that regulate function of key coagulation proteases, with a focus on the modes and consequences of interactions with natural and engineered anticoagulants.

Chapter One demonstrates the extent to which proteases in the coagulation system are resistant to their natural inhibitor AT in the context of heparinoid anticoagulants when incorporated into their activation complexes, findings that underpin the development of the DOACs. *Chapter Two* expands on the development of the thrombin exosites, which govern binding interactions important for protecting thrombin from AT inhibition, and provide a basis for new paradigms in targeting thrombin activity. *Chapter Three* emphasizes that subtle differences in DOAC structure affect inhibition rates of factor Xa, which may explain differences in global tests of coagulation. *Chapter Four* demonstrates that DOAC binding to thrombin has consequences for exosite-mediated cofactor interactions through allosteric modulation. *Chapter Five* shows that the catalytic Ser195 residue is integral to thrombin allostery, and may in fact be a key regulatory factor in determining thrombin specificity.

Collectively, these studies emphasize the role of allostery as a mechanism for regulation of the specificity and function of coagulation proteases. The use of agents that directly interact with the active site and measuring the resultant effects on exosite binding sheds light on the intricate allosteric networks that regulate enzyme activity. Understanding these regulatory systems ultimately reveal potential directions for refining future pharmacologic interventions for better specificity and efficacy.

Protection is a physiological mechanism for propagating common pathway activity

Inherent thrombogenicity of clots has been thought to contribute to recurrent and refractory thrombosis despite treatment with antithrombotic drugs. In support of this, clots have been found to harbour active coagulation enzymes (Mutch *et al.*, 2001), and clot-bound thrombin has been shown to resist inhibition by AT and retain its thrombogenic capacity in plasma (Weitz *et al.*, 1990). Interestingly, small molecule thrombin inhibitors are unaffected by fibrin-thrombin binding (Weitz *et al.*, 1998), an observation that formed the basis for development of direct thrombin inhibitors (Perzborn *et al.*, 2005, Pinto *et al.*, 2007, Van Ryn *et al.*, 2013). The protection phenomenon was later observed upstream at the level of factor Xa and the prothrombinase complex (Brufatto *et al.*, 2003, Rezaie, 2001).

Our current data confirm observations with prothrombinase. Factor Xa in complex with factor Va on a phospholipid surface is protected from AT inhibition. Addition of prothrombin further decreases the AT inhibition rate in a dose-dependent manner, an effect that does not occur without assembly of the prothrombinase complex. This suggests that prothrombinase assembly capacitates factor Xa for interaction with the substrate prothrombin, allowing prothrombin to then act as a competitive inhibitor for AT. This protection phenomenon is largely localized at the level of prothrombinase. Factor IXa assembly into the intrinsic tenase complex only yielded moderate protection, and factor VIIa assembly into the extrinsic tenase complex did not protect the enzyme. Furthermore, addition of factor X to the tenase complexes did not change the AT inhibition rates.

Protection phenomena likely reflects a substrate specificity switch that evolved to enable rapid clot propagation. The coagulation system exhibits multiple levels of redundancy, which necessitate negative and positive feedback. As threshold levels of pro-thrombotic stimuli are reached, the system becomes committed to clot generation (Hoffman and Monroe, 2007). This occurs through switching of substrate specificity, engagement of positive feedback loops, and dampening of negative feedback pathways. With factor Xa, binding to factor Va on the platelet surface modifies its pathway of interaction with prothrombin (Betz *et al.*, 1997, Betz and Krishnaswamy, 1998, Bianchini *et al.*, 2005, Krishnaswamy *et al.*, 1993, Nesheim *et al.*, 1979b). With thrombin, binding with fibrinogen via exosites 1 and 2 modify its interaction with AT through steric and long range allosteric interactions (Becker *et al.*, 1999, Fredenburgh *et al.*, 2008). Thus, complex assembly causes steric and allosteric changes that enforce procoagulant reactions and make

the binding of inhibitors such as AT unfavourable. These phenomena are likely part of a conserved regulatory mechanism for promoting clot propagation once thrombin generation has reached an appropriate threshold (Jesty and Beltrami, 2005).

