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INTERACTION OF THROMBIN WITH PROTHROMBIN FRAGMENT 2, HEPARIN COFACTOR II, AND FIBRIN

By

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ABSTRACT

Thrombin is a multifunctional serine protease that plays a central role in hemostasis. Unlike related serine proteases of the hemostatic system, thrombin is unique in that it has both procoagulant and anticoagulant activities. Structural features defined by X-ray crystallographic studies of thrombin provide a molecular basis for the enzyme's specificity. These features include the active site cleft and two anion-binding electropositive exosites located on opposite poles of the thrombin molecule. What is less evident from crystallographic studies is the thrombin's flexibility and its capacity to undergo conformational changes upon ligand binding to the exosites.

These studies were undertaken to explore different but interrelated aspects of thrombin regulation. The first goal of this thesis was to determine how prothrombin fragment 2 (F2), a prothrombin activation fragment, binds to thrombin and modulates its activity. Cocrystallographic studies have shown that the interaction F2 with thrombin involves the formation of salt bridges between the kringle inner loop of F2 and anion-binding exosite II of thrombin. When F2 binds to thrombin, it has been shown to evoke conformational changes at the active site and at exosite I of the enzyme. Using plasma, recombinant, and synthetic F2 peptides (F2, rF2, and sF2, respectively) we have further localized the thrombin binding domain on F2. F2, rF2(1-116), rF2(55-116), and sF2(63-116), all of which contain the kringle inner loop (residues 64-93) and the acidic C-terminal connecting peptide (residues 94-116), bind to thrombin-agarose. In contrast, analogues of

the kringle inner loop, sF2(63-90), or the C-terminal connecting peptide, sF2(92-116), do not bind. Thus, contrary to predictions from the crystal structure, the C-terminal connecting peptide as well as the kringle inner loop are involved in the interaction of F2 with thrombin. F2 and sF2(63-116) bind saturably to fluorescently labelled-active-site-blocked-thrombin with K_d values of 4.1 and 51.3 μM, respectively. The affinity of sF2(63-116) for thrombin increases about 5-fold (K_d=10 μM) when Val at position 78 is substituted with Glu. F2 and sF2(63-116) bind to exosite II on thrombin because both reduce the heparin-catalyzed rate of thrombin inhibition by antithrombin - 4-fold. In contrast, only F2 slows the uncatalyzed rate of thrombin inactivation by antithrombin. Like F2, sF2(63-116) induces allosteric changes in the active site and exosite I of thrombin because it alters the rates of thrombin-mediated hydrolysis of chromogenic substrates and displaces fluorescently-labelled $hirudin_{54-65}$ from active-site-blocked thrombin, respectively. Both peptides also prolong the thrombin clotting time of fibrinogen in a concentration-dependent fashion reflecting their effects on the active site and/or exosite I. The different functional changes evoked by F2 and sF2(63-116) likely reflect additional contacts of F2 relative to the smaller sF2(63-116) and suggest that ligand binding to various subdomains within exosite II may have different effects on thrombin function. The important implication of these findings is that distinct allosteric effects evoked by ligand binding to subdomains of exosites may contribute to the diversity of thrombin function at the molecular level.

The activity of thrombin is also regulated by blood-borne protease inhibitors. The second goal of this work was to gain insight into the mechanism by which thrombin is

inactivated by heparin cofactor II (HCII), a serine protease inhibitor (serpin) in plasma that selectively inhibits thrombin in a reaction that is accelerated ≥1000-fold by glycosaminoglycans (GAGs) such as dermatan sulfate (DS) and heparin. Current thinking is that GAG binding to HCII disrupts ionic bonds between the amino-terminal acidic domain and the GAG-binding domain of HCII, thereby permitting the acidic domain to interact with exosite I on thrombin. Based on this allosteric activation model, we predicted that substitution of basic residues in the GAG-binding domain of HCII with neutral ones would mimic the catalytic effect of GAGs. Compared with wild-type recombinant HCII expressed in BHK cells (wt rHCII), mutation of Arg^{184}, Lys^{185}, Arg^{192}, Arg^{193} (Mut C) or Arg^{184}, Lys^{185}, Arg^{189}, Arg^{192}, Arg^{193} (Mut D) reduced the affinity for heparin-Sepharose and increased the uncatalyzed rate of thrombin inactivation ~130-fold (from 4.6×10^4 M^{-1} min^{-1} to 6.2×10^6 and 6.0×10^6 M^{-1} min^{-1}, respectively). Furthermore, unlike wt rHCII or plasma-derived HCII (pHCII), neither heparin nor dermatan sulfate increased the rate of thrombin inhibition by Mut C or Mut D. The increased basal rate of thrombin inhibition by these mutants reflects displacement of their amino-terminal acidic domains because (a) they inhibit γ-thrombin at a 65-fold slower rate than α-thrombin, (b) the exosite 1-binding fragment hirudin-(54-65) decreases the rate of thrombin inhibition, and (c) deletion of the amino-terminal acidic domain (-del74) of Mut D reduces the rate of thrombin inhibition ~100-fold. To determine whether GAG-mediated bridging of thrombin to HCII contributes to accelerated thrombin inhibition, we compared the catalytic effects of longer heparin or dermatan sulfate chains with those of shorter chains. Heparin chains comprised of 30 or more saccharide units

produced an ~5-fold greater increase in the rate of thrombin inhibition by pHCII, wt rHCII, and wt-del74 than heparin chains comprised of 20 or fewer saccharide units. In contrast, dermatan sulfate and a low molecular weight fragment of dermatan sulfate stimulated thrombin inhibition by pHCII and wt rHCII to the same extent, and neither agent affected the rate of thrombin inhibition by wt-del74. Our findings support the concept that heparin and dermatan sulfate activate HCII by releasing the acidic amino-terminal domain from intramolecular connections with the GAG-binding domain. Since both GAGs produce ≥1000-fold increase in the rate of thrombin inhibition by HCII, our observation that only heparin serves as a template raises the possibility that dermatan sulfate induces more extensive allosteric changes than heparin.

In addition to regulation by serpins, thrombin function is also modulated by its incorporation into forming thrombi. Despite being catalytically active, fibrin-bound thrombin is protected from inactivation by inhibitors, notably antithrombin (AT)/heparin. The resistance of fibrin-bound thrombin to inactivation by AT is thought to reflect formation of a productive ternary thrombin-fibrin-heparin complex in which thrombin is protected from inactivation by AT. The anchoring of thrombin in a productive ternary complex is mediated by thrombin's exosites, fibrin via exosite I and heparin via exosite II. It has been proposed that productive ternary complex assembly is dependent on binary interactions between thrombin-heparin, thrombin-fibrin, and heparin-fibrin. Unlike heparin, DS inhibits soluble and fibrin-bound thrombin equally well, however the explanation for this phenomenon is unclear.

The third goal of this work was to determine why fibrin-bound thrombin is susceptible to inactivation by the HCII/DS complex but not by the AT/heparin complex. The results of this study indicate that, unlike heparin, DS does not promote the formation of a productive ternary thrombin-fibrin-DS complex. This concept is supported by three lines of evidence. First, in the presence of fibrin monomer (Fm), thrombin is protected from inhibition by HCII/heparin, but not by HCII/DS as quantified by protease inhibition assays under pseudo first-order conditions. Second, DS does not promote the binding of radiolabeled active site-blocked thrombin (^{125}I-FPR-thrombin) to fibrin. In contrast, heparin augments ^{125}I-FPR-thrombin binding to fibrin in a concentration-dependent manner. Third, DS does not interact with fibrin and binds to thrombin with a 22-fold lower affinity than heparin (K_d values of 2.6 μM and 117 nM, respectively). These results reveal that, although exosite I and exosite II of thrombin can be ligated by fibrin and DS, respectively, productive ternary complex does not occur because DS is unable to bridge thrombin to fibrin. These findings indicate that all three binary interactions are essential for productive ternary complex formation. We also examined the protective effect of the thrombin-fibrin-heparin complex on thrombin inhibition by Mut D. Whereas Fm alone has little effect on the uncatalyzed rate of thrombin inhibition by Mut D, addition of heparin decreases the rate of thrombin inhibition by Mut D ~ 30 fold (from 6.0 x 10^6 M^{-1} min^{-1} to 2.1 M^{-1} min^{-1}). Furthermore, in the presence of Fm, heparin causes a dose-dependent decrease in the DS-catalyzed rate of thrombin inhibition by HCII. These observations reveal that the protective effect of heparin results from the anchoring of thrombin in a productive thrombin-fibrin-

heparin complex in which exosite I is inaccessible to the amino-terminus of HCII.

Collectively, these studies illustrate different modes of regulating thrombin function, all of which are intricately interrelated. The remarkable diversity of thrombin activity allows thrombin to serve multiple functions in highly controlled processes in hemostasis.

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ABBREVIATIONS

A	absorbance
ANS	anilinonapthalene-6-sulfonic acid
AT	antithrombin
AP	α2-antiplasmin
BHK	Baby Hamster Kidney cell line
CNBr	cyanogen bromide
COOH	carboxy
Desmin	a 5.6 kDa low molecular weight dermatan sulfate
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DS	dermatan sulfate
FITC	fluorescein-5-isothiocyanate
Fm	fibrin monomer
FPA	fibrinopeptide A
FPB	fibrinopeptide B
ϵ	extinction coefficient
E. coli	Escherichia coli
ECM	extracellular matrix
F1	prothrombin fragment 1
F2	prothrombin fragment 2

FPR	D-Phe-Pro-Arg chloromethyl ketone
GAG	glycosaminoglycan
Gla domain	gamma-carboxyglutamic acid domain
GPRP-NH_2	Gly-Pro-Arg-Pro-amide
g	gravitational force
HBSE	20 mM Hepes, pH 7.0, 100 mM NaCl, 1 mM EDTA
Hepes	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HCII	heparin cofactor II
I/Io	ratio of intensity at time t to intensity at time 0
IPTG	isopropyl-1-thio-β-D-galactoside
k_1	pseudo first-order rate constant
k_2	apparent second-order rate constant
K_d	dissociation constant
K_m	Michaelis constant
k_{cat}	turnover number
kDa	kiloDalton
PCR	polymerase chain reaction
polybrene	hexadimethrine bromide
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	20 mM Tris-HCL, pH 7.4, 150 mM NaCl
Tris	tris-(hydroxymethyl)-aminomethane

TSP	20 mM Tris-HCL, pH 7.4, 150 mM NaCl, 0.6% PEG 8000
TSPTw	TSP, 0.01% Tween 20

CHAPTER 1- INTRODUCTION

The hemostatic system serves to maintain intravascular blood fluidity as well as to prevent hemorrhage. Hemostasis is achieved by a delicate balance of procoagulant, anticoagulant, and fibrinolytic activities. The fundamental mechanism of coagulation and fibrinolysis is the sequential proteolytic activation of circulating zymogens, strictly controlled by both positive and negative feedback loops. Acquired or inherited abnormalities that impair hemostasis can cause bleeding or thrombotic tendencies. Thrombosis can produce a spectrum of clinical manifestations depending on which vascular bed is involved. Thrombosis in the venous system produces deep vein thrombosis and pulmonary embolism, whereas clotting in arterial beds may manifest as myocardial infarction or stroke (Colman et al., 1994).

One of the key regulatory enzymes of hemostasis is thrombin, a multifunctional trypsin-like serine protease that is involved in diverse physiologic processes such as inflammation, wound healing, and brain development in addition to hemostasis (for review, see Fenton, 1995). As a procoagulant enzyme, thrombin activates blood clotting factors V, VIII, and XIII, as well as platelets (for review, see Mann 1994). In addition, thrombin converts soluble plasma fibrinogen into insoluble fibrin, a major component of the matrix of thrombi (for review, see Hantgan et al., 1994). In contrast, when complexed with thrombomodulin (TM), an endothelial cell membrane glycoprotein, thrombin acquires anticoagulant properties through its ability to activate protein C (for review, see Esmon,

1991). Activated protein C limits the amplification and progression of the coagulation cascade by inactivating factors Va and VIIIa. Recently, the thrombin/thrombomodulin complex has also been shown to inhibit fibrinolysis by activating a thrombin-activatable fibrinolysis inhibitor (TAFI) which down-regulates the formation of plasmin, an enzyme that degrades fibrin (Bajzar et al., 1996; Bajzar et al., 1998). Thus, the partitioning of thrombin between procoagulant, anticoagulant, and antifibrinolytic pathways allows thrombin to participate in the fine control of hemostasis.

Because of its central role in hemostasis, thrombin activity is strictly regulated. There are a number of ways in which thrombin function is controlled. These include (a) the proteolytic activation of prothrombin to thrombin, (b) allosteric modulation of thrombin activity, (c) inhibition of thrombin by plasma serine protease inhibitors (serpins), and (d) interaction of thrombin with fibrin. In this thesis, three different aspects of the modulation of thrombin were investigated, all of which have biochemical and physiological relevance to the regulation of thrombin in hemostasis. First, I determined how prothrombin fragment 2 (F2), a ligand for thrombin, modulates thrombin's function (Chapter 3). Second, I investigated the mechanism by which thrombin is inactivated by heparin cofactor II (HCII), a serine protease inhibitor (serpin) in human plasma that selectively inhibits thrombin in a reaction that is catalyzed by glycosaminoglycans (GAGs) (Chapter 4). Third, I examined why thrombin bound to fibrin is protected from inactivation by heparin but not dermatan sulfate (Chapter 5). The following sections describe the current knowledge of thrombin's role in hemostasis with emphasis given to the regulation of thrombin through modulation of thrombin generation and inhibition.

1.1 Proteolytic Activation of Prothrombin to Thrombin

Human prothrombin, a 581-residue plasma glycoprotein synthesized by the liver, is converted from an inactive zymogen to the serine protease thrombin in the final stages of the blood coagulation cascade (for review, see Mann, 1994). Prothrombin is the most abundant vitamin K-dependent coagulation protein, present in the circulation at concentrations ranging from 1 to 2 μM (McDuffie et al., 1979). Prothrombin consists of an amino-terminal Gla domain, which contains 10 γ-carboxyglutamic acid residues that mediate the Ca^{2+}-dependent interaction of prothrombin with negatively charged phospholipid surfaces, followed by two contiguous kringle domains, and a carboxy-terminal serine protease domain (Mann, 1976).

Under physiologic conditions, prothrombin activation is catalyzed by the prothrombinase complex, composed of the serine protease, factor Xa, the cofactor, factor Va, Ca^{2+}, together with a phospholipid surface (for review, see Mann et al., 1988). The phospholipid membrane surface, provided by injured endothelial cells, activated platelets, or monocytes, localizes the coagulation response to sites of vascular damage (Mann et al., 1990). Although factor Xa alone can activate prothrombin, the incorporation of factor Xa into the prothrombinase complex increases the catalytic efficiency (Kcat/Km) of factor Xa by ~ 10^5-fold (Nesheim et al., 1979; Rosing et al., 1980). It has been postulated that the interaction of factor Xa with factor Va increases the turnover number (Kcat) of factor Xa about 3000-fold (Rosing et al., 1980), while the interaction of prothrombin with anionic phospholipid is responsible for a ~100-fold decrease in the Km for prothrombin activation (Giesen et al., 1991; Billy et al., 1995).

In vivo, Factor Xa in the prothrombinase complex initially cleaves prothrombin at

Arg^{320}-Ile^{321} to yield the active, but transient, intermediate meizothrombin (Fig. 1) (Owen et al., 1974; Krishnaswamy et al., 1986; Rosing et al., 1989; Doyle and Mann, 1990). Further cleavage by factor Xa at Arg^{271}-Thr^{272} yields active thrombin, which consists of two polypeptides linked by a disulfide bond as well as the amino terminal activation peptide termed fragment 1.2 (Fig. 1). Fragment 1.2 can then be cleaved at Arg^{155}-Ser^{156} by thrombin or by meizothrombin to liberate fragment 1 (F1), consisting of the Gla domain and the kringle 1 domain, and fragment 2 (F2), consisting of the kringle 2 domain (Mann, 1976). Thrombin and meizothrombin are also capable of cleaving the Arg^{155}-Ser^{156} bond of meizothrombin to release F1, thereby generating another transient intermediate, meizothrombin desF1 (Morita et al., 1976).

Meizothrombin has been shown to be generated during the clotting of whole blood *in vitro* (Bovill et al., 1995) and both meizothrombin and meizothrombin desF1 have been detected when prothrombin is activated on endothelial cell surfaces (Tijburg et al., 1991). Although these intermediates have catalytic efficiencies towards tripeptide substrates similar to that of thrombin, they have reduced activities towards macromolecular substrates such as factor V, fibrinogen, and platelets (Doyle and Mann, 1990; Cote et al., 1997). Recently, meizothrombin was shown to exhibit only 10% of the antifibrinolytic activity of thrombin, as expressed through TM-dependent activation of TAFI, but was 6-fold more potent than thrombin in the activation of protein C (Cote et al., 1997). These data suggest that, relative to thrombin, meizothrombin has greater profibrinolytic and less coagulant activity.

Prothrombin activation fragment F2 also appears to play a physiologic role in hemostasis. Human F2 is a 116-residue kringle domain that binds saturably to fluorescently-

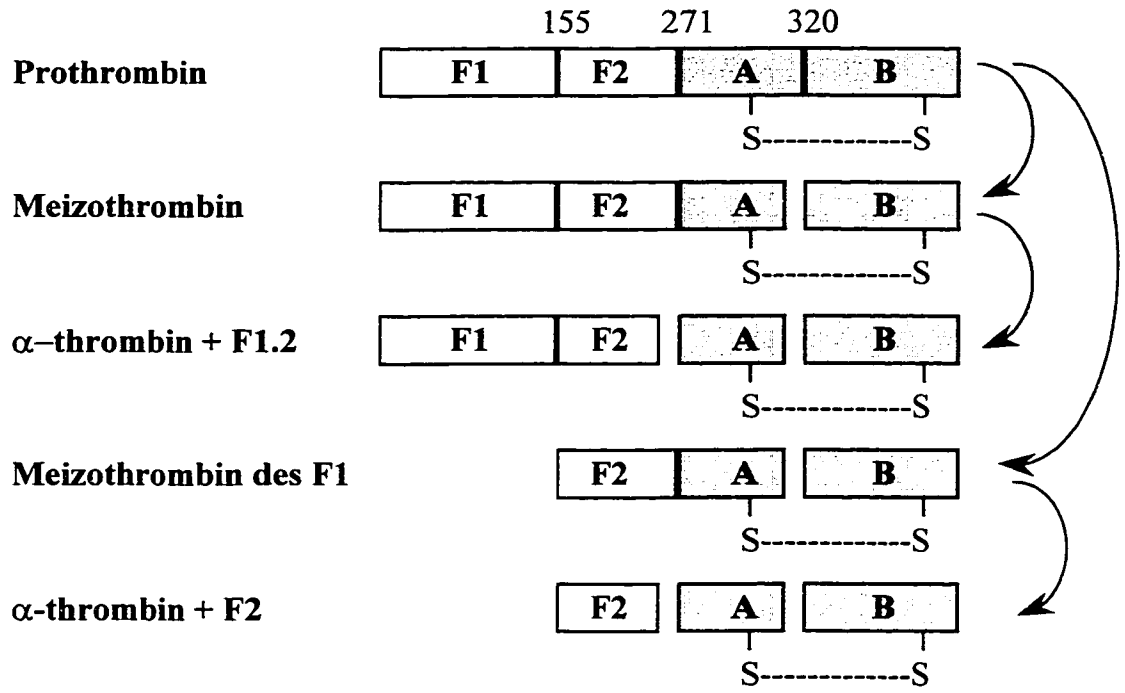


Fig. 1. **Schematic diagram of pathways and intermediates in the activation of human prothrombin** (adapted from Mann, 1994). Human prothrombin consists of fragment 1(F1), fragment 2 (F2), and the serine protease domain. A and B represent thrombin A and B chains, respectively, linked by a disulfide bond. Two pathways of prothrombin activation are indicated by arrows on the right side.

labelled, active site-blocked thrombin with a Kd value of ~ 4 μM (Bock, 1992; Liaw et al., 1998). Although F2 has also been shown to interact with factor Va (Bajaj et al., 1975), F2-factor Va interactions do not improve the catalytic efficiency of factor Xa within the prothrombinase complex. Instead, the role of F2 may be to alter the conformation of prothrombin so that its scissile bond(s) is more complementary to the active site of factor Xa (Krinshnaswamy and Walker, 1997). After release from prothrombin, F2 retains its ability to bind thrombin and can modulate thrombin's function (see section 1.3.1). The F1 fragment of prothrombin appears to regulate the accessibility of anion-binding exosite I (Wu et al., 1994).