Prothrombin exosites I and II are sequentially capacitated for binding to physiologic substrates, a steric and allosteric process driven by proteolysis

The two thrombin exosites are known for their exquisite ability to direct thrombin function by binding to a myriad of cofactors (Lane *et al.*, 2005). These interactions are thought to be responsible for generating specificity in coagulation, fibrinolysis, inflammation, and immunity. For nearly all known exosite interactions, the cofactors bind with much higher affinity to thrombin than to prothrombin. This is thought to be one way in which thrombin is kept in quiescent in its proenzyme state. Indeed, exosite I in prothrombin may be allosterically inactivated, while exosite II is sterically hindered by F2 (Adams and Huntington, 2016, Krishnaswamy, 2013).

Our group showed that the DNA-aptamer HD1 binds prothrombin exosite I with the same high affinity as it binds exosite I on thrombin. Consequently, HD1 not only attenuates thrombin activity, but also blocks prothrombin activation (Kretz *et al.*, 2006), an effect verified in thrombin generation assays and in interactions with FV and FVIII (Kretz *et al.*, 2010). The mechanism by which exosite I is regulated during proteolysis is important because exosite-exosite interactions are critical for prothrombin activation (Huntington, 2009, Krishnaswamy, 2013). How is ligand specificity of the (pro)thrombin exosites governed? Furthermore, is exosite I on prothrombin capable of binding TM or fibrin? If so, then the catalytically inert prothrombin may be capable of competing with thrombin, thereby modulating its function.

Chapter Two systematically evaluates the direct binding of prothrombin and its transitional intermediates to numerous exosite I, exosite II, or bivalent (both exosites) ligands. In keeping with previous observations (Anderson *et al.*, 2003, Anderson and Bock, 2003), exosite-mediated binding to the physiological ligands TM, γA-fibrin, γ′-fibrin, and FVa occurred sequentially with proteolysis. Our SPR data support the conformational remodeling of prothrombin exosites during proteolysis as cleavage of F1 to

form prethrombin 1 capacitates exosite I function. However, the greatest effect comes from cleavage of Arg320 to form meizothrombin. Steric effects from the cleavage of F2 are also evident because prethrombin-2 binds FVa with a higher affinity than prethrombin-1. Although structural studies have suggested that cleavage of Arg320 modifies the F2 binding site on the catalytic domain, our direct binding data do not support this as prethrombin 2 binds to univalent and bivalent exosite ligands with similar affinity to meizothrombin, which has an intact F1.2 (Adams and Huntington, 2016). Our data also supports the notion that meizothrombin is not the terminal point of exosite capacitation because cleavage of F1 further increases its affinity for exosite I ligands (Kroh *et al.*, 2007).

The concept of "proexosite" was first proposed to explain an immature domain that does not bind to $Hir^{54\text{-}65}(SO_3^-)$ (Anderson *et al.*, 2000a, Anderson *et al.*, 2000b). The differences in binding of various ligands to exosite I in this extensive catalogue support the notion that subsites within the exosites may govern certain binding interactions (Kretz *et al.*, 2006). Crystallographic studies have shown that HD1 (Padmanabhan *et al.*, 1993a) and $Hir^{54\text{-}65}(SO_3^-)$ bind to distinct subdomains (Chen *et al.*, 1995, Pica *et al.*, 2013). Thus, HD1 binds to the cationic surface residues of exosite 1, while $Hir^{54\text{-}65}(SO_3^-)$ makes contact with the hydrophobic surface depression. This explains the capacity for HD1 to block exosite I mediated prothrombin docking, as well as thrombin function (Kretz *et al.*, 2010). In contrast, $Hir^{54\text{-}65}(SO_3^-)$ only binds to thrombin exosite I. These exosite subdomains may also undergo variable degrees of allosteric modulation during proteolysis, explaining differences between proteolytic capacitation of ligands for binding. Indeed, crystal structures of prethrombin-2 suggest that the hydrophobic pocket of exosite I remains cryptic until Arg320 cleavage, whereas the cationic residues are exposed for binding (Malkowski *et al.*, 1997). Together, these studies emphasize the impact of subtle differences in molecular conformation that occur during the conversion of prothrombin to thrombin on exosite-ligand interaction.

Subsite interactions at the factor Xa active site may explain the differential effects of DOACs on global tests of coagulation

Although the overall binding affinities of the factor Xa inhibitors rivaroxaban and apixaban are similar (Furugohri *et al.*, 2008, Luettgen *et al.*, 2011, Perzborn *et al.*, 2005), head-to-head measurement of

their effect on global tests of plasma coagulation show that rivaroxaban is by far more potent at delaying the PT and aPTT (Baglin, 2013, Barrett *et al.*, 2010, Garcia *et al.*, 2013). These differences can be attributed to the faster on-rate of rivaroxaban for factor Xa binding compared with apixaban (*Chapter Three*). Because the global tests of coagulation flood either the extrinsic or intrinsic system with excessive TF or negatively charged surface, respectively, enzyme turnover is the main rate-limiting step. Since the on-rate of rivaroxaban is faster than that of apixaban in the same timeframe of the global assays as shown in real-time chromogenic assays, rivaroxaban causes the greatest decrease in the kcat of prothrombin cleavage by prothrombinase.

These effects are likely mediated by subtle differences in the interaction of each DOAC with the active site. Unique interactions of the substrate with the active site affect binding affinity and catalytic rate of both factor Xa and thrombin, concepts that have guided the design of the DOACs (Le Bonniec *et al.*, 1996, Ludeman *et al.*, 2003, Rumthao *et al.*, 2004). Indeed, crystal structures of rivaroxaban or apixaban with factor Xa reveal they exhibit distinct interactions with subsites S1 and S4 (Pinto *et al.*, 2007, Roehrig *et al.*, 2005, Steinberg and Becker, 2014, Straub *et al.*, 2011) and may account for the disparate association rate constants and effects on prothrombin activation. These results emphasize the impact of subtle differences in the mode of binding on enzyme function.

Since assembly into the prothrombin complex does not affect DOAC inhibition of factor Xa, this suggests that DOACs are not sensitive to allosteric effects of factor Va on factor Xa (*Chapter Three*). This is analogous to previous observations that exosite ligands of thrombin are able to disrupt AT binding, but not the binding of small active site inhibitors (Fredenburgh *et al.*, 2004, Fredenburgh *et al.*, 2008) and that prothrombinase assembly affects AT inhibition of factor Xa (*Chapter One*) (Rezaie, 2001). These observations decrease the likelihood that DOAC-binding causes allosteric changes that decrease cofactor binding to factor Xa, because allosteric effects are likely to be reciprocal.

What is the clinical relevance of these “fast” and “faster” new generation of direct and rapid acting anticoagulants? Although the DOACs were designed to effect rapid anticoagulation, the major side effect is hemorrhage. Could a “slower” rapid onset inhibitor such as apixaban allow for a small amount of thrombin formation to provide hemostasis? Indeed, this may be an explanation for why apixaban has been shown to

have less relative risk of intracranial hemorrhage compared with warfarin in large randomized controlled trials for stroke prophylaxis in patients with atrial fibrillation (Granger *et al.*, 2011, Patel *et al.*, 2011). Alternatively, a "faster" inhibitor may be more effective in the setting of acute coronary syndromes, where the rapid thrombus formation is the cause of myocardial infarction.

The specificity of long range allostery is governed by an allosteric network

Numerous studies have described allosteric effects of exosite ligation on the thrombin active site as a mechanism to direct thrombin activity. These include increasing the overall catalytic activity of thrombin through the conserved Na^+ binding site (Gohara and Di Cera, 2011, Pozzi *et al.*, 2011), or direct specific functions through exosite I-mediated binding of PAR-1 (Chen *et al.*, 2015, Gandhi *et al.*, 2008, Lechtenberg *et al.*, 2010), TM (Adams *et al.*, 2009, Gasper *et al.*, 2012), γA-fibrin (Fredenburgh *et al.*, 2008), and exosite II-mediated binding of GpIb (Li *et al.*, 2001, Sabo and Maurer, 2009), prothrombin fragment 2 (Malovichko *et al.*, 2013), HD22 (Petrera *et al.*, 2009), heparin cofactor II (Baglin *et al.*, 2002) hypersulfated low molecular weight heparins (Henry *et al.*, 2007, Henry and Desai, 2014) or the γ′-fibrin peptide (Sabo *et al.*, 2006). Few studies however, show the allosteric effects of AT-binding on exosite ligation (Li *et al.*, 2010). Long range inter-exosite allostery has also been observed between exosite I and II, and in the bivalent binding interaction of thrombin with γ′-fibrin (Fredenburgh *et al.*, 2008, Nimjee *et al.*, 2009, Petrera *et al.*, 2009).