1.2 Structure of Thrombin

Thrombin is a glycosylated serine protease consisting of a 36-residue A chain and a 259-residue B chain, covalently linked by a disulfide bond (Degen et al., 1983). Three dimensional X-ray crystallographic determinations of human thrombin complexed with various substrates (Stubbs et al., 1992; Mathews et al., 1994), ligands (Martin et al., 1992; Arni et al., 1993; Padmanabhan et al., 1993; Mathews et al., 1994), and inhibitors (Bode et al., 1989; Rydel et al., 1990; Bode et al., 1992; Karshikov et al., 1992) have provided a wealth of information on the structure of thrombin.

1.2.1 Overall Structure

Thrombin, an ellipsoid molecule of approximate dimensions 45 Å x 45 Å x 50 Å, consists of two six-stranded antiparallel barrel domains, five helical segments, one helical turn, and several surface turn structures (Bode et al., 1992). A schematic diagram of the functional domains of thrombin is shown in Figure 2. A convenient nomenclature system

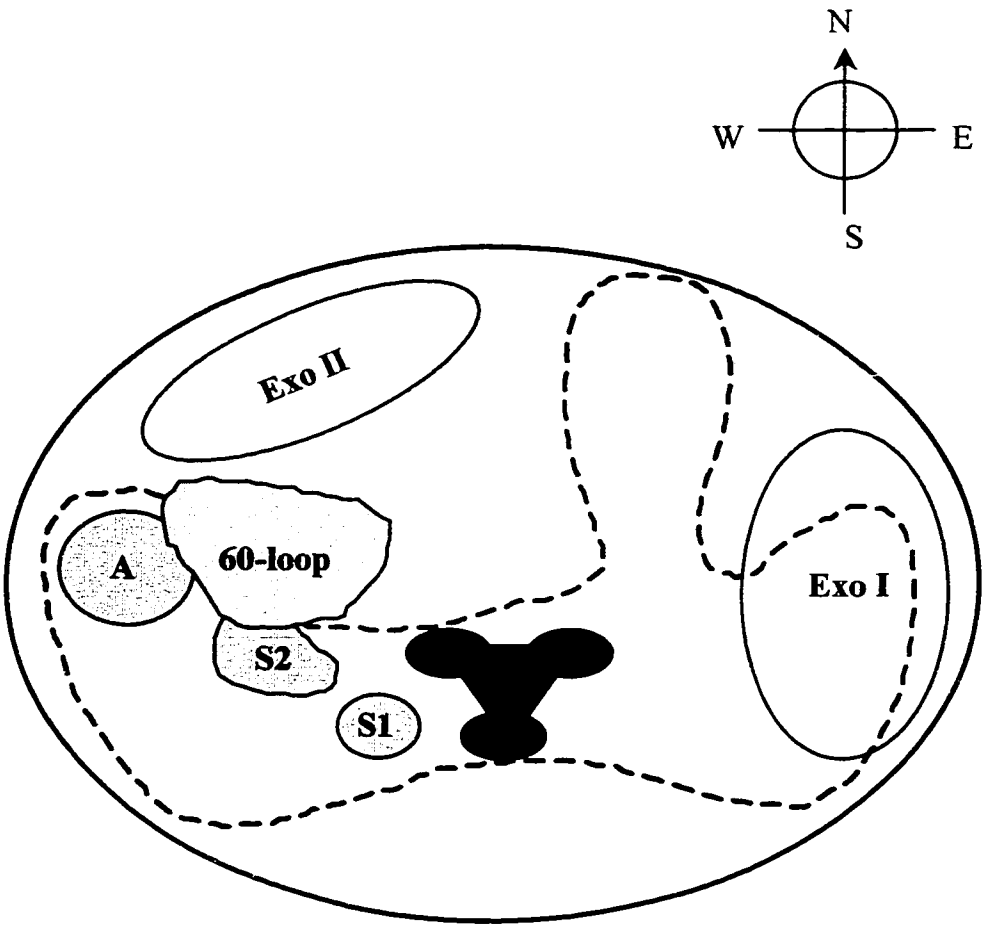


Fig. 2. **Schematic diagram of the functional domains of thrombin** (adapted from Stubbs and Bode, 1993). The active site canyon is depicted with dotted lines. Symbols: ▼, catalytic triad (Ser^{195}, His^{57}, and Asp^{102}); S1 and S2, specificity sites amino-terminal to cleavage; A, aryl binding site; 60-loop, hydrophobic nine-residue insertion loop; Exo I, anion-binding exosite I; Exo II, anion-binding exosite II.

for numbering thrombin residues is based on topological similarities between thrombin and bovine chymotrypsin (Bode et al., 1989). This system allows direct comparison of the structure of thrombin with that of other serine proteases. The A-chain, positioned along the back side of the thrombin molecule, is likely flexible because its conformation varies in different crystals (Stubbs and Bode, 1993).

1.2.2 Thrombin's Active Site Cleft

The hydrogen-bonded active site residues (Ser^{195}, His^{57}, and Asp^{102}) are located at the junction between the two barrel domains and form the base of the deep canyon-like active site cleft, which runs from "west" to "east" (Fig. 2). In this cleft, the peptide chain of the inhibitor or substrate is positioned to optimally align the site of cleavage with the active site. The residues within a substrate or inhibitor's reactive centre loop are numbered from amino- to carboxy-terminus as follows: P_n-...P_2-P_1-P_1'-P_2-...-P_n', where proteolysis occurs at the P_1-P_1' bond (Schechter and Berger, 1967). The complementary binding sites on the protease are numbered S_n-...-S_2-S_1-S_1'-S_2'-...-S_n'. The channel-shaped S1 pocket is the principal determinant of substrate specificity. Asp^{189}, at the bottom of the pocket, forms ionic bonds with the basic P1 residue (Bode et al., 1992). Mutations in the P1 residue of several serine protease inhibitors (serpins) demonstrate the importance of this residue for enzyme specificity (Owen et al., 1983; Heeb et al., 1990; Derechin et al., 1990). The hydrophobic S2 pocket, formed by residues His^{57}, Ser^{214}, Leu^{99}, Tyr^{60A}, and Trp^{60D}, binds to P2 residues, which are typically nonpolar, medium-sized residues such as proline (Bode et al., 1992). Adjacent to the S2 pocket is the hydrophobic aryl binding site formed by Ile^{174}, Trp^{215}, segment 97-99, and Tyr^{60H}, which binds to aromatic residues (Bode et al., 1992).

The geometry of thrombin's active site triad is almost identical to that of other trypsin-like serine proteases (Bode and Huber, 1992). However, when compared with chymotrypsin, the prototypical serine protease, thrombin has several peptide insertions or "loops". Two of the most notable loops extend above and below the active site, serving to restrict the approach of most macromolecular substrates and inhibitors. The upper loop is a hydrophobic and rigid 9-residue 60-loop or the YPPW (Tyr^{60A}-Pro^{60B}-Pro^{60C}-Trp^{60D}) loop located on the northern side of the active site cleft (Bode et al., 1992). Mutagenesis studies have shown that deletion of only three residues (Pro^{60B}, Pro^{60C}, and Trp^{60D}) within this loop improves the accessibility of thrombin to bovine pancreatic trypsin inhibitor but does not affect thrombin's specificity toward small synthetic inhibitors (Le Bonniec et al., 1993). Interestingly, this thrombin variant exhibits poorer activity with fibrinogen, protein C, antithrombin (AT), and hirudin, findings that may be explained by the fact that residues within the 60-loop also contribute to the S2 pocket and the aryl binding site (Le Bonniec et al., 1993). The 9-residue lower loop, also known as the autolysis loop, consists of a five-residue insertion (Ala^{149a}-Asn^{149b}-Val^{149c}-Gly^{149d}-K^{149e}) that opposes the 60-loop (Bode et al., 1992). Upon binding to hirudin, this loop assumes a different conformation to that observed in the PPACK-thrombin structure (Grutter et al., 1990; Rydel et al., 1990; Rydel et al., 1991).

1.2.3 Anion-binding Exosites

The recognition of macromolecular substrates by thrombin also involves secondary binding sites or "exosites" located on the surface of the thrombin molecule. Both are positively charged patches and lie on opposing sides of the active site. Anion-binding exosite I, located on the "eastern" surface of thrombin, consists of the exposed side chains

of nine basic residues (Lys^{149E}, Arg^{67}, Arg^{73}, Arg^{75}, Arg^{77A}, Lys^{81}, Lys^{36}, Lys^{109}, and Lys^{110}). Exosite I interacts with the carboxy-terminal 17 residues of the leech-derived thrombin inhibitor hirudin (Grutter et al., 1990; Rydel et al., 1990; Rydel et al. 1991), the fifth and sixth epidermal growth factor-like domains of thrombomodulin (Kurosawa et al., 1988), the thrombin receptor (Herbert et al., 1994), the central E domain of fibrinogen (Berliner and Sugawara, 1985; Kaminski and McDonagh, 1987; Naski and Shafer, 1990; Stubbs et al., 1992), the D domain on the γ' chains of fibrin(Meh et al., 1996), and the amino-terminal acidic domain of HCII (Hortin et al., 1989). Site-directed mutagenesis studies of exosite I indicates that residues with procoagulant or anticoagulant functions are not spatially separated, but instead interdigitate with amino acids of opposite functions (Tsiang et al., 1995).

Anion-binding exosite II, extending over the north-western surface of thrombin, encompasses eight basic residues (Arg^{97}, Arg^{101}, Arg^{165}, Lys^{169}, Arg^{173}, Arg^{175}, Arg^{233}, and Lys^{235}) (Bode et al., 1992). This exosite binds heparin (Church et al., 1994; Gan et al., 1994; Sheehan and Sadler, 94), the chondroitin sulfate moiety of thrombomodulin (Ye et al., 1993; Liu et al., 1994), and prothrombin fragment 2 (F2) (Arni et al., 1993). The two basic exosites, located at opposite ends of the thrombin molecule, are separated by a patch of negatively charged amino acids that extend around the active site of thrombin and influence the orientation of approaching macromolecular substrates and inhibitors (Bode et al., 1992). Both anion-binding exosites on thrombin are involved in the recognition of factor V and factor VIII (Esmon and Lollar, 1996).

1.3 Allosteric Regulation of Thrombin Activity

Although X-ray diffraction studies have provided much detail into thrombin's structure, many functional studies indicate that thrombin is not a rigid molecule, but rather undergoes allosteric transitions linked to the binding of different ligands. This capacity to undergo conformational changes allows thrombin to participate in seemingly antagonistic roles in hemostasis and represents another strategy for molecular recognition.

1.3.1 Modulation of Thrombin Activity by Macromolecules

Thrombomodulin (TM), an integral membrane glycoprotein found on the endothelial cell surface, plays an important role in inhibiting blood coagulation (for review, see Esmon et al., 1991). TM binds to exosite I of thrombin via its fifth and sixth epidermal growth factor-like repeats (Ye et al., 1992), an interaction that is enhanced by the binding of the chondroitin sulfate moiety of TM to exosite II of thrombin (Liu et al., 1994). The binding of thrombin to TM evokes a change in the macromolecular specificity of thrombin that converts the procoagulant enzyme into an anticoagulant enzyme. Thrombin bound to TM functions as an anticoagulant by activating protein C, which, along with protein S, proteolytically degrades and inactivates factors Va and VIIIa, thereby inhibiting further thrombin generation (Esmon, 1991). Although binding of epidermal growth factor-like domains five and six to exosite I on thrombin alters thrombin's specificity as shown by changes in both k_{cat} and K_m, epidermal growth-factor-like domains four through six are also required for TM to accelerate protein C activation (Zushi et al., 1989; Hayashi et al., 1990; Ye et al., 1991).

Structure-function studies using nitroxide spin labels (Musci et al., 1988) and

fluorescent dyes (Ye et al., 1991) positioned at different locations within thrombin's active site have shown that thrombomodulin functions as an allosteric modulator by altering the conformation of thrombin's active site environment. It has been proposed that TM induces a conformational change in thrombin's active site that alleviates the inhibitory effects that arise from charge repulsion between the acidic residues at the S3 and S3' sites of thrombin and the acidic residues at the P3 and P3' positions of protein C (Le Bonniec and Esmon, 1991; Le Bonniec et al., 1991). Unlike protein C, the P3 and P3' sites of fibrinogen are occupied by non-acidic residues, thereby serving as an excellent substrate for unbound thrombin. Other studies have shown that the carboxy-terminus of hirudin also affects the conformation of thrombin's active site and alters the rates of hydrolysis of chromogenic substrates (Jackman et al., 1992; Duffy et al., 1997; Fredenburgh et al., 1997). Thus, exosite I may serve as an allosteric modulator of thrombin structure and function.

F2, a prothrombin activation fragment that interacts with exosite II on thrombin, can also influence thrombin's structure and function. The binding of F2 to thrombin has been shown to slow the rate of thrombin inhibition by AT ~ 3-fold (Walker and Esmon, 1979), change the environment of thrombin's active site (Bock, 1992), stimulate the esterolytic activity of thrombin on synthetic substrates (Myrmel et al., 1976; Fredenburgh et al., 1997), inhibit the fibrinogen clotting activity of thrombin (Jakubowski et al., 1986), and shift the Ca^{2+}-dependence of protein C activation (Liu et al., 1994). These studies highlight the similarities between exosite II and exosite I in terms of their ability to regulate thrombin. Furthermore, there is also direct allosteric linkage between the two exosites since ligand binding at one exosite affects the binding properties of the opposing exosite (Fredenburgh

et al., 1997).

1.3.2 Modulation of Thrombin Activity by Na^+ ions

Na^+ ions can also bind to thrombin and affect its function. In addition to inducing major conformational changes as monitored by intrinsic fluorescence measurements, kinetic studies indicate that Na^+ ions alter the rates of hydrolysis of synthetic substrates, with the slow (Na^+-free) form of thrombin showing a ~ 10-fold reduced catalytic efficiency compared with the fast (Na^+-bound) form of thrombin (Wells and Di Cera, 1992). The slow form of thrombin is anticoagulant because it cleaves protein C with higher specificity (Dang et al., 1995), while the fast form of thrombin is procoagulant because it has increased fibrinogen clotting activity (Mathur et al., 1993).

The molecular basis for Na^+-induced allosteric regulation of catalytic activity in serine proteases depends primarily on the residue at position 225 (Dang and Di Cera, 1996), which lies near Asp^{189} of the S1 pocket within a water solvent channel (Di Cera et al., 1995). Substitution of Tyr^{225} with Pro in thrombin results in a mutant that loses the ability to bind Na^+ and behaves like the allosteric slow form in terms of its reduced specificity towards fibrinogen and enhanced rate of protein C activation (Dang and Di Cera, 1996; Dang et al., 1997). Site-directed mutagenesis studies have shown that residues Glu^{39}, Trp^{60D}, Glu^{192}, Asp^{221}, and Asp^{222} in thrombin form part of the structural network that enables the Na^+ binding site to communicate allosteric changes to the active site and to exosite I (Guinto et al., 1995).

In vivo, the slow and fast forms are almost equally populated because the K_d for Na^+ binding (113 mM) is similar to the Na^+ concentration in the blood (145 mM) (Wells and Di

Cera, 1992). It has been proposed that the role of Na^+ is to facilitate the switching mechanism upon binding to macromolecules such as fibrinogen and thrombomodulin (Ayala and Di Cera, 1994). First, ligand binding at exosite I of thrombin induces the release of a Cl^- ion from the thrombin surface. Second, a conformational change occurs that increases the accessibility of the catalytic site through movement of the Trp^{148} loop. This conformational change is linked to Na^+ binding and to the slow to fast transition of thrombin. In the case of fibrinogen, Na^+ uptake is followed by the interaction of the E domain with the catalytic site. However, the allosteric nature of thrombin is likely more complex as demonstrated by the finding that a synthetic compound, designated LY254603, mediates a change in thrombin specificity through a mechanism that appears to be independent of allosteric changes induced by either sodium ions or TM (Berg et al., 1996).

1.4. Inhibition of Thrombin by Plasma Serpins

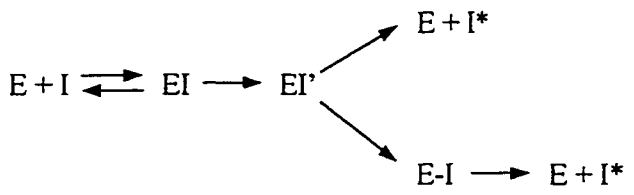
The activity of thrombin is also influenced by the balance between its activation and inhibition. Three prominent inhibitors exist in the circulation to limit thrombin activity. In plasma, 74% of the free thrombin generated is inhibited by the serpin AT, 17% by α2 macroglobulin, and 9% by the serpin HCII (Mitchell et al., 1991). α2 macroglobulin, an inhibitor with broad specificity, will not be discussed further in this work.

1.4.1 Serpins

Serpins are a superfamily of structurally related proteins that regulate proteolytic events in a wide variety of biological processes such as coagulation, fibrinolysis, complement activation, inflammation, and angiogenesis (Church et al., 1997). Mammalian serpins are large proteins (> 400 residues) consisting of three beta-sheets surrounded by alpha-helices.

Serpins inactivate their target proteases by forming equimolar, covalent serpin-protease complexes. The covalent serpin-protease complex arises from the cleavage of the P1-P1' bond in the serpin's reactive center loop by the cognate protease, and the formation of a stable acyl-enzyme intermediate (Lawrence et al., 1995; Wilczynska et al., 1995). Cleavage of the P1-P1' bond is followed by a rapid insertion of the reactive centre loop into the body of the protein as a central strand of β-sheet A (Loebermann et al., 1984; Wilczynska et al., 1997). This insertion leads to an increase in thermal stability of the serpin (Creighton et al., 1992; Lee et al., 1996).

Serpins inactivate their target proteases by a branched suicide-substrate inhibition mechanism (Patson et al., 1991):



In this scheme, the protease (E) and the serpin (I) initially form a non-covalent, Michaelis complex (EI), which rapidly converts to an intermediate (EI'). EI' likely represents an acyl-enzyme intermediate in which the carbonyl group of the P1 residue and the hydroxyl group of the active site serine are linked via an ester bond (Lawrence et al., 1995; Olson et al., 1995; Wilczynska et al., 1995). Depending on the reaction conditions, EI' can then proceed either along the substrate pathway, in which a cleaved inactive serpin (I*) and a free enzyme (E) are generated, or the inhibitory pathway, in which the serpin-protease complex (E-I) is generated. E-I may dissociate slowly to yield cleaved inactive serpin and free protease.