Despite this evidence of the dynamic nature of thrombin activity, there are discrepancies in the degree and specific directionality of allosteric effects. Whereas some studies did not observe allostery with certain ligands like the sulfated hirudin peptide or fragment 2 (Anderson *et al.*, 2003), others found that ternary structures could be formed when all thrombin binding domains bound simultaneously (Verhamme *et al.*, 2002) and conclude that allosteric effects with multi-valent ligands were likely steric in origin. Still others observed divergent effects of ligands that bound at the same site (Sabo and Maurer, 2009), and divergent long range allosteric cooperativity and competition (Nimjee *et al.*, 2009). Indeed, our group has observed that interexosite allostery can be competitive or cooperative, as in the reciprocal effect of HD22

on thrombin clotting of fibrinogen (Petrera *et al.*, 2009) versus the bivalent interaction of thrombin with γ′-fibrin (Fredenburgh *et al.*, 2008).

How do we reconcile these divergent results? *Chapter Four* (1) addresses the gap in evidence of active site to exosite allostery by using small molecules that bind directly to the thrombin active site, and (2) catalogues these effects by performing head-to-head measurement with multiple univalent and bivalent ligands of the thrombin exosites. We used SPR to directly measure these binding reactions in fluid phase, and correlate them to functional measurements. This strategy allows studies of direct binding observations in a dynamic environment unlike the rigid environment of crystallographic studies. In addition, it permits simultaneous direct comparison of multiple ligand-ligand and ligand-exosite interactions. These methods also circumvent the need to fluorescently modify the ligands, which could alter their binding and influence allostery.

We show that subtle differences in the interactions of dabigatran and argatroban with the thrombin active site can divergently affect both univalent and bivalent ligand interactions at exosites I and II. Whereas dabigatran decreases thrombin exosite I-binding to γ_A-fibrin and bivalent factor Va binding while having no effect for GP1bα269-286ppp, argatroban increases binding to all of these ligands (*Chapter Four*).

First, these results shed light on the potential secondary anticoagulant mechanisms of dabigatran. By not only inhibiting the activity of thrombin protected within clots, dabigatran may be able to disable the thrombogenicity of a clot by causing clot-bound thrombin to diffuse out where it can be rapidly inhibited by AT and cleared. Second, the allosteric modulation of the exosites by active site ligands is dependent on subsites of the active site. How do these subsites exert such fine control over thrombin function? Insights from amide proton hydrogen-deuterium exchange with MALDI-TOF mass spectrometry, mutagenesis, isothermal titration calorimetry, and NMR spectroscopy have allowed for greater understanding of thrombin allostery as a a spectrum of possible conformations in an equilibrium state (Fenwick *et al.*, 2011, Lechtenberg *et al.*, 2012, Malovichko *et al.*, 2013, Pineda *et al.*, 2004, Qureshi *et al.*, 2009). Ligation at any site modifies the thermodynamics of entire molecule to favour a specific equilibrium of conformations.

To model the complicated systems interactions, computational methods (Panjkovich and Daura, 2012) have been employed for numerous other biologic systems including G-coupled protein receptors (LeVine and Weinstein, 2015), and cyclic nucleotide-binding domains (Malmstrom *et al.*, 2015). The surface residues of thrombin are likely energetically linked through the primary structure as well as secondary and tertiary interactions, linkages which can form specific scaffolds in an allosteric network within the molecule that are important for determining affinity (Gasper *et al.*, 2012). Indeed, some active site ligands appear to "zymogenize" thrombin by deactivating the exosites (Huntington, 2009) while others may shift thrombin to a "proteinase-like" state and potentiate binding (Kamath *et al.*, 2010). In *Chapter Four*, dabigatran ligation may zymogenize, while argatroban may shift toward proteinase states using distinct scaffolds within the thrombin molecule. Consideration of these allosteric scaffolds may be important in drug discovery, since inhibitors like argatroban may inhibit clot-bound thrombin function, but be unable to reduce clot thrombogenicity by displacing thrombin as was seen with dabigatran. Indeed, drug discovery efforts are now exploiting allosteric modulation as a strategy for anticoagulation (Lu *et al.*, 2014, Tsai and Nussinov, 2014, Woodruff and Sullenger, 2015).

Key residues in the allosteric network control subsite-specific long range communication

Our previous studies showed subtle differences in surface contacts of ligands affect both binding (*Chapter Three*) and long range allosteric effects (*Chapter Four*). Since the capacitation of thrombin exosites for binding proceeds through the steric and allosteric consequences of proteolysis (*Chapter Two*), and proteolysis produces structural modifications in the active site that enable catalytic activity (Huntington and Esmon, 2003), is there a role of the catalytic serine 195 residue in regulating allostery? In head-to-head direct binding comparisons of the S195A-thrombin with plasma derived α-thrombin to γA-fibrin, γ′-fibrin and factor Va, we show that S195A-thrombin has significantly higher capacity to bind ligands via one or both exosites. Thus, global kinetic modeling of the SPR profiles show that while plasma-derived thrombin binding is single-site with fast on- and off-rates, S195A-thrombin is multi-site with multiple on- and off-rates. The multi-site binding evolves as the molecule binds to immobilized ligand, as evidenced by the non-

linear binding profile. Together, these findings suggest that mutation of the active site serine residue unlocks the allosteric potential of the molecule.

The observation that neutralization of Ser195 increases the flexibility of the thrombin molecule is in keeping with the mechanism of molecular rearrangement that potentiates the thrombin active site for catalysis (Khan and James, 1998). During prothrombin activation, cleavage at Arg320 frees the new N-terminal Ile321 residue and enables its insertion into the activation pocket, thereby forming the primary substrate binding S1 subsite flanking Ser195. This rearrangement is thought to evoke proteinase-like equilibrium conformations in thrombin (Bradford and Krishnaswamy, 2012, Huntington, 2009), a phenomenon that may be modulated by exosite ligation (Kamath *et al.*, 2010). Neutralization of the Ser195 interaction may remove the restriction on mobility exerted by this activation conformation change. In line with these concepts, we also show that FPR-thrombin binds to exosite ligands with lower affinity than plasma-derived or S195A-thrombin (*Chapter Five*). Since active site engagement with FPRck is thought to shift thrombin toward a "proteinase" conformation (Kamath *et al.*, 2010), it follows that overall flexibility of the thrombin allosteric network for exosite binding is decreased. Thus, neutralization of Ser195 removes a barrier for specificity for thrombin's proteinase-like conformations.

Does S195A increase thrombin flexibility since the molecule becomes more ambiguous toward proteinase-specific ligands? To investigate this, we measured effect of active site inhibitors on exosite-mediated univalent and bivalent binding using direct binding studies. In keeping with increased flexibility of the thrombin molecule, dabigatran was much more potent in displacement of S195A-thrombin than plasma-derived thrombin. Interestingly, where argatroban was previously shown to increase exosite binding, its effect on S195A-thrombin was entirely reversed, displacing the mutant from all ligands. Interexosite allostery was also more apparent, as HD22 binding at exosite II robustly displaced S195A-thrombin binding to γA-fibrin at exosite I with greater potency than α -thrombin. Together, these observations suggest that S195A-thrombin has greater flexibility than thrombin, an effect that may be explained by loss of stability in the interaction between the S1 pocket and Ser195.

The influence of the expression system for S195A-thrombin remained to be investigated. To examine this, we compared the binding of wild-type α-thrombin expressed in *E.Coli*, with that of plasma

derived α-thrombin or FPR-thrombin. We demonstrated that α-thrombin expressed in *E.coli* bound to exosite ligands with significantly increased affinity compared with plasma-derived or FPR-thrombin. Because bacterial expression omits carbohydrate on the 60s-loop flanking the active site, steric and allosteric effects may be at play. Our data are reassuring that this carbohydrate domain largely leads to differences in steric hindrance and not in changes to the binding mode as evidenced by global kinetic analysis of the binding profile and displacement studies with active site inhibitors. Thus, the mode of binding of the wild-type α-thrombin expressed in *E. coli* was similar to plasma α-thrombin and FPR-thrombin, and there was no change in the effect of dabigatran or argatroban on the allosteric scaffolds (*Chapter Five*).