1.4.2 Inhibition of Thrombin by AT

AT, a member of the serpin superfamily of proteins, is the principal plasma inhibitor of proteases involved in the coagulation cascade, especially thrombin and factor Xa (for review, see Bjork and Olson, 1997). The importance of AT in regulating the coagulation system is highlighted by the observation that individuals with AT deficiency or dysfunction are subject to recurrent thrombosis (Olds et al., 1994). AT is a 58 kDa single-chain glycoprotein consisting of 432 residues, three disulfide bonds, and four asparagine-linked carbohydrate side chains (Bock et al., 1982; Prochownik et al., 1983; Franzen et al., 1980). The reactive centre peptide bond (Arg^{393}-Ser^{394}) of AT is located near the carboxy-terminus of the protein (Bjork et al., 1982). The presence of Arg^{393} at the P1 position results in the broad specificity of AT, enabling all of the proteases of the intrinsic coagulation pathway, as well as plasmin, to be inhibited (Rosenberg, 1987).

Compared with most other serpin-protease reactions, the rate of thrombin inhibition by AT is slow (~2×10^5 M^{-1} min^{-1}), but is accelerated by 3 orders of magnitude (~ 2×10^8 M^{-1} min^{-1}) in the presence of heparin (Griffith, 1982), a pharmaceutical sulfated polysaccharide consisting of alternating D-glucuronic or L-iduronic acid and N-sulfated D-glucosamine (Casu, 1989) (Fig. 3). The physiological source of cofactor activity is heparan sulfate, a related glycosaminoglycan (GAG) found in the luminal and abluminal surface of blood vessels (De Agostini et al., 1990; Felsch et al., 1994), which localizes AT activity to the site of vessel wall injury where activated coagulation proteases are generated.

The heparin-mediated potentiation of thrombin inhibition results from the simultaneous binding of AT and thrombin to the same heparin molecule, a process that

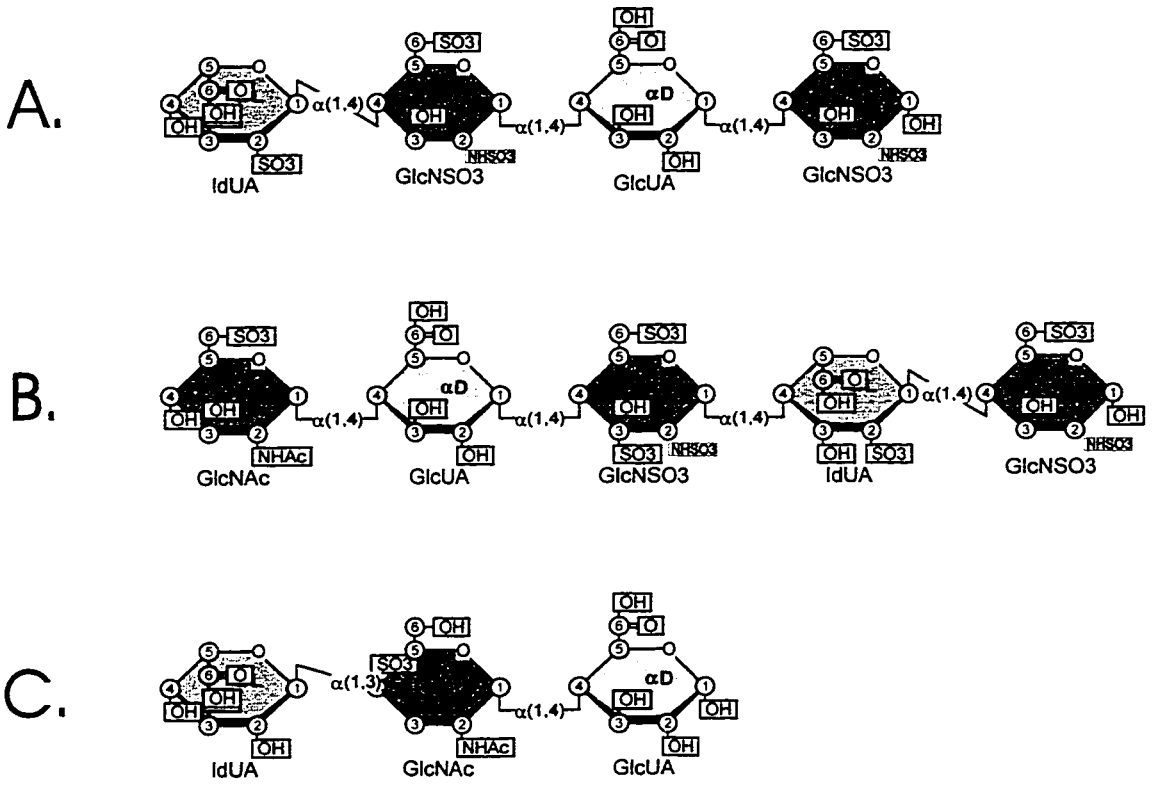


Fig. 3. **Comparison of glycosaminoglycan structures** (adapted from Tollefsen et al., 1987). Panel A, common repeating structure of heparin; panel B, high-affinity AT-binding pentasaccharide sequence of heparin; panel C, common repeating structure of dermatan sulfate.

bridges AT and thrombin in a ternary complex (Olson and Bjork, 1991). The sulfate and carboxyl groups of heparin form salt bridges with basic residues in thrombin exosite II (Church et al., 1989; Gan et al., 1994; Sheehan et al., 1994) and with basic residues in the A and D helices of AT (Liu and Chang, 1987; Peterson et al., 1987; Sun and Chang, 1990; Ersdal-Badju, 1997). The high affinity AT-binding region of heparin, present in about one-third of all heparin chains in commercial preparations, consists of a unique, highly sulfated pentasaccharide sequence (Fig. 3) (Lindahl et al., 1980; Casu et al., 1981; Choay et al., 1983). Approximation of AT and thrombin requires pentasaccharide-containing heparin molecules of at least 18 saccharide units. In contrast with this mechanism, a conformational change in AT induced by binding to the pentasaccharide is sufficient for factor Xa inhibition (Olson et al., 1992). This conformational change is reflected in the increased fluorescence displayed by buried tryptophans in the heparin-AT complex (Olson and Shore, 1981). It has been proposed that the binding of the pentasaccharide region of heparin to AT leads to an expulsion of the reactive centre loop from β-sheet A, thereby increasing its reactivity with factor Xa (van Boeckel et al., 1994).

1.4.2 Inhibition of Thrombin by HCII

HCII, also known as human leuserpin-2 (Ragg and Preibisch, 1988), is a second GAG-catalyzed inhibitor of thrombin in plasma (Tollefsen et al., 1982). HCII selectively inhibits thrombin in a reaction that is catalyzed ≥1000-fold by GAGs such as heparin, dermatan sulfate, and heparan sulfate (Tollefsen et al., 1983). Dermatan sulfate, a naturally occurring sulfated GAG found in the extracellular matrix of connective tissue (Rosenberg et al., 1985; Choi et al., 1989), consists of a repeating polymer of D-glucuronic or L-iduronic

acid and N-acetyl-D-galactosamine (Maimone and Tollefsen, 1990). The finding that cultured fibroblasts and vascular smooth muscle cells, but not endothelial cells, acclerate thrombin inhibition by HCII suggests that HCII has evolved to inhibit thrombin at extravascular sites where dermatan sulfate is present (McGuire and Tollefsen, 1987; Hiramoto and Cunningham, 1988). This concept is supported by the observation that, unlike AT, deficiencies in HCII are not associated with thrombotic disease (Tollefsen et al., 1997). However, *in vitro* studies indicate that the anticoagulant activities of therapeutic dermatan sulfate and some of the anticoagulant properties of heparin are mediated by HCII (Parker and Tollefsen, 1985; Sie et al., 1988; Fernandez et al., 1986; Merton and Thomas, 1987).

HCII is a 66 kDa, single-chain glycoprotein found in human plasma at a concentration of 1.2 μM (Tollefsen and Pestka, 1985). The serpin consists of 480 amino acid residues with three potential asparagine-linked glycosylation sites at Asn^{30}, Asn^{169}, and Asn^{368} and two potential sulfation sites at Tyr^{60} and Tyr^{73} (Hortin et al., 1986; Kim et al., 1988). The P1 residue, Leu^{444}, is inconsistent with thrombin specificity for basic residues which likely explains the observation that the uncatalyzed rate of thrombin inactivation by HCII (~ $2 \times 10^4\ M^{-1}\ min^{-1}$) is about 10-fold slower than that for AT (Griffith et al., 1985).

The amino-terminus of HCII contains a duplicated sequence from residues 55 to 75, Glu-Asp-Asp-Asp-Tyr-X-Asp (where X=Leu and Ile), and has been shown to interact with exosite I of thrombin (Hortin et al., 1989). The GAG-binding domain of HCII, consisting of residues Arg^{184}, Lys^{185}, Arg^{189}, Arg^{192}, and Arg^{193} in the D-helix, was identified by analysis of natural and recombinant variants of HCII (Blinder et al., 1989; Blinder and Tollefsen, 1990; Ragg et al., 1990a, 1990b). Deletion of the amino-terminal acidic domain of HCII

increases the affinity of HCII for heparin-Sepharose, suggesting that in native HCII the GAG-binding domain interacts with the acidic domain (van Deerlin and Tollefsen, 1990).

An allosteric model has been proposed to rationalize GAG-mediated catalysis of thrombin inhibition by HCII (Ragg et al., 1990a; van Deerlin and Tollefsen, 1991; Sheehan et al., 1994). In this model, binding of GAGs to the electropositive GAG-binding domain on HCII displaces the amino-terminal acidic domain so that it can interact with exosite I of thrombin. This promotes enzyme-inhibitor complex formation prior to active site interaction (Fig. 4). The importance of the amino-terminal acidic domain in mediating GAG-catalyzed thrombin inhibition by HCII is supported by the observations that (a) deletion of this domain markedly decreases the rate at which HCII inhibits thrombin in the presence of heparin or dermatan sulfate (van Deerlin and Tollefsen, 1991) and (b) certain GAG-binding domain HCII mutants exhibit increased uncatalyzed thrombin inhibitory activities, an observation that is postulated to reflect increased mobility of the amino-terminal acidic domain through disruption of ionic interactions (Ragg et al. 1990a; Liaw et al., 1998). This mechanism is representative of dermatan sulfate-mediated acceleration of thrombin inhibition by HCII. In the case of heparin, maximal stimulation requires a combination of allosteric changes and a templating effect in which heparin binds simultaneously to exosite II of thrombin and the GAG-binding domain of HCII (Rogers et al., 1992; Sheehan et al., 1994; Liaw et al., 1998).

1.5 Interaction of Thrombin with Fibrin

Thrombin activity is also influenced by saturable and reversible binding of the enzyme to the growing fibrin network, a process that can limit thrombin's reactivity with macromolecular inhibitors outside the clot (Liu et al., 1979). However, fibrin-bound

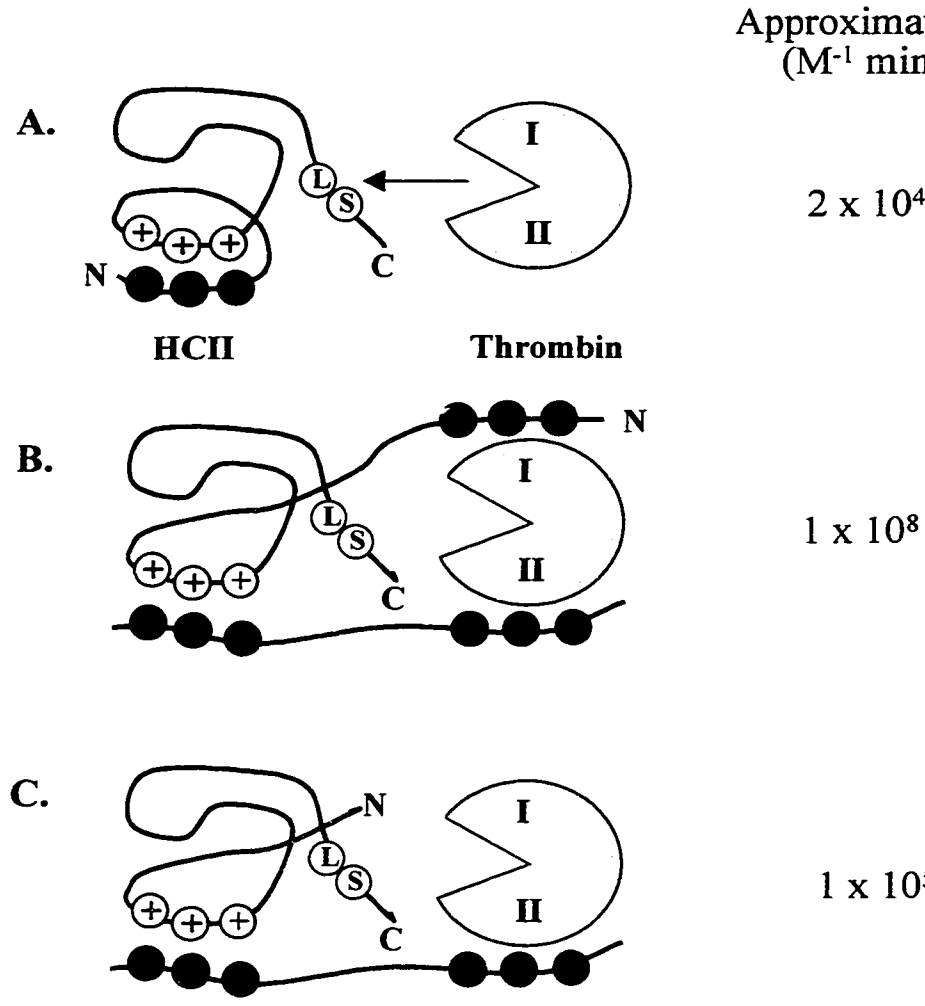


Fig. 4. **Model of thrombin inhibition by HCII** (adapted from Tollefsen, 1994; Liaw et al., 1998). **A**, in the absence of GAGs, the amino-terminal acidic domain of HCII (-) forms ionic bonds with the basic GAG-binding domain (+) and is unable to react with thrombin. **B**, DS displaces the amino-terminus of HCII, thereby permitting its interaction with exosite I of thrombin. In the case of heparin, maximal stimulation requires a combination of allosteric and templating effects. **C**, a mutant of HCII which lacks the amino-terminus is shown. Complex formation can be stimulated by heparin chains comprised of 30 or more saccharide units via the template mechanism. Approximate second-order rate constants are shown.

thrombin remains functionally active as shown by its ability to catalyze the release of FPA from fibrinogen (Francis et al., 1983; Weitz et al., 1990), hydrolyze synthetic substrates (Francis et al., 1983; Kaminski and McDonagh, 1987), and sustain the feedback activation of factors V and VIII as well as activate platelets (Kumar, 1994). Plasmic lysates of fibrin also exhibit thrombin activity, at a level similar to the fibrin from which the lysates were prepared, suggesting that thrombin binding sites on fibrin are not disrupted during fibrinolysis (Francis et al., 1983; Weitz et al., 1998). The presence of enzymatically active thrombin on fibrin thus contributes to the *in vivo* thrombogenicity of clots and is of pathogenetic importance in vascular thrombotic disease.

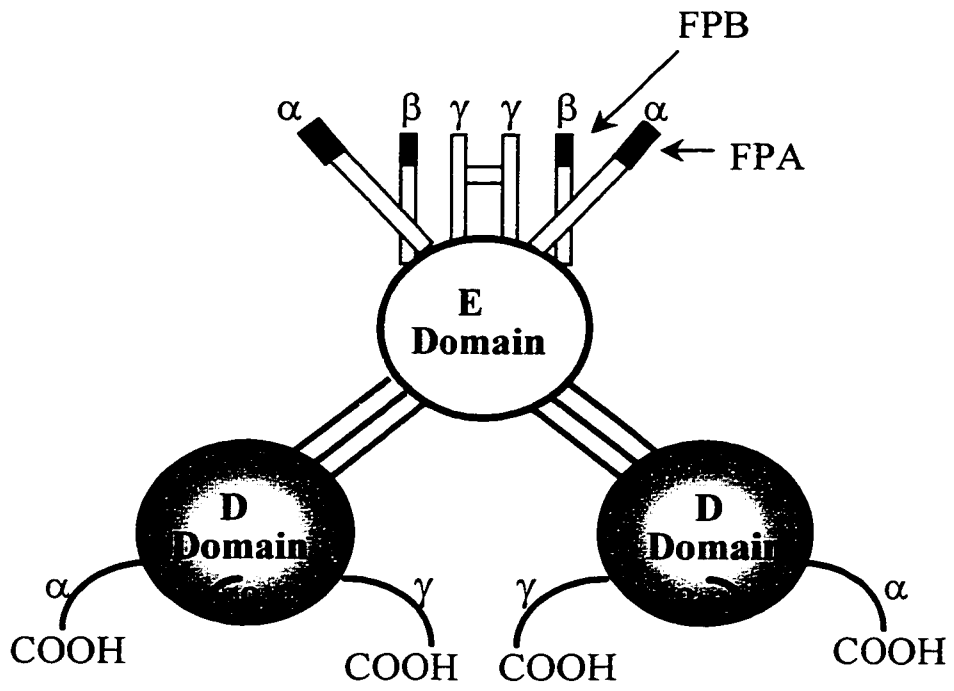
1.5.1 Structure of Fibrinogen

Fibrinogen is a 340 kDa plasma protein composed of three pairs of polypeptide chains, designated Aα (610 residues), Bβ (461 residues), and γ (411 residues) (for review, see Hantgan et al., 1994). The polypeptide chains are linked by 29 disulfide bonds and assemble to form two independently folded symmetrical domains (Fig. 5). The globular central E domain consists of the amino-terminal regions of all six chains. The six amino termini emerge from the E domain to form two lateral bundles, each containing single Aα, Bβ, and γ chains. The carboxy-terminal region of each bundle form the globular E domains. Electron microscopic images reveal that fibrinogen has a trinodular, elongated structure with three globular domains connected by two spacer regions.

1.5.2 Conversion of Fibrinogen to Fibrin

The conversion of soluble fibrinogen into an insoluble fibrin network is initiated by the cleavage of the Arg^{16}-Gly^{17} bond on the Aα chains by thrombin (Blomback et al., 1978).

A.



B.

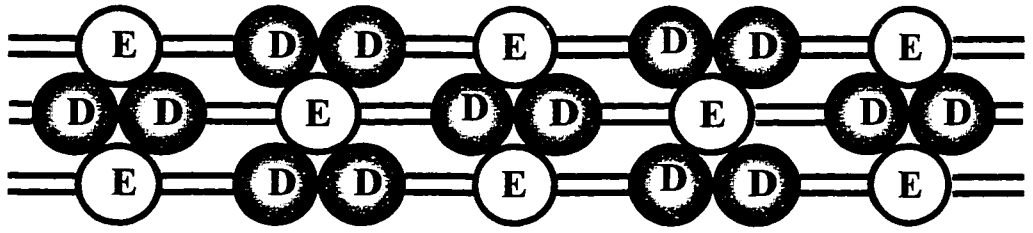


Fig. 5. **Schematic diagram of fibrinogen (panel A) and fibrin (panel B)** (adapted from Hantgan et al., 1994). The Aα, Bβ, and γ chains of fibrinogen are shown along with the globular domains (D and E) and fibrinopeptides A (FPA) and B (FPB). Polymerization of fibrin yields half-staggered, non-covalent complexes.

This cleavage releases highly acidic fibrinopeptide A (FPA) molecules and converts fibrinogen to fibrin I monomer. Cleavage of fibrinogen by thrombin requires the interaction between the central E domain of fibrinogen and exosite I of thrombin, as well as recognition of residues 6 to 16 of the Aα chain by thrombin's catalytic site (Berliner and Sugawara, 1985; Kaminski and McDonagh, 1987; Naski and Shafer, 1990; Stubbs et al., 1992). Subsequent thrombin-mediated cleavage of the Arg^{14}-Gly^{15} bond on the Bβ chains by thrombin results in the release of highly acidic fibrinopeptide B (FPB) molecules, thereby generating fibrin II monomer. FPA release exposes a new binding site on the E domain, termed "A", which interacts with a complementary preexisting site, termed "a", on the γ chain of the D domain of another molecule of fibrin monomer (Olexa et al., 1980). Similarly, release of FPB unmasks a "B" site on the E domain that interacts with a "b" site on the γ chain of the D domain of another fibrin monomer molecule (Shainoff and Dardik, 1979; Weisel, 1986).

During the fibrin assembly process, fibrin I monomer formation is followed by end-to-end oligomerization, whereas fibrin II formation facilitates lateral association of fibrin I strands (Blomback et al., 1978). Polymerization of fibrin II into protofibrils thus yields half-staggered noncovalent complexes (Fig. 5). The protofibrils are further stabilized by intermolecular cross-links introduced by factor XIIIa, a plasma transglutaminase that is generated by proteolytic cleavage of the inactive precursor, factor XIII, by thrombin (for review, see McDonagh, 1994). Factor XIIIa cross-links the side chains of lysine and glutamine of α- and γ-chains in adjacent fibrin strands, thus increasing the mechanical strength of clots.

1.5.3 Fibrin-bound Thrombin

During the polymerization of fibrin, thrombin is incorporated into the growing clot via a site distinct from its catalytic centre (Seegers et al., 1945; Liu et al., 1979; Wilner et al., 1981; Berliner et al., 1985; Kaminski and McDonagh, 1987; Vali and Scheraga, 1988). Current thinking is that thrombin interacts with fibrin via exosite I. This concept is supported by observations that hirugen, an exosite I-directed competitive inhibitor, blocks the binding of fibrin to thrombin (Naski et al., 1990) and γ-thrombin, a proteolytic derivative of thrombin lacking exosite I, does not incorporate into fibrin clots (Wilner et al., 1981). Two classes of thrombin binding sites on fibrin have been identified (Liu et al., 1979; Hogg and Jackson, 1990; Meh et al., 1996). The high affinity site is located on γ' chains in the fibrin D domains (Kd ~200 nM), whereas the low affinity binding site is located in the fibrin E domain (Kd ~3.4 μM) (Meh et al., 1996).

Despite being catalytically active, fibrin-bound thrombin displays impaired reactivity with selected inhibitors. Notably, the rate of heparin-catalyzed inactivation of thrombin by AT is reduced by ~300-fold in the presence of soluble fibrin II monomer (Hogg and Jackson, 1989) and a 20-fold higher concentration of heparin is needed to inactivate fibrin-bound thrombin compared with fluid phase thrombin (Weitz et al., 1990). The limitations of heparin have been attributed, at least in part, to the inability to inactivate fibrin-bound thrombin (Hogg and Jackson, 1989; Weitz et al., 1990). Thus, heparin is limited in clinical situations characterized by extensive thrombin and fibrin formation such as arterial thrombosis (Weitz and Hirsh, 1993), angioplasty (Popma et al., 1995), and reocclusion of coronary arteries after thrombolytic therapy (Gold et al., 1986; Galvani et al., 1994; Merlini

et al., 1995).

The molecular basis for the protective effect of fibrin on the inactivation of thrombin by heparin-AT involves the incorporation of thrombin into a ternary thrombin-fibrin-heparin complex (Fig. 6) (Hogg and Jackson, 1990a, 1990b). Heparin, in the ternary complex, presumably interacts with exosite II of thrombin (Church et al., 1989; Gan et al., 1994; Sheehan and Sadler, 1994) and the β15-42 region of fibrin (Odrljin et al., 1996). It has been proposed that heparin bridges thrombin and fibrin, thereby enhancing the interaction between fibrin and exosite I of thrombin (Hogg and Jackson, 1990a; Hogg et al., 1996). Quantitative binding studies of binary complexes indicate that heparin binds thrombin with the highest affinity (Kd~15 nM), whereas fibrin polymer binds thrombin and heparin with similar but lower affinities (Kd ~301 nM and ~280 nM, respectively) (Hogg and Jackson, 1990a).

The limitations of heparin have prompted the search for more effective antithrombotic agents. Recently, it has been shown that dermatan sulfate, despite having no effect on AT, has antithrombotic properties in experimental animal models (Fernandez et al., 1986; Maggi et al., 1987; Hoppensteadt et al., 1990). In contrast to heparin, dermatan sulfate is equipotent in inhibiting both fluid-phase and fibrin-bound thrombin (Okwusidi et al., 1990; Bendayan et al., 1994). Furthermore, when given in doses with similar inhibitory activity against thrombin, dermatan sulfate has been shown to be more effective than heparin in preventing fibrin accretion onto existing thrombi in experimental animal models (van Rynn-McKenna et al., 1989; Okwusidi et al., 1990; Carrie et al., 1992). Although these studies suggest that dermatan sulfate is better than heparin at inactivating fibrin-bound thrombin, the explanation for this phenomenon is unclear.

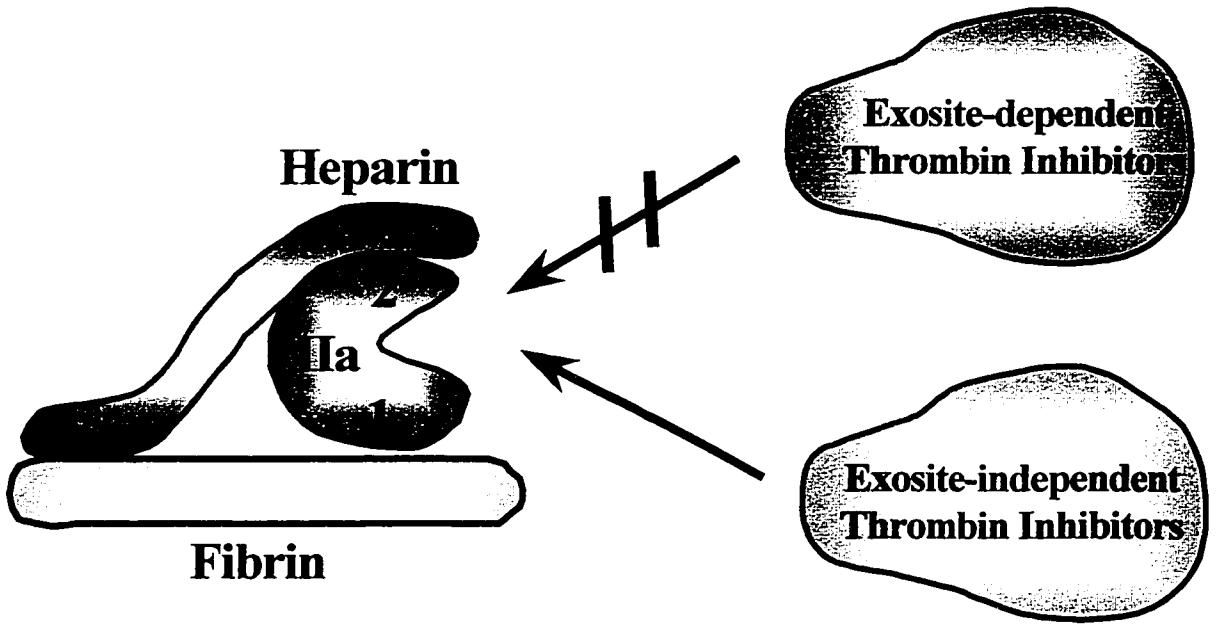


Fig. 6. **Ternary thrombin-heparin-fibrin complex.** Thrombin (IIa) within this complex is bound to fibrin via exosite 1 (1), whereas heparin binds to both fibrin and exosite 2 (2) on thrombin. Impaired access to thrombin's exosites contributes to protecting thrombin from inactivation from exosite-dependent thrombin inhibitors but not exosite-independent thrombin inhibitors. Adapted from Becker et al., 1998.

1.6 Objectives

The overall goal of this thesis work was to examine different aspects of the functional properties of thrombin in the context of hemostasis. The first objective was to identify the thrombin-binding domain on F2 using plasma, recombinant, and synthetic peptides. The thrombin-binding derivatives of F2 were then used in functional studies to examine how ligand binding at exosite II modulates thombin's function.

The second objective was to gain further insight into the mechanism by which GAGs catalyze thrombin inhibition by HCII. Using structure-function studies, I explored the possibility that conformational activation of HCII by GAGs can be mimicked by disrupting intramolecular interactions between the amino-terminal acidic domain of HCII and the GAG-binding domain. I also distinguished between the catalytic mechanisms employed by heparin and by dermatan sulfate in the acceleration of thrombin inhibition by HCII.

The third objective of this study was to determine why fibrin-bound thrombin is susceptible to inactivation by the HCII/dermatan sulfate complex, but not the AT/heparin complex. In these studies, dermatan sulfate was used as a tool to better understand how ternary thrombin-fibrin-GAG complex formation protects thrombin from inactivation by inhibitors.

CHAPTER 2- EXPERIMENTAL PROCEDURES

Preface

This chapter is a compilation of the experimental procedures outlined in chapters 3, 4, and 5. This chapter represents a more thorough description of the methodology.

2.1 Materials

Oligonucleotides were synthesized by the Institute for Molecular Biology and Biotechnology, McMaster University. Fluorescein-5-isothiocyanate (FITC) was from Molecular Probes Inc. (Eugene, OR). D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (FPR) was from Novabiochem Intl. (San Diego, CA). FITC-FPR and biotin-FPR was from Haematologic Technologies Inc. (Essex Junction, VT). N-methylsulfonyl-D-Phe-Gly-Arg p-nitroanilide (Chz-tPA) and N-Methoxycarbonyl-Nle-Gly-Arg-4-nitroanilide acetate (Chz-Xa) were from Boehringer Mannheim Canada (Laval, PQ). Benzyloxycarbonyl-Ile-Glu-(OR)-Gly-Arg p-nitroanilide (S-2222), H-D-Phe-Pip-Arg p-nitroanilide (S2238), pyro-Glu-Gly-Arg p-nitroanilide (S-2444), L-pyroglutamyl-L-Pro-L-Arg-p-nitroaniline hydrochloride (S2366), and H-D-Ile-Pro-Arg p-nitroanilide (S-2288) were from Chromogenix (Helena Laboratories, Mississauga, ON). Prothrombin, HCII, and AT, isolated from human plasma by affinity chromatography, and monospecific polyclonal IgGs against human prothrombin, HCII, and AT were from Affinity Biologicals Inc. (Hamilton, ON). Hexadimethrine bromide (polybrene) was obtained from Aldrich Chemical Company (Milwaukee, WI). Heparin, hirudin$_{54-64}$, anti-sheep IgG alkaline phosphatase, streptavidin-

agarose, benzamidine-agarose, N-p-tosyl-Gly-Pro-Arg-p-nitroanilide (Chz-Th), and Gly-Pro-Arg-Pro-amide (GPRP-NH_2) were from Sigma Chemical Co. (St. Louis, MO). Heparin-Sepharose CL-6B resin, CNBr-activated Sepharose 4B, sulfopropyl C-50, deoxynucleotides (dNTPs), and RNAguard ribonuclease inhibitor were from Pharmacia Biotech (Uppsala, Seden). Human α- and γ-thrombin, factor Xa, factor IXa, factor XIa, fibrinogen, and size-restricted heparin fractions of 18 kDa, 9kDa, 6kDa, and 4 kDa were from Enzyme research Laboratores (South Bend, IN). Dermatan sulfate was obtained from Mediolanum Farmaceutici (Milan, Italy). Desmin, a 5.6 kDa low molecular weight dermatan sulfate fraction obtained by limited depolymerization (Harenburg et al., 1996), was generously provided by Dr. Giancarlo Agnelli (Universita di Perugia, Perugia, Italy). A Baby Hamster Kidney (BHK) cell line was generously provided by Dr. William Sheffield (McMaster University, Hamilton, ON). The recombinant thrombin mutant with Arg^{93}, Arg^{97}, and Arg^{101} substituted with Ala (RA-thrombin) (Ye et al., 1994) was generously provided by Dr. Charles Esmon (Howard Hughes Medical Institute, Oklahoma Medical research Foundation, Oklahoma City, OK). Dulbecco's modified Eagle's medium, Geneticin, and Superscript RNase H reverse transcriptase were from Gibco BRL (Gaithersburg, MD). Fetal bovine serum was obtained from HyClone Laboratories Inc. (Logan, UT). Cellulose ester dialysis membranes were from Spectra/Por (Houston, TX). All other chemicals were of the highest grade commercially available.

2.2 Methods

2.2.1 Synthesis of Synthetic F2 Peptides: Cyclized synthetic peptides were prepared by

Chiron Mimotopes Peptide Systems (San Diego, CA). Peptides were synthesized on polyethylene pins that had been radiation-grafted with hydroxyethylmethacrylic acid as described by Bray et al. (1995). The crude peptides were lyophilized after deprotection, cleaved from the solid support, and purified by reverse phase high pressure liquid chromatography (HPLC). To cyclize the peptides, the peptides were dissolved at a final concentration of 0.2 mg/ml in 50 mM ammonium bicarbonate, pH 8.05, 30% acetonitrile, covered in aluminum foil, mixed on an orbital shaker for 2 days at 23°C, and then lyophilized. The cyclized peptides were purified by preparative reverse phase HPLC. The peptides displayed > 90% purity by HPLC and yielded the expected composition on quantitative amino acid analysis, and the identities were confirmed by mass spectrometry. The cyclized peptides were resuspended in TBS (20 mM Tris-HCl, pH 7.4/150 mM NaCl), and titrated to pH 7.0 with NaOH. sF2(63-116) corresponds to residues 63-116 of human F2 except that Cys^{76} and Cys^{93} were replaced with serine residues. A peptide variant of sF2(63-116) with a scrambled sequence but retaining the original positions of the two Cys residues (Cys^{64} and Cys^{88}) also was prepared. sF2(63-94) and sF2(63-90), truncated derivatives of sF2(63-116), also were synthesized.

2.2.2 Construction of Recombinant F2 Peptides: DNA manipulations were carried out using standard cloning techniques (Sambrook et al., 1989). Plasmid pBS-hFII containing the entire coding region of human prothrombin as a *Hind*III-*Sst*1 fragment in pBluescript (Stratagene Ltd., La Jolla, CA) was generously provided by Dr. Ross MacGillivray (University of British Columbia, Vancouver, BC). Full-length and truncated constructs of rF2 were prepared by PCR amplification (35 PCR cycles for 1 min at 94°C, 1 min at 55° C,

and 1 min at 72 ° C) using plasmid pBS-hFII as the template. The primers used in the PCR reactions contained terminal restriction sites and are shown in Table 1 (Chapter 3). The PCR products were gel-purified (QIAGEN Inc., Chatsworth, CA), cleaved with *Nco*1 and *Xho*1, and cloned as *Nco*1-*Xho*1 fragments into the bacterial expression vector pET22b(+) (Novagen Inc., Madison, WI). The fidelity of each PCR-amplified construct was confirmed by double-stranded DNA sequencing using dideoxy chain termination and Sequenase 2.0 (United States Biochemical, Cleveland, OH).

To ensure proper disulfide bond formation and to prevent the formation of intermolecular complexes, PCR mutagenesis was used to convert Cys^{76} and Cys^{93} to serine residues. Briefly, plasmid pBS-hFII was PCR amplified using primer B (Table 1, Chapter 3), which hybridizes downstream of the multiple cloning site of plasmid pBS-hFII, and the mutagenesis primer F (Table 1, Chapter 3), which contains a point mutation to convert Cys^{76} and Cys^{93} to serine residues (35 PCR cycles for 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C). The PCR product was gel-purified and used as a primer on plasmid pBS-hFII for one PCR cycle (1 min at 94°C, 30 s at 60°C, and 40 s at 72°C). Primers C and D (Table 1) were immediately added to the PCR reaction and 35 more cycles of PCR were carried out (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C). Primer C hybridizes upstream of the multiple cloning site of pBS-hFII while primer D hybridizes downstream of primer B. To convert Cys^{93} to serine, the mutagenesis procedure was repeated with primer G. The authenticity of the mutations, as well as the fidelity of the PCR-amplified constructs, were confirmed by double-stranded DNA sequencing.

2.2.3 Expression and Purification of Recombinant F2 Peptides: Recombinant F2

peptides were expressed in *E. coli* BL21(DE3) by isopropylthio-β-D-galactoside (IPTG) induction using the histidine-tag pET system (Novagen, Inc., Madison, WI). The expression vector used, pET22b(+), directs the recombinant proteins to the periplasmic space thereby promoting proper folding and disulfide bond formation. The periplasmic fraction was obtained using the cold osmotic shock method described by Ausubel et al. (1989). Histidine-binding Ni^{2+} resin (Novagen, Inc., Madison, WI) was used to purify the recombinant proteins. Cells were grown in Luria broth (2 L) containing 100 μg/ml ampicillin at 37°C to an optical density at 600 nm of 0.6. Expression of F2 peptides was induced by adding IPTG to a final concentration of 0.4 mM. The cells were harvested after 2.5 h and resuspended in 240 ml of 20% (w/v) sucrose, 30 mM Tris-HCl, pH 8.0, 1 mM EDTA. The cells were mixed at 23 °C for 10 min, pelleted, and resuspended in 240 ml of ice-cold 5 mM $MgSO_4$. After stirring the cells on ice for 10 min, the cells were pelleted, and the supernatant was applied to 2.5 ml of histidine-binding Ni^{2+} resin. The proteins were purified on the resin as desribed by the supplier (Novagen Inc., Madison, WI). The isolated proteins at a concentration of 1 mg/ml were subjected to electrophoresis in 15% SDS-polyacrylamide gels (Laemmli, 1970) under reducing conditions, transferred to nitrocellulose, and visualized by Ponceau S staining and by immunoblotting as described by Austin et al. (1995).

2.2.4 Purification of F2: F2 was obtained from a terminal prothrombin activation reaction as described by Stevens and Nesheim (1993). Human prothrombin (20 mg) was activated in 20 ml of TBS containing 2 mM $CaCl_2$, 23 μM phosphatidylcholine:phosphatidylserine, 4 nM factor Va, and 5 nM factor Xa. After 2 h the reaction was stopped by the addition of EDTA to 5 mM. The sample was diluted with an equal volume of H_2O and applied to a sulfopropyl C-50 (5 ml) column to adsorb the thrombin. Unbound protein was applied to a

benzamidine-agarose column (2 ml) to remove residual factor Xa and thrombin. The flow-through was made 1 μM in FPR and, after the addition of 0.025 volumes of 20 x concentrated TBS, the sample was applied to a Mono Q (HR 5/5) column on an FPLC system (Pharmacia Biotech Inc.). After washing the sample with 20 ml of TBS, a 30-ml linear gradient from 150 to 500 mM NaCl in 20 mM Tris-HCl, pH 7.4, was run at a flow rate of 1 ml/min. Fractions of 1 ml were collected and the A_{280} was determined. Typically, the elution profile demonstrated base-line separation of two peaks that were identified as prothrombin fragments 1 and 2 by comparison of their mobilities with molecular weight standards on SDS-polyacrylamide gel electrophoresis. F2 was dialyzed against TBS buffer and the concentration was determined spectrophotometrically using $\epsilon_{280}^{0.1\%}$ of 1.1 (Church et al., 1991).