Site-directed mutagenesis studies helped decipher the regulators of enzyme binding and function. We show that single-site mutation at Ser195 changes the effect of ligand binding on allosteric scaffolds. This observation is important for the interpretation of mutagenesis data. Indeed, mutagenesis studies at α-thrombin Cys191 (Bush-Pelc *et al.*, 2007) and Glu 192 (Marque *et al.*, 2000) have been shown to cause allosteric changes, and has informed our understanding of the regulation of thrombin allostery (Lechtenberg *et al.*, 2012, Qureshi *et al.*, 2009). Our data encourage caution to be taken with the extrapolation of functional data, especially in the context of allostery, with thrombin mutants.

FUTURE DIRECTIONS

Confirmation of the lack of direct competition between factor X and AT for the intrinsic and extrinsic tenase complexes

Rationale:

Using our system, FXa was confirmed to be protected from AT inhibition by assembly into the prothrombinase complex on PCPS vesicles, and the addition of prothrombin further protected FXa from AT inhibition. Addition of prothrombin decreases the rate of AT inhibition of FXa in a dose dependent manner, suggesting that prothrombin competes with AT for access to FXa.

With FIXa, assembly of the intrinsic tenase complex conferred modest protection to the enzyme from AT inhibition. For FVIIa, assembly of the extrinsic tenase complex increases the rate of AT inhibition, as previously shown.

Interestingly, the addition of the natural substrate of tenase, FX, does not appear to decrease the rate of AT inhibition of the enzyme moiety for either the intrinsic or extrinsic complex. Given that prothrombin can compete with AT for access to FXa in prothrombinase, it is expected that FX also competes in a similar fashion.

A potential explanation for the lack of protection from AT inhibition conferred by FX is a technical limitation due to the use of TAP as a FXa inhibitor. TAP binds to the active site of FXa, but derives its FXa specificity through binding to residues in the autolysis and sodium binding loops that are not conserved in other coagulation proteases. (Rezaie, 2004, Waxman *et al.*, 1990) Because interaction of the FX zymogen with the tenase enzyme complexes may also be dependent on these sites, the use of TAP may preclude FX from effectively competing with AT for FXa.

Future studies: analysis of competition with S195A-factor X

In order to address this, we (1) repeated inhibition experiments with a synthetic direct FXa inhibitor, apixaban, (2) used SPR and SDS PAGE to show that TAP weakly binds FX and only modestly affects the rate of FX cleavage, respectively, (3) used SDS PAGE to show that AT inhibition occurs

concomitantly with FX activation by either tenase complex, and finally (4) will examine the use of recombinant active site inactivated S195A factor X as a substrate for the tenases.

Allosteric regulators in the activation of factor X by tenase complexes

Rationale:

Surprisingly, our preliminary data shows that S195A-factor X proteolysis is faster than WT with extrinsic tenase and slower than WT with intrinsic tenase (SDS-PAGE analysis of FX activation with tenases, not shown). Since the analogous prothrombin proteolysis by prothrombinase is a concerted, sequential reaction that relies on exosites flanking the active site (Bianchini *et al.*, 2005, Kamath *et al.*, 2010), our data suggests that an S195A mutation disrupts allosteric scaffolds within the molecule that regulate these interactions (*Chapter Five*), does the S195A mutation also disrupt exosite-mediated interactions of factor X with the tenase complexes? These studies may also explain why S195A-prothrombin mutants used in studies competing against AT-heparin for prothrombinase binding differed from those using AT mutants (Brufatto *et al.*, 2003, Brufatto and Nesheim, 2001, Rezaie, 2001).

Future studies: Activation of factor X by factor IXa or factor VIIa in the absence of cofactors

To address whether exosite-mediated binding is critical to the differences in the rate of factor X activation, we will compare S195A-factor X cleavage by factor IXa or factor VIIa to WT-factor X using SDS-PAGE. If this effect is limited to assembly in the tenase complex, this suggests that exosite-mediated interactions are necessary for differences seen between proteolysis of WT- and S195A-factor X.