Thrombin-agarose Affinity Chromatography: Thrombin-agarose affinity chromatography was used to compare the affinities of plasma- and recombinant-F2 peptides for thrombin. 58 μM thrombin was incubated for 30 min at 37°C with a 10-fold molar excess of biotin-FPR in TBS. After dialysis against TBS, the biotin-FPR-thrombin was passed over streptavidin agarose in TBS and excess binding sites were blocked with biotin. 0.2 ml of purified F2 peptide at a concentration of 1 mg/ml was applied at 4°C to 0.5 ml of a streptavidin-agarose column containing 125 nmoles of biotin-FPR-thrombin. The column had previously been equilibrated in buffer containing 20 mM Tris-HCl, pH 7.4, 10 mM NaCl. All subsequent steps were performed at 4°C. Elution of the protein was performed with 1 ml of 20 mM Tris-HCl, pH 7.4 containing increasing concentrations of NaCl (from 10 mM to 500 mM). Aliquots from the flowthrough and eluates were analyzed using SDS-PAGE and immunoblotting as described above. The protein elution profiles were obtained by laser

densitometry scans of immunoblots using the UltroScanTM XL laser densitometer (Pharmacia LKB Biotechnology). The density of F2 in each fraction was expressed as a percentage of the total F2 density in the complete elution profile.

2.2.6 Reduction and Alkylation of F2 and rF2(1-116): The disulfide bonds in F2 and rF2(1-116) were reduced and alkylated as described by Bulleid and Freidman (1988). The peptides were resuspended in 50 mM DTT at a concentration of 1 mg/ml and placed at room temperature for 30 min. Iodoacetamide (made up fresh in sterile distilled water) was then added to 50 mM and the reaction mixture was placed at room temperature for 45 min in the dark. The reaction mixture was dialyzed against 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.01% Tween-20 using Spectra/Por cellulose ester dialysis membrane (15 kD cutoff) and the protein concentration determined as described above for intact F2.

2.2.7 Determination of the Affinity of F2 Peptides for Thrombin: The association between F2 peptides and thrombin was monitored by the F2-dependent fluorescence intensity change of anilinonaphthalene-6-sulfonic acid-thrombin (ANS-FPR-thrombin). ANS-FPR-thrombin was prepared using ATA-FPR (N-((Acetylthio)acetyl)-D-Phe-Pro-Arg-CH_2Cl) as described by the supplier (Molecular Innovations Inc., Royal Oak, MI). Briefly, 10.6 μM of thrombin was incubated for 30 min at room temperature with 2.5-fold molar excess ATA-FPR in HBSE buffer (20 mM HEPES, pH 7.0, 100 mM NaCl, 1 mM EDTA). The reaction mixture was dialyzed in HBSE buffer, incubated with 10-fold molar excess IAANS (2-(4-(Iodoacetamide)anilino)naphthalene-6-sulfonic acid) (Molecular Probes Inc., Eugene, OR) in the presence of hydroxylamine for 60 min at room temperature in the dark, and then redialyzed. 500 μl of 100 nM ANS-FPR-thrombin was added to the same buffer in a semi-micro quartz cuvette. Using a Perkin Elmer LS50B luminescence spectrometer with

excitation and emission wavelengths set to 328 nm and 450 nm, respectively, and excitation and emission slit widths set to 12 nm, and an emission filter of 390 nm, readings were taken of ANS-FPR-thrombin alone (Io). Known quantities of F2 peptides (3.8 mM) were then added to the cuvette and, after mixing, the change in fluorescence was monitored (I). As a control, a scrambled variant of the 54 amino acid peptide was titrated to the same concentration, and any change in fluorescence due to non-specific binding was used to correct the binding curve. The K_d values were calculated by plotting I/Io versus F2 peptide concentration. The parameters K_d and α were calculated by nonlinear regression ("Tablecurve", Jandel Scientific, San Rafael, CA) using the equation $I/Io=(1+(K_d+[F2])/[ANS\text{-}IIa]-((1+((K_d+[F2])/([ANS\text{-}IIa])))^2-(4\times[F2]/([ANS\text{-}IIa])))^{0.5})\times(\alpha/2)+1$ where α is the maximum fluorescence change and assuming a stoichiometry of 1 (Fredenburgh et al., 1997).

2.2.8 Displacement of Fluorescein-hirudin$_{54\text{-}65}$ from FPR-thrombin by F2 and by sF2(63-116): Sulfated hirudin$_{54\text{-}65}$ was labelled with FITC as described by Liu et al. (1991). 2 mL of TBS containing 10 nM of FITC-hirudin$_{54\text{-}65}$ was added to a 1 cm x 1 cm quartz cuvette in a Perkin Elmer LS50B luminescence spectrometer. Excitation and emission wavelengths were set at 492 nm and 522 nm, respectively, and excitation and emission slit widths were both set to 8 nm. The fluorescence intensity of 15 nM FPR-thrombin was measured before (Io) and after (I) the addition of either 20 to 40 µl aliquots of 161 µM F2 or 2 to 5 µl aliquots of 3.8 nM sF2(63-116). Both F2 and sF2(63-116) solutions contained 10 nM FITC-hirudin$_{54\text{-}65}$ so that the concentration of FITC-hirudin$_{54\text{-}65}$ remained constant. Titration was continued up to 43 µM F2 or 300 µM sF2(63-116). After the experiment, intensity values were read from time drive profiles and I/Io values were calculated and plotted versus the peptide

concentration. As a control, the fluorescence intensity was measured before and after the addition of up to 100 μM reduced and alkylated sF2(63-116).

2.2.9 Effect of F2 on Thrombin-mediated Clotting of Fibrinogen: Thrombin, at a final concentration of 0.6 nM, was mixed with either F2 (0 to 50 μM) or sF2(63-116) (0 to 300 μM) in TSTW buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.01% Tween-20) in a final volume of 75 μl. 25 μl of human fibrinogen and $CaCl_2$ were then added to final concentrations of 4 μM and 2 mM, respectively, and the times for half maximal increase in turbidity ($T_{1/2}$) were determined at 23°C by continuous measurement of absorbance at 405 nm using a Molecular Devices plate reader.

2.2.10 Effect of F2 on Thrombin-mediated Hydrolysis of Chromogenic Substrates: The chromogenic activity of 20 nM thrombin in TBS was determined with 0.7 to 14 mM of various thrombin substrates in the presence of 25 μM F2 or 150 μM sF2(63-116). The rates of thrombin-mediated hydrolysis were monitored at 405 nm in a plate reader and the percentage changes from control rates were determined.

2.2.11 Effects of F2 on the Rate of Thrombin Inhibition by AT: The second-order rate constant (k_2) for the inhibition of thrombin by AT was determined under pseudo first-order conditions (Olson et al., 1993) in the absence or presence of 1 U/ml heparin. In a multiwell plate, aliquots (10 μl) of thrombin (final concentration of 10 nM) were incubated for varying intervals with 10 μl of 500 nM AT in TBSP (TBS containing 0.6% polyethylene glycol-8000) in the absence or presence of F2 or sF2(63-116) in concentrations ranging from 10 to 500 μM. All reactions were terminated by the addition of 200 μM chromogenic substrate (N-p-tosyl-Gly-Pro-Arg-p-nitroanilide) in 200 μl TBSP containing 10 mg/ml polybrene. Thrombin inhibitory activity in the presence of 1 U/ml heparin was determined using

thrombin and AT concentrations of 5 nM and 50 nM, respectively. Residual thrombin activity was then calculated by measuring absorbance for 5 min at 405 nm using a Molecular Devices plate reader. The pseudo-first-order rate constants (k_1) for thrombin inhibition were determined by fitting the data to the equation $k_1 \bullet t = \ln([P]_o/[P]_t)$, where $[P]_o$ is initial thrombin activity and $[P]_t$ is thrombin activity at time t. The second-order rate constant, k_2, was determined by dividing k_1 by the AT concentration.

2.2.12 Construction of Recombinant HCII Variants: Human HCII cDNA was cloned from HepG2 cells by reverse transcription- polymerase chain reaction. Briefly, total RNA was isolated from HepG2 liver cells using the RNeasy total RNA kit (Qiagen Inc., Chatsworth, CA) and reverse transcribed using primer A (5' AAG GCA CTT CAG ACA CCT AGA CCT CCA 3') which hybridizes to the 3' untranslated region of HCII cDNA (24). Reverse transcription was done by first heating 1 μg of total RNA and 50 ng of primer A for 10 min at 70°C and then placing the mixture on ice. The volume was brought to 20 μl by adding 4 μl of 5X reverse transcriptase buffer (Gibco BRL), 2 μl 0.1 M dithiothreitol, 0.5 mM of each dNTP, 37.5 U RNAguard ribonuclease inhibitor, and 200 U Superscript RNase H reverse transcriptase. cDNA synthesis was performed at 42°C for 60 min. The reaction mixture was then heated to 75°C for 10 min and chilled on ice. HCII cDNA was PCR amplified using primer A and primer B (5' AGC TCC GCC AAA ATG AAA CAC TCA TTA AAC GCA 3') which hybridizes to the 5' untranslated region of HCII cDNA (24). The PCR product was purified on a 1% agarose gel, digested with *Eco*RV, and initially subcloned into the *Eco*RV site of pBluescript (KS) (Stratagene Ltd., La Jolla, CA). HCII cDNA was then cloned in the forward orientation into the *Eco*RI site of the phagemid vector pALTER-1 (Promega, Madison, WI). *In vitro* mutagenesis to generate and select oligonucleotide-

directed mutants was performed using single-stranded phagemid DNA as described by the supplier. The sequence of the HCII cDNA and the authenticity of the mutations were confirmed by double-stranded sequencing using dideoxy chain termination (25) and Sequenase 2.0 (United States Biochemical, Cleveland, OH).

2.2.13 Stable Expression of Wild Type and Variant Forms of HCII in BHK Cells: cDNAs encoding the wildtype and variant forms of HCII were cloned into the *Eco*RI site of the eukaryotic expression vector pcDNA3.1(+) (Invitrogen, San Diego, CA). In the resulting plasmid, the expression of HCII cDNA is under the control of the human cytomegalovirus immediate-early promoter. Transfection of BHK cells was performed in Dulbecco's Modified Eagle Medium using Qiagen-purified pcDNA3.1 constructs employing the SuperFect transfection reagent for 3 h as described by the supplier (Qiagen). The medium was then changed to DMEM containing 10% fetal bovine serum and 1 mg/ml Geneticin. After 2 weeks of selection, in which the medium was changed every 3 days, drug-resistant colonies were isolated and levels of recombinant protein expression were determined by immunoblotting with sheep anti-HCII antibody. Clones secreting the highest level of recombinant protein were seeded into roller bottles and cultured in serum-free DMEM.

2.2.14 Recombinant Protein Purification: Sheep anti-HCII antibody was coupled to CNBr-activated Sepharose 4B matrix as described by Cuatrecasas (1970). All subsequent steps were done at room temperature. The conditioned medium of the transfected BHK cells was applied to anti-HCII resin pre-equilibrated in (TBS). The column was washed with 5 column volumes of 20 mM Tris-HCl, 0.8 M NaCl, pH 7.4 followed by 5 column volumes of 20 mM Tris-HCl, 0.05 M NaCl, pH 7.4. Bound protein was eluted with Gentle Ag/Ab Elution Buffer (Pierce, Rockford, IL), dialyzed at 4°C overnight against two changes of 500 ml TBS,

and then concentrated using a Centriprep-30 ultrafiltration apparatus (Amicon, Inc., Beverly MA). Protein concentration was measured using $\epsilon_{280}^{0.1\%}$ =9.1 (Enzyme Research Labs) and protein purity was determined by SDS-PAGE analysis (Laemmli, 1970).

2.2.15 Rates of Thrombin Inhibition by Wildtype and Variant HCIIs: The second-order rate constants (k_2) for inhibition of thrombin by the various HCII variants were determined under pseudo first-order conditions (Olson et al. 1993) in the absence or presence of 3.3 μM GAGs. In a multiwell plate, 10 μl aliquots of thrombin (final concentration 2 nM) were incubated for varying intervals with 40 nM HCII suspended in 10μl TBS containing 0.6 % polyethylene glycol-8000 (TBSP). All reactions were terminated by the addition of 200 μM chromogenic substrate (N-p-tosyl-Gly-Pro-Arg-p-nitroanilide) in 200 μl TBSP containing 10 mg/ml polybrene. Residual thrombin activity was calculated by measuring absorbance at 405 nm for 5 min using a Molecular Devices plate reader. The pseudo-first-order rate constants (k_1) for thrombin inhibition were determined by fitting the data to the equation $k_1 \bullet \ln([P_o]/[P]_t)$, where $[P]_o$ is initial thrombin activity and $[P]_t$ is thrombin activity at time t (Olson et al., 1993). The second-order rate constant, k_2, was then calculated by dividing k_1 by the HCII concentration.

2.2.16 Heparin-Sepharose Affinity Chromatography: Heparin-Sepharose affinity chromatography was used to compare the affinities of HCII variants for heparin. 0.2 ml of purified HCII, at a concentration of 10 μg/ml, was batch adsorbed with 0.2 ml of heparin-Sepharose resin for 1 h at 4°C. Adsorbed proteins were eluted with 1 ml of HP buffer (20 mM HEPES, pH 7.4, 0.1 % polyethylene glycol-8000) containing NaCl in concentrations ranging from 30 mM to 1M. Aliquots from the flowthrough and eluates were analyzed by SDS-PAGE followed by immunoblotting with sheep anti-HCII antibody. Protein elution

profiles were obtained by laser densitometry scans of immunoblots using the UltroScanTM XL laser densitometer (Pharmacia LKB Biotechnology). The density of HCII in each fraction was expressed as a percentage of the total HCII density in the complete elution profile.

2.2.17 Preparation of Soluble Fibrin Monomer (Fm): Human fibrinogen was subjected to gelatin-agarose adsorption to remove fibronectin. 15 ml of fibrinogen (130 μM) was mixed in a tube for 30 min with 5 ml gelatin-agarose. The suspension was centrifuged for 10 min, and then the supernatant was removed and exposed to the same treatment one more time. The fibrinogen concentration was determined using a molecular weight of 340 kDa and by measuring the absorbanve using $\epsilon_{280}^{0.1\%}$ of 15.1 (Dellenback, 1970) after correction for light scatter at 320 nm using the relationship $Acorr_{280}=A_{280}-1.7 \times A_{320}$ (Bloom et al., 1979). Fm was prepared by clotting fibrinogen (60 to 100 μM) at 37°C for 4 to 6 h with 2 nM thrombin (Hogg and Jackson, 1989). The resultant fibrin was sedimented by centrifugation at 2000 x g for 5 min and transferred to dialysis tubing (12 000 to 14 000 molecular weight cut-off). After dialysis against distilled water (>200 vol) at 4 °C overnight to remove fibrinopeptides A and B, the material was dialyzed against 20 mM acetic acid for approximately 8 h until dissolved. A molecular weight of 340 kDa and $\epsilon_{280}^{0.1\%}$ value of 14.0 was used to calculate the Fm concentration (Lewis et al., 1985). Aliquots were stored at -70°C. In all experiments, Fm was prevented from polymerizing by 5 mM GPRP-NH_2 (Kawasaki et al., 1992) and neutralized with 40% vol/vol 1 M Tris-HCl, pH 7.5.

2.2.18 Preparation of Fibrin Monomer-Sepharose: Fibrinogen was dialyzed into a coupling buffer (0.1 M NaHCO3, 0.5M NaCl, pH 8.3) and the fibrinogen concentration was determined. CNBr-activated Sepharose 4B (10 mg/ml gel vol) was swollen in 1 mM HCl

for 15 min and washed with 200 ml of 1mM HCl through a sintered glass funnel, and then with 5 ml coupling buffer. Approximately 35 mg fibrinogen (~1.35 ml) was immediately added to the Sepharose resin and the suspension was mixed on an end-over-end mixer in a 15 ml conical tube at 23 °C for 2 h. In order to block unreacted remaining groups, the resin was packed in a small column (0.7 x 9 cm) and washed over 2 h with 15 ml coupling buffer, and then with 10 ml TBS. Finally, the washed resin was removed from the column, diluted 1:2 with TBS, and the immobilized fibrinogen was converted to Fm by addition of thrombin at a final concentration of 2 nM. The reaction was allowed to proceed for 3 h with gentle end-over-end mixing in a 15 ml conical tube at 23 °C (Heene and Matthias, 1973). The Fm-Sepharose was transferred to a column (0.7 x 9 cm) and washed with 20 nM Tris-HCI, 1 M CaCl2 (10 vol) followed by TBS (10 vol). A final protein concentration was determined using a BCA Protein Assay (Pierce, Rockford, IL) according to the manufacturer's instructions.

2.2.19 Rates of Thrombin Inhibition by HCII and MUT D in the Absence or Presence of Fm, or GAGs, or Both: The second-order rate constants (k_2) for inhibition of thrombin by HCII variants were determined under pseudo first-order conditions in the absence or presence of 3.3 μM heparin or DS, or 4 μM Fm, or both. Thrombin (10 nM) was incubated for 5 minutes at room temperature in TSP (20 mM Tris-HCl, 150 mM NaCl, 0.6% PEG-8000, pH 7.4) containing various concentrations of heparin or DS (0 to 100 μM) and Fm (0 to 12 μM), 10 mM GRRP-NH_2, and 15 mM Tris-HCl, pH 7.5. Reaction mixtures (10 μl) were aliquotted to 96-well round bottom microtitre plates and an equal volume of either HCII or MUT D (in a concentration at least 10-fold higher than that of thrombin) was added to each well at various time intervals (2 seconds to 5 minutes). All reactions were terminated

by the addition of 200 μM chromogenic substrate (N-p-tosyl-Gly-Pro-Arg-p-nitroanilide) in 200 μl TBSP containing 10 mg/ml polybrene. Residual thrombin activity was calculated by measuring absorbance at 405 nm for 5 min using a Molecular Devices plate reader. The pseudo-first-order rate constants (k_1) for thrombin inhibition were determined by fitting the data to the equation $k_1 \bullet \ln([P_o]/[P]_t)$, where $[P]_o$ is initial thrombin activity and $[P]_t$ is thrombin activity at time t. The second-order rate constant, k_2, was then determined by dividing k_1 by the HCII concentration (Olson et al., 1993).

2.2.20 Effect of heparin and DS on the Binding of ^{125}I-FPR-thrombin to Fibrin Clots: Active site-blocked thrombin (FPR-thrombin) was prepared as described by Fredenburgh et al. (1997). Iodination of FPR-thrombin was performed using Iodo-beads iodination reagent as described by the supplier (Pierce Co., Rockford, IL). The binding of ^{125}I-FPR-thrombin to fibrin clots in the absence or presence of either heparin or DS was studied in 20 mM Tris-HCl, pH. 7.4, 150 mM NaCl, containing 0.6% PEG-8000 and 0.01% Tween-20 at room temperature. Fibrinogen (7.5 μM) was incubated with increasing concentrations of either heparin or DS (0 to 2.5 μM) in a total volume of 40 μl in a series of 400 μl nipple tubes. Clotting was initiated by addition of 10 μl of stock A containing 10 mM $CaCl_2$, 500 nM ^{125}I-FPR-thrombin, and 10 nM thrombin. After 45 min incubation at room temperature, fibrin was pelleted by centrifugation for 5 min at 15, 000 x g, and aliquots of supernatant were removed for gamma counting. The fraction of thrombin bound to fibrin was calculated as the change in ^{125}I-FPR-thrombin binding compared with controls lacking GAGs.

2.2.21 Effect of Ternary Thrombin-fibrin-GAG Complexes on Thrombin-mediated Hydrolysis of Chromogenic Substrates: The chromogenic activity of 10 nM thrombin in TBS was determined with 500 μM of various thrombin substrates in the presence of 4 μM

Fm and either 250 nM heparin, or 250 nM DS. The rates of thrombin-mediated hydrolysis were monitored at 405 nm in a plate reader and the percentage changes from control rates were determined.

2.2.22 Determination of the Affinities of FITC-heparin, FITC-DS, and FITC-FPR-thrombin for Fibrin Clots: Heparin and DS were labelled with FITC as described by Nagasawa and Uchiyama (1978). FITC-labelled active site-blocked thrombin (FITC-FPR-thrombin) was prepared as described by Fredenburgh et al. (1997). The affinity of FITC-labelled ligands for fibrin was determined by measuring unbound ligand in supernatants of clots prepared by clotting varying concentrations of fibrinogen clotted with 1 nM thrombin. Briefly, 200 µl of 2 mM $CaCl_2$, 100 nM FITC-labelled ligand, various concentrations of fibrinogen (30 to 3000 nM), and 1 nM thrombin were mixed in a series of eppendorf tubes. After 1 hour incubation at 23 °C, fibrin was pelleted by centrifugation for 5 minutes at 10,000 x g. 100 µl of supernatant was removed and added to 300 µl of TS buffer. The fluorescence intensity of the samples was measured using a Perkin-Elmer LS50B luminescence spectrometer with excitation and emission wavelengths set to 492 and 522 nm, respectively, and excitation and emission slit widths both set to 15 nm. The K_d values were calculated by plotting I/Io versus fibrinogen concentration, where I and Io represent the fluorescence intensities before and after adding a fixed quantity of fibrinogen, respectively. The parameters K_d and α were calculated by nonlinear regression ("Tablecurve", Jandel Scientific, San Rafael, CA) using the equation $I/Io=(1+(K_d+[Y])/[X])-((1+((K_d+[Y])/[X]))^2-(4\times[Y]/([X]))^{0.5})\times(\alpha/2)+1$ where α is the maximum fluorescence change, Y is FITC-heparin, FITC-DS, or FITC-FPR-thrombin, X is fibrinogen, and assuming a stoichiometry of 1 (Fredenburgh et al., 1997).

2.2.23 Determination of the Affinities of Thrombin for Heparin and DS: The affinities of heparin or DS for thrombin were determined by monitoring GAG-dependent intrinsic protein fluorescence change of thrombin. 2 ml of 100 nM thrombin in TBS (20 mM Tris-HCl, pF 7.4, 150 mM NaCl) was added to a semi-micro quartz cuvette. Using a Perkin-Elmer LS50B luminescence spectrometer with excitation and emission wavelengths set to 280 and 340 nm, respectively, and excitation and emission slit widths set to 6 nm, readings were taken of thrombin alone (Io). Known quantities of either heparin or DS were then added to the cuvette and, after mixing, the change in fluorescence was monitored (I). The Kd values were calculated by plotting I/Io versus GAG concentration and the data were fit by nonlinear regression to the equation given above.

CHAPTER 3- LOCALIZATION OF THE THROMBIN-BINDING DOMAIN ON PROTHROMBIN FRAGMENT 2

Preface

This manuscript has been published in the Journal of Biological Chemistry (Vol. 273, No. 15, Issue of April 10, pp 8932-8939, 1998). The authors are: Patricia C. Y. Liaw, James C. Fredenburgh, Alan R. Stafford, Alexander Tulinsky, Richard A. Austin, and Jeffrey I. Weitz. The corresponding author is Dr. Weitz. Written permission to include copyright material has been obtained from the Journal of Biological Chemistry (p. 48).

Most of the experiments were performed by me. Alan Stafford assisted in the determination of dissociation constants for the interaction of F2 peptides with ANS-FPR-thrombin (Table II) and in the determination of the influence of F2 peptides on the chromogenic activity of thrombin (Fig. 6).

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[1]The abbreviations used are: F1, prothrombin fragment 1; F2, prothrombin fragment 2; sF2, synthetic F2 peptides; rF2, recombinant F2 peptides; AT, antithrombin; ANS, anilinonaphthalene-6-sulfonic acid; FPR, D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone; FITC, fluorescein-5-isothiocyanate. [2]Sequential numbering of amino acids in F2

begins at the first amino acid of F2 (corresponds to amino acid 218 of prothrombin). The chymotrypsinogen numbering system is used for thrombin (Bode et al., 1992).

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3.1 Summary

Cocrystallographic studies have shown that the interaction of human prothrombin fragment 2 (F2) with thrombin involves the formation of salt bridges between the kringle inner loop of F2 and anion-binding exosite II of thrombin. When F2 binds to thrombin, it has been shown to evoke conformational changes at the active site and at exosite I of the enzyme. Using plasma, recombinant, and synthetic F2 peptides (F2, rF2, and sF2, respectively) we have further localized the thrombin binding domain on F2. F2, rF2(1-116), rF2(55-116), and sF2(63-116), all of which contain the kringle inner loop (residues 64-93) and the acidic C-terminal connecting peptide (residues 94-116), bind to thrombin-agarose. In contrast, analogues of the kringle inner loop, sF2(63-90), or the C-terminal connecting peptide, sF2(92-116), do not bind. Thus, contrary to predictions from the crystal structure, the C-terminal connecting peptide as well as the kringle inner loop are involved in the interaction of F2 with thrombin. F2 and sF2(63-116) bind saturably to fluorescently labelled-active-site-blocked-thrombin with K_d values of 4.1 and 51.3 μM, respectively. The affinity of sF2(63-116) for thrombin increases about 5-fold (K_d=10 μM) when Val at position 78 is substituted with Glu. F2 and sF2(63-116) bind to exosite II on thrombin because both reduce the heparin-catalyzed rate of thrombin inhibition by antithrombin - 4-fold. In contrast, only F2 slows the uncatalyzed rate of thrombin inactivation by antithrombin. Like F2, sF2(63-116) induces allosteric changes in the active site and exosite I of thrombin because it alters the rates of thrombin-mediated hydrolysis of chromogenic substrates and displaces fluorescently-labelled $hirudin_{54-65}$ from active-site-blocked thrombin, respectively. Both peptides also prolong the thrombin clotting time of fibrinogen in a concentration-dependent

fashion reflecting their effects on the active site and/or exosite I. These studies provide further insight into the regions of F2 that evoke functional changes in thrombin.

3.2 Introduction

Prothrombin, a 581 amino acid plasma glycoprotein, is converted to the serine protease thrombin in the final stages of the blood coagulation cascade. The proteolytic conversion of prothrombin to thrombin is catalyzed by prothrombinase, an enzyme complex composed of the serine protease factor Xa, the cofactor Va, phospholipids, and calcium (Hemker et al., 1967; Mann et al., 1988). During this reaction, prothrombin is cleaved into three fragments: fragment 1 (F1)[1] (consisting of a γ-carboxyglutamic acid (Gla) domain and the kringle 1 domain), fragment 2 (F2) (consisting of the kringle 2 domain), and the catalytic domain (Mann, 1976). The Gla domain of F1 facilitates calcium-dependent binding of the proenzyme to phospholipid surfaces (Soriano-Garcia et al., 1992). Although the F2 domain has been shown to interact with factor Va (Bajaj et al., 1975), recent studies indicate that F2-factor Va interactions are not required for factor Va to enhance the catalytic efficiency of factor Xa within the prothrombinase complex (Krishnaswamy and Walker, 1997). Rather, the function of F2 in prothrombin may be to alter the conformation of the proenzyme so that its scissile bond(s) is more complementary to the active site of factor Xa (Krishnaswamy and Walker, 1997).

After release from prothrombin, F2 retains its ability to bind thrombin and influences thrombin function. Thus, F2 has been reported to slow the rate of thrombin inactivation by antithrombin (AT) (Walker and Esmon, 1979), alter the environment of the catalytic site (Bock, 1992), enhance the esterolytic activity of thrombin (Myrmel et al., 1976), inhibit the clotting activity of thrombin (Jakubowski et al., 1986) and modulate the calcium dependence of protein C activation (Liu et al., 1994). Crystallographic and chemical modification studies

have shown that F2 interacts with a highly electropositive region in the COOH-terminus of thrombin, also known as anion-binding exosite II or the heparin-binding region of thrombin (Arni et al., 1993; Arni et al., 1994; Church et al., 1989). The crystallographic structure of F2 complexed with active site-blocked thrombin reveals numerous ionic interactions between the anionic inner loop of the F2 kringle and the COOH-terminal helix of thrombin, a region abundant in arginine and lysine residues (Arni et al., 1993; Arni et al., 1994; Church et al., 1989; Bode et al., 1992; Sheehan and Sadler, 1994).

In this study, we set out to further characterize the thrombin-binding domain within F2. Using plasma, recombinant, and synthetic F2 peptides (F2, rF2, and sF2, respectively), we report that, contrary to what would have been predicted from the crystallographic data, residues in the COOH-terminal connecting peptide as well as the inner loop of the F2 kringle are necessary for F2 interaction with thrombin. In addition, to identify the regions in F2 that evoke functional changes in thrombin, we also compared sF2(63-116)^2, a thrombin-binding analogue encompassing the kringle inner loop and the C-terminal connecting peptide, with F2 in terms of their ability to modulate thrombin function.

3.3 Experimental procedures

Materials: Oligonucleotides were synthesized by the Institute for Molecular Biology and Biotechnology, McMaster University. D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (FPR) was from Novabiochem Intl. (San Diego, CA). Biotin-FPR was from Haematologic Technologies Inc. (Essex Junction, VT). Monospecific polyclonal IgGs against human prothrombin and AT, isolated from human plasma by affinity chromatography, were from Affinity Biologicals Inc. (Hamilton, ON). Heparin, hirudin$_{54\text{-}64}$, anti-sheep IgG alkaline phosphatase, streptavidin-agarose, and N-p-tosyl-Gly-Pro-Arg-p-nitroanilide were from Sigma Chemical Co. (St. Louis, MO). Fluorescein-5-isothiocyanate (FITC) was from Molecular Probes Inc. (Eugene, OR). N-methylsulfonyl-D-Phe-Gly-Arg p-nitroanilide (Chz-tPA) was from Boehringer Mannheim Canada (Laval, PQ). Benzyloxycarbonyl-Ile-Glu-(OR)-Gly-Arg p-nitroanilide (S-2222), H-D-Phe-Pip-Arg p-nitroanilide (S2238), pyro-Glu-Gly-Arg p-nitroanilide (S-2444), L-pyroglutamyl-L-Pro-L-Arg-p-nitroaniline hydrochloride (S2366), and H-D-Ile-Pro-Arg p-nitroanilide (S-2288) were from Chromogenix (Helena Laboratories, Mississauga, ON). Cellulose ester dialysis membranes were from Spectra/Por (Houston, TX). All other chemicals were of the highest grade commercially available.

Synthetic peptides: Cyclized synthetic peptides, purified by preparative reverse phase high performance liquid chromatography, were prepared by Chiron Mimotopes Peptide Systems (San Diego, CA) as described by Fredenburgh et al. (1997). The cyclized peptides were resuspended in TBS (20 mM Tris-HCl, pH 7.4/150 mM NaCl), and titrated to pH 7.0 with NaOH. The peptides yielded the expected composition on quantitative amino acid analysis,

and their identities were confirmed by mass spectrometry. sF2(63-116) corresponds to residues 63-116 of human F2 except that Cys^{76} and Cys^{93} were replaced with serine residues. A peptide variant of sF2(63-116) with a scrambled sequence but retaining the original positions of the two Cys residues (Cys^{64} and Cys^{88}) also was prepared. sF2(63-94) and sF2(63-90), truncated derivatives of sF2(63-116), also were synthesized.

DNA construction and mutagenesis: Plasmid pBS-hFII containing the entire coding region of human prothrombin as a *Hind*III-*Sst*1 fragment in pBluescript (Stratagene Ltd., La Jolla, CA) was generously provided by Dr. Ross MacGillivray (University of British Columbia, Vancouver, BC). Full-length and truncated constructs of rF2 were prepared by PCR amplification (35 PCR cycles for 1 min at 94°C, 1 min at 55° C, and 1 min at 72 ° C) using plasmid pBS-hFII as the template. The primers used in the PCR reactions contained terminal restriction sites and are shown in Table 1. The PCR products were gel-purified (QIAGEN Inc., Chatsworth, CA), cleaved with *Nco*1 and *Xho*1, and cloned as *Nco*1-*Xho*1 fragments into the bacterial expression vector pET22b(+) (Novagen Inc., Madison, WI). The fidelity of each PCR-amplified construct was confirmed by double-stranded DNA sequencing using dideoxy chain termination and Sequenase 2.0 (United States Biochemical, Cleveland, OH).

To ensure proper disulfide bond formation and to prevent the formation of intermolecular complexes, PCR mutagenesis was used to convert Cys^{76} and Cys^{93} to serine residues. Briefly, plasmid pBS-hFII was PCR amplified using primer B (Table 1), which hybridizes downstream of the multiple cloning site of plasmid pBS-hFII, and the mutagenesis primer F (Table 1), which contains a point mutation to convert Cys^{76} and Cys^{93} to serine residues (35 PCR cycles for 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C). The PCR

TABLE I
Primers used to PCR amplify recombinant F2 peptides

Primer	Sequence	Amino acid hybridization site in F2
A	5' GGA T<u>CC ATG G</u>CC GAA GGC TCC AGT GTG AAT 3' (NcoI)	1
B	5' GTG <u>CTC GAG</u> ACG CCC TTC GAT GGC CCT G 3' (XhoI)	116
C	5' GGA T<u>CC ATG G</u>CC TCA GCT GTG CAG CTG GTG GAG 3' (NcoI)	55
D	5' GTG <u>CTC GAG</u> ACA ATA GTT GAG GTC GCA GTA C 3' (XhoI)	93
E	5' GTG <u>CTC GAG</u> TGA GTT GAA GTC CTG GTG CTT GCT 3' (XhoI)	55
F	5' GGC GTG **TGG** AGC TAT GTG GCC GGG 3' (Cys76-->Ser)	76
G	5' CTC AAC **TAT** AGT GAG GAG GCC GTG (Cys93-->Ser)	93
H	5' GGA GTA CTA GTA ACC CTG GCC CCA GTC ACG ACG TTG TAA A 3'	
I	5' CAG GAA ACA GCT ATG ACC AT 3'	
J	5' GGA GTA CTA GTA ACC CTG GC 3'	

The sequences of the primers used to PCR amplify recombinant F2 peptides are shown along with the corresponding amino acid residues of the coding regions of F2 to which the primers hybridize. The restriction enzyme sites are underlined while the mutated nucleotides are indicated in bold. Primers H, I, and J were used in the PCR mutagenesis procedure as described under "Experimental Procedures".

product was gel-purified and used as a primer on plasmid pBS-hFII for one PCR cycle (1 min at 94°C, 30 s at 60°C, and 40 s at 72°C). Primers C and D (Table 1) were immediately added to the PCR reaction and 35 more cycles of PCR were carried out (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C). Primer C hybridizes upstream of the multiple cloning site of pBS-hFII while primer D hybridizes downstream of primer B. To convert Cys^{93} to serine, the mutagenesis procedure was repeated with primer G. The authenticity of the mutations, as well as the fidelity of the PCR-amplified constructs, were confirmed by double-stranded DNA sequencing.

Expression and purification of recombinant F2 peptides: Recombinant F2 peptides were expressed in *E. coli* BL21(DE3) by isopropylthio-β-D-galactoside induction using the histidine-tag pET system (Novagen, Inc., Madison, WI). The expression vector used, pET22b(+), directs the recombinant proteins to the periplasmic space thereby promoting proper folding and disulfide bond formation. The periplasmic fraction was obtained using the cold osmotic shock method described by Ausubel et al. (1989). Histidine-binding Ni^{2+} resin (Novagen, Inc., Madison, WI) was used to purify the recombinant proteins. The isolated proteins at a concentration of 1 mg/ml were subjected to electrophoresis in 15% SDS-polyacrylamide gels under reducing conditions (Laemmli, 1970), transferred to nitrocellulose, and visualized by Ponceau S staining and by immunoblotting as described previously (Austin et al., 1995).

Purification of F2: F2 was obtained from a terminal prothrombin activation reaction as described by Fredenburgh et al. (1997). F2 was dialyzed against TBS and the concentration was determined spectrophotometrically using $\epsilon_{280}^{0.1\%}$ of 1.1 (Church et al., 1991).

Thrombin-agarose affinity chromatography: Thrombin-agarose affinity chromatography was used to compare the affinities of plasma- and recombinant-F2 peptides for thrombin. 58 μM thrombin was incubated for 30 min at 37°C with a 10-fold molar excess of biotin-FPR in TBS. After dialysis against TBS, the biotin-FPR-thrombin was passed over streptavidin agarose in TBS and excess binding sites were blocked with biotin. 0.2 ml of purified F2 peptide at a concentration of 1 mg/ml was applied at 4°C to 0.5 ml of a streptavidin-agarose column containing 125 nmoles of biotin-FPR-thrombin. The column had previously been equilibrated in buffer containing 20 mM Tris-HCl, pH 7.4, 10 mM NaCl. All subsequent steps were performed at 4°C. Elution of the protein was performed with 1 ml of 20 mM Tris-HCl, pH 7.4 containing increasing concentrations of NaCl (from 10 mM to 500 mM). Aliquots from the flowthrough and eluates were analyzed using SDS-PAGE and immunoblotting as described above. The protein elution profiles were obtained by laser densitometry scans of immunoblots using the UltroScanTM XL laser densitometer (Pharmacia LKB Biotechnology). The density of F2 in each fraction was expressed as a percentage of the total F2 density in the complete elution profile.

Reduction and alkylation of F2 and rF2(1-116): The disulfide bonds in F2 and rF2(1-116) were reduced and alkylated as described (Bulleid and Freidman, 1988). The peptides were resuspended in 50 mM DTT at a concentration of 1 mg/ml and placed at room temperature for 30 min. Iodoacetamide (made up fresh in sterile distilled water) was then added to 50 mM and the reaction mixture was placed at room temperature for 45 min in the dark. The reaction mixture was dialyzed against 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.01% Tween-20 using Spectra/Por cellulose ester dialysis membrane (15 kD cutoff) and the protein

concentration determined as described above for intact F2.

Determination of the affinity of F2 peptides for thrombin: The association between F2 peptides and thrombin was monitored by the F2-dependent fluorescence intensity change of anilinonaphthalene-6-sulfonic acid-thrombin (ANS-FPR-thrombin). ANS-FPR-thrombin was prepared using ATA-FPR (N-((Acetylthio)acetyl)-D-Phe-Pro-Arg-CH_2Cl) as described by the supplier (Molecular Innovations Inc., Royal Oak, MI). Briefly, 10.6 μM of thrombin was incubated for 30 min at room temperature with 2.5-fold molar excess ATA-FPR in HBSE buffer (20 mM HEPES, pH 7.0, 100 mM NaCl, 1 mM EDTA). The reaction mixture was dialyzed in HBSE buffer, incubated with 10-fold molar excess IAANS (2-(4-(Iodoacetamide)anilino)naphthalene-6-sulfonic acid) (Molecular Probes Inc., Eugene, OR) in the presence of hydroxylamine for 60 min at room temperature in the dark, and then redialyzed. 500 μl of 100 nM ANS-FPR-thrombin was added to the same buffer in a semi-micro quartz cuvette. Using a Perkin Elmer LS50B luminescence spectrometer with excitation and emission wavelengths set to 328 nm and 450 nm, respectively, and excitation and emission slit widths set to 12 nm, and an emission filter of 390 nm, readings were taken of ANS-FPR-thrombin alone (Io). Known quantities of F2 peptides (3.8 mM) were then added to the cuvette and, after mixing, the change in fluorescence was monitored (I). As a control, a scrambled variant of the 54 amino acid peptide was titrated to the same concentration, and any change in fluorescence due to non-specific binding was used to correct the binding curve. The K_d values were calculated by plotting I/Io versus F2 peptide concentration. The parameters K_d and α were calculated by nonlinear regression ("Tablecurve", Jandel Scientific, San Rafael, CA) using the equation

$I/Io=(1+((K_d+[F2])/[ANS\text{-}IIa])-((1+((K_d+[F2])/([ANS\text{-}IIa]))^2-(4x[F2]/([ANS\text{-}IIa]))^{0.5})x(\alpha/2)+1$ where α is the maximum fluorescence change and assuming a stoichiometry of 1 (Fredenbirgh et al., 1997).