Does prothrombin activation also capacitate the allosteric network for specificity?

Rationale:

Our data suggest that the highly specific allosteric network responsible for the long range effects of active site binding on exosite function may be significantly perturbed or even reversed with a single site mutation of Ser195 to alanine. S195A-thrombin is a hyperflexible molecule (Pozzi *et al.*, 2013b) that displays degree of cooperative binding to bivalent ligands, interexosite communication, and reversal of

active site mediated effects. Since insertion of the newly generated N-terminal Ile321 following Arg320 cleavage during prothrombin proteolysis forms the S1 substrate specificity pocket flanking Ser195, the specificity of the allosteric scaffold may also be subject to regulation by prothrombin activation.

This project aims to determine whether prothrombin activation specifies the long range interactions of the exosites of thrombin by setting the constraints of the allosteric network. Understanding the role of prothrombin activation in the regulation of allostery uncovers a potential mechanism by which thrombin activity is modulated.

Future studies: BIACore interexosite displacement studies with WT-prethrombin-2 in comparison to S195A-prethrombin-2

To determine the whether prothrombin proteolysis affects interexosite allostery, we will measure the interexosite effect of a panel of ligands on the direct binding of WT-prethrombin-2 to γA-fibrin, the GP1bα peptide, and factor Va using BIACore. These will be compared head-to-head to plasma-derived thrombin binding to infer the consequences of Arg320 cleavage and catalytic activation. As a secondary goal, to test the contribution of the Ser195 residue in stabilizing the molecular rearrangement, a comparison of interexosite allostery can also be made using S195A-prethrombin-2. Both prethrombin-2 molecules can be generated using the standard procedure outlined for plasma-derived prethrombin-2.

Preliminary data shows significantly decreased maximal effect and sensitivity of HD22 for disruption of exosite I-mediated TM binding in WT-prethrombin-2 (not shown).

Do the DOACs targeting factor Xa cause allosteric effects on exosite-mediated binding

Rationale:

Exosite-mediated binding interactions are critical for prothrombinase function. The binding of factor Xa to lipid surfaces, factor Va, potentiation by Ca2+ binding, and subsequent interactions with prothrombin are all exosite-mediated (Bradford *et al.*, 2013, Rosing *et al.*, 1980). Binding to Ca2+ and Na+ cations, the factor Va heavy chain, and binding to molecular phosphatidylserine to factor Xa sites distant from the active site have been shown to increase factor Xa catalysis (Barhoover *et al.*, 2008a, Barhoover *et*

al., 2008b, Hirbawi *et al.*, 2010, Majumder *et al.*, 2005, Silva, Jr. *et al.*, 2006, Vogt *et al.*, 2010), affect the mechanism of cleavage (Bukys *et al.*, 2005, Bukys *et al.*, 2006), and cause structural changes (Srivasatava *et al.*, 2014). Thus, exosite-mediated allosteric control likely regulates factor Xa function and specificity like with thrombin exosite interactions.

Given the homology between thrombin and factor Xa, we will investigate whether binding of factor Xa-directed DOACs affects exosite-binding ligands. These can shed light on inter-exosite allosteric regulation in factor Xa and on the secondary antithrombotic mechanisms of the DOACs.

Future studies: SPR displacement studies with WT-factor Xa and S195A-factor Xa.

To determine whether rivaroxaban, apixaban, or edoxaban binding to factor Xa has long-range effects on exosite-mediated binding, we will use surface plasmon resonance to examine the effect of the DOACs on factor Xa binding to (1) phospholipid vesicles using a previously validated approach (Wikstrom and Deinum, 2007), (2) factor Va, and (3) S195A-prothrombin. We will also probe the role of Ser195 in factor Xa allostery using the S195A-factor Xa mutant.

Since recent studies have suggested that factor V activation by factor Xa is a prime driver of thrombin generation (Schuijt *et al.*, 2013). Do the DOACs inhibit factor Xa cleavage and binding to factor V? To investigate this concept, we will examine the binding of S195A-factor Xa to factor V in the presence of rivaroxaban, apixaban, and edoxaban using surface plasmon resonance.

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