Displacement of fluorescein-hirudin$_{54\text{-}65}$ from FPR-thrombin by F2 and by sF2(63-116): Sulfated hirudin$_{54\text{-}65}$ was labelled with FITC as described by Liu et al. (Liu et al., 1991). 2 mL of TBS containing 10 nM of FITC-hirudin$_{54\text{-}65}$ was added to a 1 cm x 1 cm quartz cuvette in a Perkin Elmer LS50B luminescence spectrometer. Excitation and emission wavelengths were set at 492 nm and 522 nm, respectively, and excitation and emission slit widths were both set to 8 nm. The fluorescence intensity of 15 nM FPR-thrombin was measured before (Io) and after (I) the addition of either 20 to 40 µl aliquots of 161 µM F2 or 2 to 5 µl aliquots of 3.8 nM sF2(63-116). Both F2 and sF2(63-116) solutions contained 10 nM FITC-hirudin$_{54\text{-}65}$ so that the concentration of FITC-hirudin$_{54\text{-}65}$ remained constant. Titration was continued up to 43 µM F2 or 300 µM sF2(63-116). After the experiment, intensity values were read from time drive profiles and I/Io values were calculated and plotted versus the peptide concentration. As a control, the fluorescence intensity was measured before and after the addition of up to 100 µM reduced and alkylated sF2(63-116).

Effect of F2 on thrombin-mediated clotting of fibrinogen: Thrombin, at a final concentration of 0.6 nM, was mixed with either F2 (0 to 50 µM) or sF2(63-116) (0 to 300 µM) in TSTW buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.01% Tween-20) in a final volume of 75 µl. 25 µl of human fibrinogen and $CaCl_2$ were then added to final concentrations of 4 µM and 2 mM, respectively, and the times for half maximal increase in turbidity ($T_{1/2}$) were determined at 23°C by continuous measurement of absorbance at 405

nm using a Molecular Devices plate reader.

Effect of F2 on thrombin-mediated hydrolysis of chromogenic substrates: The chromogenic activity of 20 nM thrombin in TBS was determined with 0.7 to 14 mM of various thrombin substrates in the presence of 25 μM F2 or 150 μM sF2(63-116). The rates of thrombin-mediated hydrolysis were monitored at 405 nm in a plate reader and the percentage changes from control rates were determined.

Effects of F2 on the rate of thrombin inhibition by AT: The second-order rate constant (k_2) for the inhibition of thrombin by AT was determined under pseudo first-order conditions in the absence or presence of 1 U/ml heparin. In a multiwell plate, aliquots (10 μl) of thrombin (final concentration of 10 nM) were incubated for varying intervals with 10 μl of 500 nM AT in TBSP (TBS containing 0.6% polyethylene glycol-8000) in the absence or presence of F2 or sF2(63-116) in concentrations ranging from 10 to 500 μM. All reactions were terminated by the addition of 200 μM chromogenic substrate (N-p-tosyl-Gly-Pro-Arg-p-nitroanilide) in 200 μl TBSP containing 10 mg/ml polybrene. Thrombin inhibitory activity in the presence of 1 U/ml heparin was determined using thrombin and AT concentrations of 5 nM and 50 nM, respectively. Residual thrombin activity was then calculated by measuring absorbance for 5 min at 405 nm using a Molecular Devices plate reader. The pseudo-first-order rate constants (k_1) for thrombin inhibition were determined by fitting the data to the equation $k_1Ct=\ln([P]_o/[P]_t)$, where $[P]_o$ is initial thrombin activity and $[P]_t$ is thrombin activity at time t (Olson et al., 1993). The second-order rate constant, k_2, was determined by dividing k_1 by the AT concentration.

3.4 Results

Purification of F2 peptides: F2 is a 116 amino acid prothrombin activation fragment that consists of a 14-residue interkringle peptide (region A), a 79-residue kringle (regions B, C, D, and E), and a 25-residue acidic C-terminal kringle-catalytic domain connecting peptide (region F) (Fig. 1, panel A). For simplicity, the latter is referred to as the C-terminal connecting peptide. We mapped the thrombin-binding domain of F2 using deletion and point mutants prepared by recombinant and synthetic techniques. Schematic diagrams of the F2 peptides are shown in Fig. 1 (panel B) where F2, sF2, and rF2 denote plasma, synthetic, and recombinant F2 peptides, respectively. The deletion mutants encompass various portions of F2 including the kringle inner loop (residues 63-90)[2], the kringle outer loop (residues 1-55), the C-terminal connecting peptide (residues 92-116), and the C-terminal half of F2 (residues 55-116).

The predicted molecular masses of F2, rF2(1-116), and the three deletion derivatives rF2(1-55), rF2(55-116), and rF2(1-93) are 14, 15, 6, 7, and 11 kDa, respectively. These predicted values are consistent with the apparent molecular masses of the peptides as determined by SDS-PAGE and Western blot analysis (Fig. 2). No immunoreactive material was found in lysates of *E.coli* transformed with pET22b(+), the parent vector lacking the F2 cDNA (data not shown). The presence of disulfide bonds in the F2 peptides was verified by comparing the electrophoretic mobilities of reduced versus non-reduced peptides. Disulfide bond-containing peptides migrated more slowly under reducing conditions than under non-reducing conditions (data not shown).

Thrombin-agarose affinity chromatography: The affinities of F2 peptides for thrombin

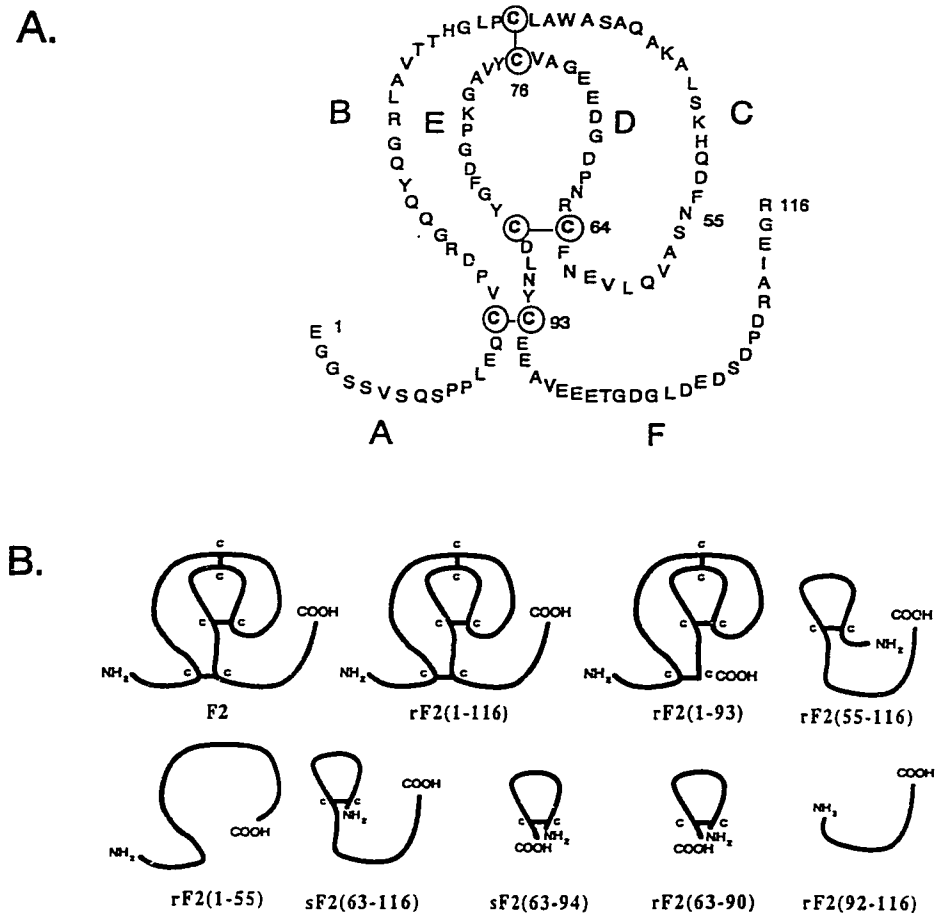

Fig. 1. **Diagram of F2 and F2 derivatives.** *Panel A*, amino acid sequence of human F2. Numbering of amino acids is from the amino terminus of human F2. The cysteine residues involved in disulfide bond formation are circled. The regions A and F correspond to the 14-residue interkringle peptide and the 25-residue C-terminal kringle-catalytic domain connecting peptide, respectively. Loops of the kringle are designated B (first outer loop), C (second outer loop), D (first inner loop), and E (second inner loop). *Panel B*, schematic diagram of F2 and F2 derivatives used in the current study. F2 refers to full-length, plasma F2. rF2(1-116), rF2(1-93), rF2(55-116), and rF2(1-55) are recombinant F2 peptides expressed in *E. coli*. sF2(63-116), sF2(63-94), sF2(63-90), and sF2(92-116) are synthetic peptides.

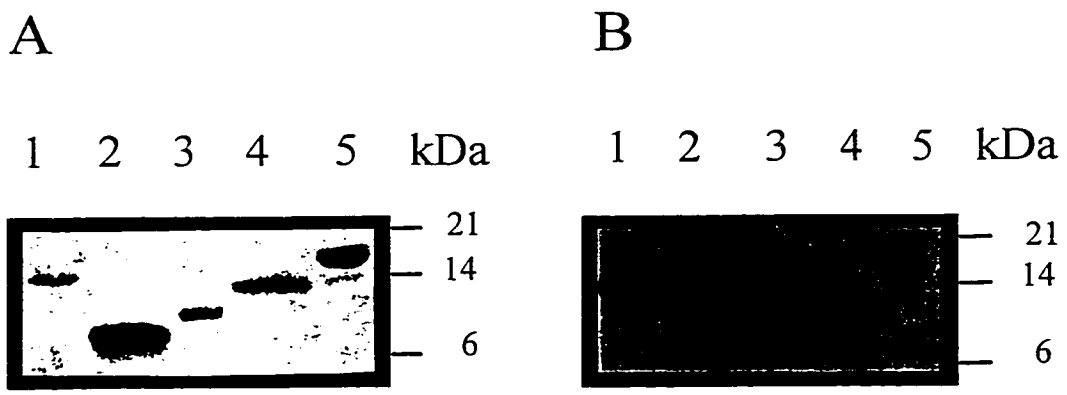

Fig. 2. **SDS-PAGE and immunoblot analysis of plasma and recombinant F2 peptides.** Purified F2 peptides (~5 μg each) were subjected to electrophoresis in 15% SDS-polyacrylamide gels under reducing conditions and transferred to nitrocellulose. *Panel A*, ponceau S stain of plasma and recombinant F2 peptides immobilized on nitrocellulose membrane: *lane 1*, F2; *lane 2*, rF2(1-55); *lane 3*, rF2(55-116); *lane 4*, rF2(1-93); *lane 5*, rF2(1-116). *Panel B*, immunoblot of plasma and recombinant F2 peptides probed with an IgG against prothrombin. The lanes in *panel B* contain the same samples as those in *panel A*.

were qualitatively assessed by subjecting the peptides to thrombin-agarose affinity chromatography. Biotin-FPR-treated thrombin was coupled to streptavidin-agarose as described in "Experimental Procedures". This directed coupling procedure ensures that all surface basic residues are unmodified and that all the thrombin molecules are coupled in the same fashion. F2 peptides were applied to the column in 10 mM NaCl, 20 mM Tris-HCl, pH 7.4 and the column was then washed with increasing concentrations of NaCl. Aliquots from the eluates were analyzed by SDS-PAGE and immunoblotting. The protein elution profiles were determined by laser densitometry scans of immunoblots. Intact F2 and rF2(1-116) were retained on the thrombin-agarose column at concentrations of NaCl up to 300 mM and 250 mM NaCl, respectively (Fig. 3, panel A). Upon reduction and alkylation of the disulfide bonds, both F2 and rF2(1-116) eluted from the thrombin agarose column as sharp peaks at 80 mM NaCl (Fig. 3, panel A). Bovine serum albumin, used as a negative control for thrombin-agarose binding, eluted from the column at 60 mM NaCl (data not shown). Like rF2(1-116), the deletion derivatives rF2(55-116) and sF2(63-116) were retained on the thrombin-agarose column at NaCl concentrations up to 250 and 300 mM NaCl, respectively (Fig. 3, panel B). Both of these peptides contain the kringle inner loop (residues 63-93) as well as the C-terminal connecting peptide (residues 94-116). In contrast, deletion derivatives that contain only the kringle inner loop, sF2(63-90), or the C-terminal connecting peptide, sF2(92-116), did not bind to thrombin-agarose. Furthermore, neither the N-terminal interkringle peptide (residues 1-14) nor the outer loop of the kringle (residues 15-55) is required for thrombin-agarose binding since rF2(1-55) and rF2(1-93) bound weakly to thrombin-agarose (Fig. 3, panel B).

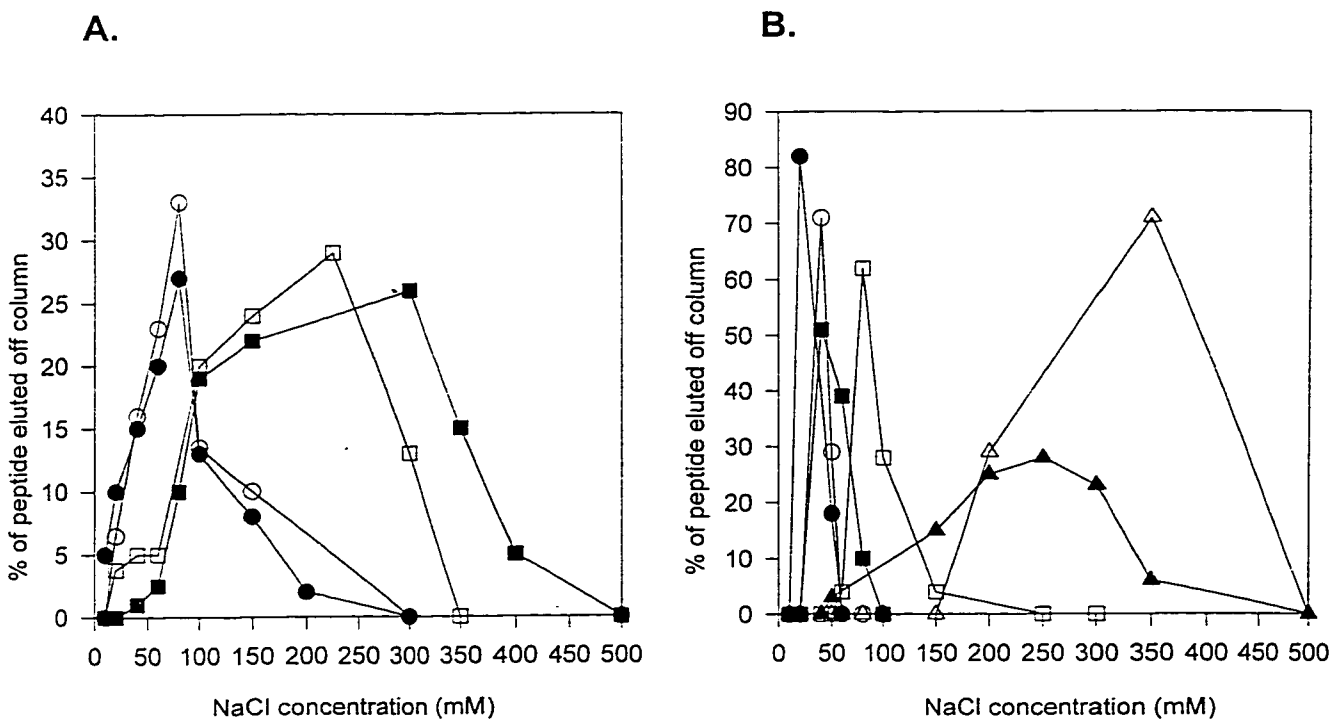

Fig. 3. **Thrombin-agarose affinity chromatography.** *Panel A*, elution of native and reduced forms of F2 and rF2(1-116) from thrombin-agarose. F2 peptides were subjected to thrombin-agarose affinity chromatography and eluted with an NaCl gradient (from 10 mM to 500 mM). Aliquots from the eluates were analyzed by SDS-PAGE and immunoblotting. The protein elution profiles were obtained by laser densitometry where the density of each lane was expressed as a percentage of the combined density of all the lanes in a given elution. Symbols: (●) reduced F2, (O) reduced rF2(1-116), (■) native F2, and (□) native rF2(1-116).

Panel B, elution of F2 derivatives from thrombin-agarose. F2 derivatives were subjected to thrombin-agarose affinity chromatography and eluted with an NaCl gradient as described above. Symbols: (●) sF2(63-90), (O) sF2(92-116), (■) rF2(1-55), (□) rF2(1-93), (▲) rF2(55-116), and (Δ) sF2(63-116).

Determination of the affinities of F2 peptides for thrombin: The affinities of F2 peptides for ANS-FPR-thrombin were determined quantitatively by monitoring the change in probe fluorescence during peptide titration as described by Bock (1992). After titration, the I/Io values were plotted versus F2 peptide concentration and the data analyzed by nonlinear regression. Because of the low yields of rF2 peptides, K_d values were only determined for F2 and sF2 peptides. F2 and sF2(63-116), both of which contain the kringle inner loop as well as the C-terminal connecting peptide, bind saturably to ANS-FPR-thrombin with K_d values of 4.1 and 51.3 μM respectively (Fig. 4, Table 2). The binding of F2 and sF2(63-116) to ANS-FPR-thrombin is specific because there was no change in fluorescence intensity of ANS-FPR-thrombin in the presence of a scrambled variant of sF2(63-116) (data not shown). Previous results, demonstrating (a) competitive binding of F2 and sF2(63-116) to FPR-thrombin and (b) binding of both F2 and sF2(63-116) to γ-thrombin, a proteolytic derivative of thrombin lacking exosite I, reveal that F2 and sF2(63-116) bind to exosite II of thrombin (Fredenburgh et al., 1997).

Further truncation of sF2(63-116) to peptides which only span the kringle inner loop, sF2(63-90) and sF2(63-94), or only encompasses the C-terminal connecting peptide, sF2(92-116), results in derivatives that do not bind to ANS-FPR-thrombin (Table 2). A mixture of sF2(63-90) and sF2(92-116) also does not affect the fluorescence intensity of ANS-FPR-thrombin, indicating that the C-terminal connecting peptide must be contiguous with the kringle inner loop for effective interaction with thrombin (Table 2).

In an attempt to modify the number of salt bridges formed with exosite II of thrombin, five separate amino acid substitutions were introduced into sF2(63-116), and the

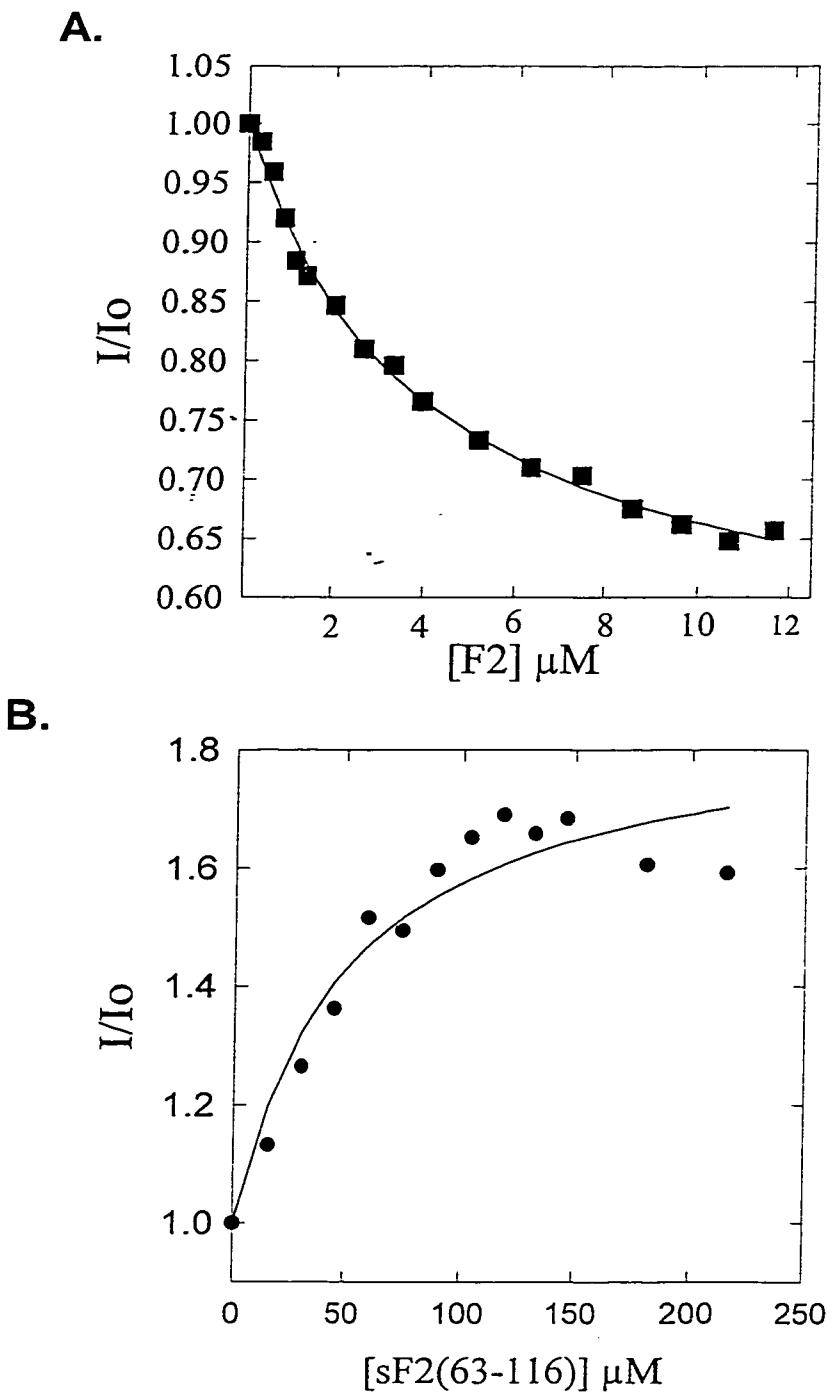

Fig. 4. **Binding of F2 and sF2(63-116) to ANS-thrombin.** Increasing amounts of F2 (panel A) or sF2(63-116) (panel B) were mixed with 100 nM ANS-thrombin and changes in fluorescent intensity were monitored. I/Io is plotted versus peptide concentration , where I is the fluorescent intensity at a given peptide concentration and Io is the initial fluorescence intensity. The K_d values were determined by non-linear regression analyses of the data (line).

TABLE 2

Dissociation constants for the interaction of F2 peptides with ANS-FPR-thrombin.

Peptide	Kd (μM)
F2	4.1
sF2(63-116)	51.3
sF2(63-90)	no binding
sF2(63-94)	no binding
sF2(92-116)	no binding
sF2(63-90) and sF2(92-116)	no binding
V78E	10.0
K81E	no binding
Y87E	40.7
F85E	99.8
K81Q	94.0

The affinities of F2 peptides for ANS-FPR-thrombin were determined by monitoring the F2 peptide-dependent fluorescence intensity changes of ANS-FPR-thrombin as described under "Experimental Procedures".

affinities of the mutant peptides for ANS-FPR-thrombin were measured (Table 2). Substitution of Val^{78} with Glu increased the affinity of sF2(63-116) for ANS-FPR-thrombin 5-fold (K_d decreased from 51.3 to 10.0 μM), whereas substitution of Tyr^{87} with Glu produced only a marginal increase (K_d decreased from 51.3 to 40.7 μM). In contrast, F85E and K81Q mutations decreased the affinity of sF2(63-116) for ANS-FPR-thrombin - 2-fold, whereas substitution of Lys^{81} with Glu abolished binding. These studies indicate that specific amino acid substitutions within the kringle inner loop can affect the affinity of F2 for thrombin.

Functional comparison of F2 and sF2(63-116): To identify the F2 domains that evoke conformational changes in thrombin, we compared the effects of F2 and sF2(63-116) on (a) the rates of thrombin inhibition by AT, (b) the rates of thrombin-mediated hydrolysis of chromogenic substrates, (c) thrombin clotting times, and (d) displacement of fluorescein-labelled $hirudin_{54-65}$ from FPR-thrombin. Like F2, saturating amounts of sF2(63-116) reduce the heparin-catalyzed rate of thrombin inhibition by AT approximately 4-fold, indicating that both peptides compete with heparin for binding to exosite II on thrombin (Fig. 5). In the absence of heparin, however, only F2 slows the rate of thrombin inhibition by AT (Fig. 5). Upon reduction and alkylation, neither F2 nor sF2(63-116) has any inhibitory effect on the rate of thrombin inhibition by AT either in the absence or presence of heparin (data not shown). As shown in Fig. 6, both F2 and sF2(63-116) increase the chromogenic activity of thrombin with substrates S2238, ChtPA, and S2222, whereas activity was reduced with S2444. With chromogenic substrates S2366 and S2288, opposing effects were induced by the two peptides.

Both F2 and sF2(63-116) also cause a concentration-dependent increase in the

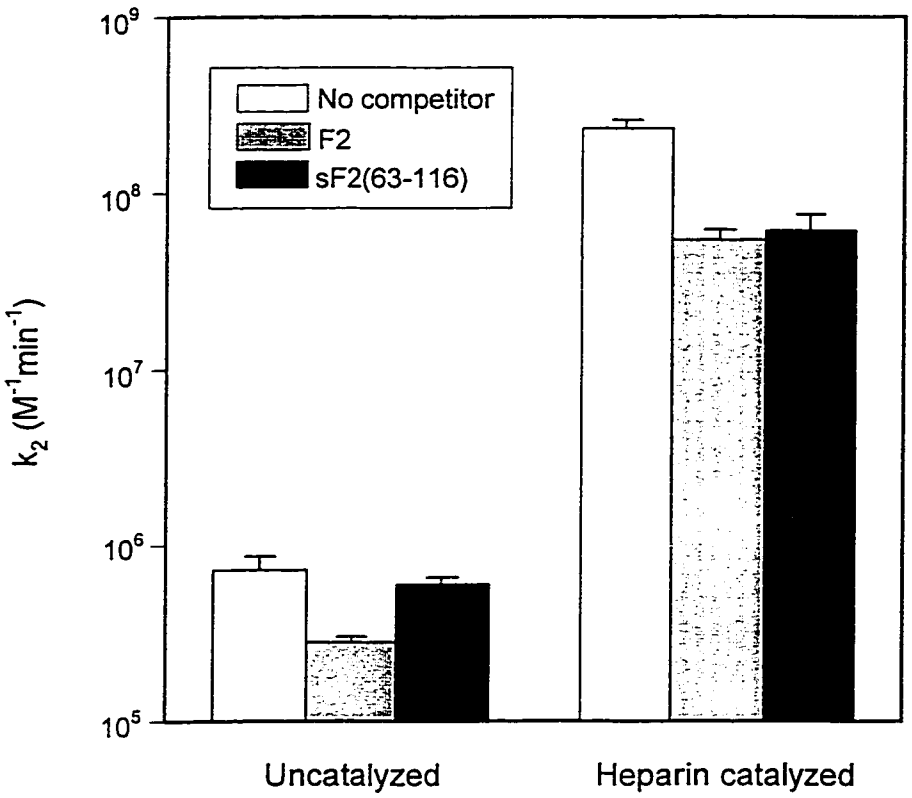

Fig. 5. **Effects of F2 and sF2(63-116) on the rates of thrombin inhibition by AT.** The second- order rate constants for the inhibition of thrombin (10 nM) by 500 nM AT were determined under pseudo first-order conditions in the absence or presence of 1 U/ml heparin. Experiments were performed in the absence or presence of saturating concentrations of F2 or sF2(63-116), 40 μM and 300 μM, respectively. The values represent the mean and SEM of three determinations.

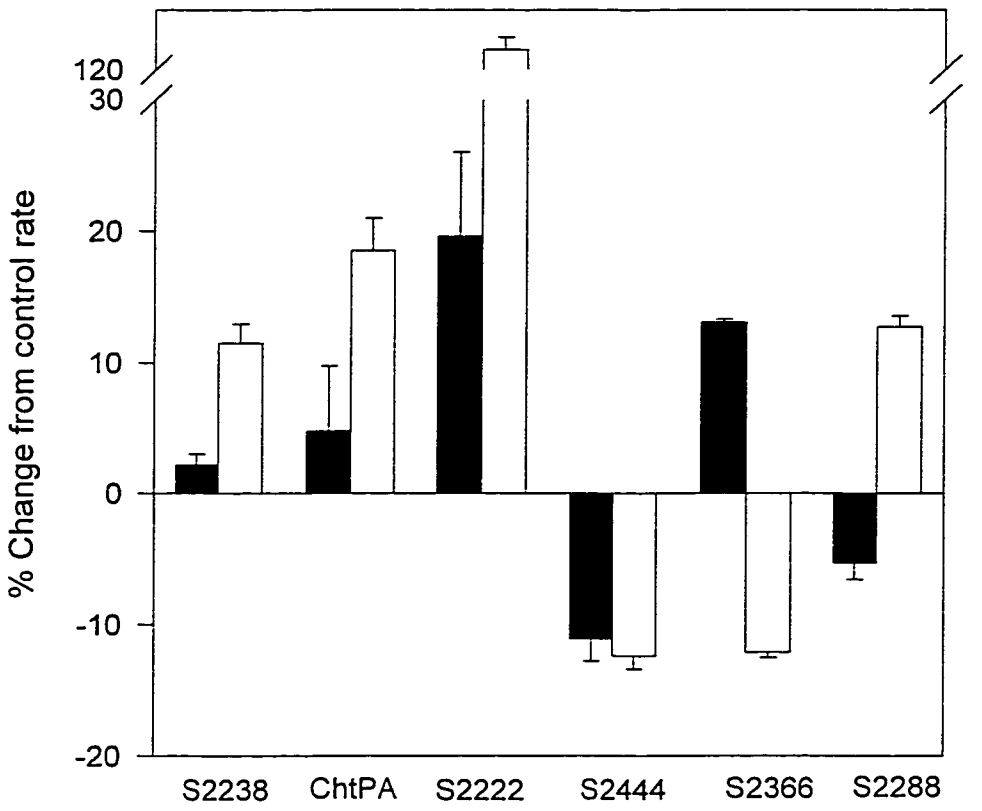

Fig. 6. **Influence of F2 and sF2(63-116) on the chromogenic activity of thrombin.** The rates of thrombin-mediated hydrolysis of chromogenic substrates were determined in the absence or presence of 25 μM F2 (G) or 150 μM sF2(63-116) (_) and the percentage changes from the control rate were calculated.

thrombin clotting time of fibrinogen (Fig. 7). Upon reduction and alkylation, sF2(63-116) not only loses its ability to increase the thrombin clotting times, but appears to promote clotting to a small extent (Fig. 7). These data confirm previous reports that ligand binding to exosite II alters the conformation and function of the active site. However, the effect of the peptides on thrombin clotting times may also reflect ligand-induced changes at exosite I since both F2 and sF2(63-116) displace fluorescein-labelled hirudin$_{54-65}$ from FPR-thrombin. As shown in Table 3, titration of FPR-thrombin with up to 300 μM sF2(63-116) causes complete displacement of fluorescein-hirudin$_{54-65}$ from FPR-thrombin. Nonlinear regression analysis of the data reveals a K_d of 200 μM for the sF2(63-116)/FPR-thrombin interaction, as monitored by fluorescein-hirudin$_{54-65}$ binding. Titration of FPR-thrombin with up to 40 μM F2 causes 45% displacement of fluorescein-hirudin$_{54-65}$ from FPR-thrombin, whereas no displacement was observed with reduced and alkylated sF2(63-116). These data establish that, although F2 and sF2(63-116) bind to the same site on thrombin, they do not evoke identical effects.

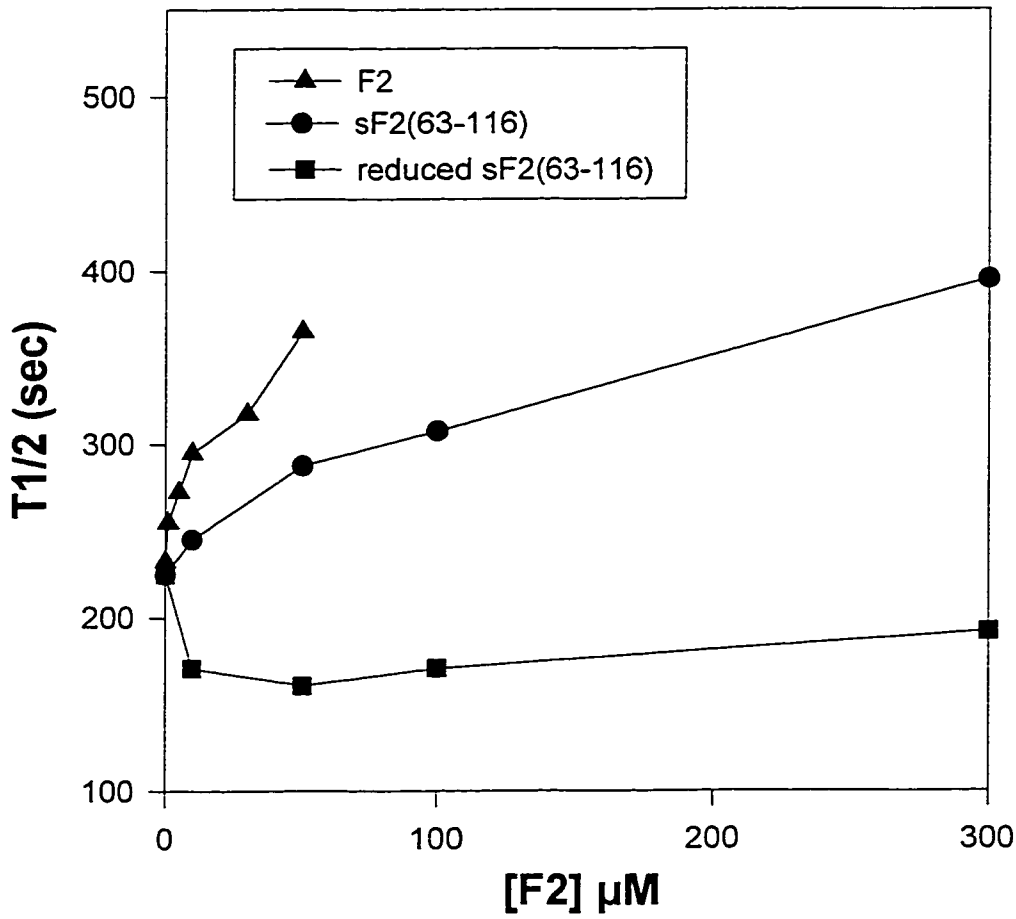

Fig. 7. **Effect of F2 and sF2(63-116) on thrombin clotting times.** Fibrinogen (4 µM) was clotted with 0.8 nM thrombin and 2 mM $CaCl_2$ in the absence or presence of F2 (▲) or sF2(63-116) (●) at the concentrations indicated. The times for half-maximal increase in turbidity ($T_{1/2}$) were determined spectrophotometricaliy at 405 nm. As a control, the effect of reduced and alkylated sF2(63-116) (■) on thrombin clotting times also was determined.

TABLE 3

Displacement of hirudin$_{54-65}$ from FPR-thrombin by F2 and sF2(63-116).

Peptide	Concentration (μM)	% displacement of fluorescein-hirudin$_{54-65}$ from FPR-thrombin	Kd (μM)
sF2(63-116)	300	100	200
F2	41	45	N.D.*
reduced sF2(63-116)	100	0	No binding

The effect of F2 and sF2(63-116) on hirudin$_{54-65}$ binding to thrombin was examined by binding fluorescein-hirudin$_{54-65}$ to FPR-thrombin and monitoring changes in the fluorescence intensity when the FPR-thrombin was titrated with F2, sF2(63-116), and reduced and alkylated sF2(63-116). N.D.= not determined.

3.5 DISCUSSION

Previous cocrystallization studies of F2 with active-site-blocked thrombin revealed that F2 makes contacts of less than 4 D with Arg^{93}, Arg^{97}, Arg^{101}, and Arg^{175} of the heparin-binding region of thrombin[2] (Arni et al., 1993; Arni et al., 1994). These positively charged residues on thrombin form salt bridges with the anionic motif DGDEE (residues 68-72) in the kringle inner loop of F2. In this study, we experimentally defined the regions of F2 required for thrombin binding. Our data indicate that, in addition to the kringle inner loop (residues 64-93), the C-terminal connecting peptide (residues 94-116) also plays a role in the interaction of F2 with thrombin. The requirement of the C-terminal connecting peptide of F2 for F2-thrombin interactions would not have been predicted from the F2-thrombin cocrystallized structure because the F2 interkringle connecting peptides (regions A and F, Fig. 1) were disordered in the crystal (Arni et al., 1993; Arni et al., 1994). However, recent molecular modelling suggests that the C-terminal connecting peptide is in contact with basic residues within exosite II (van de Locht et al., 1996), a concept supported by our data.

The interaction of F2 with thrombin is conformation-dependent and ionic in nature because the binding of F2 and rF2(1-116) to thrombin-agarose is abolished when the peptides are reduced and alkylated and binding of F2 deletion mutants to thrombin-agarose is dependent on the NaCl concentration. In support of the ionic nature of the F2/thrombin interaction, the reported K_d values for the binding of F2 to thrombin in low ionic strength buffer (0.05 M Tris-HCl, pH 8.1) and in physiologic ionic strength buffer (0.05 M Tris-HCl, pH 7.5/ 150 mM NaCl) are 770 pM (Myrmel et al., 1976) and 5 μM (Bock, 1992), respectively. In the physiologic ionic strength buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) used in our

studies, F2 and sF2(63-116) bind saturably to ANS-FPR-thrombin with K_d values of 4.1 and 51.3 μM, respectively. The K_d values determined by fluorescence (4.1 μM in this work, 5 μM reported by Bock (1992)), are in agreement with K_i values reported for the influence of F2 on AT inhibition (Walker and Esmon, 1979), fibrinogen clotting (Jakubowski et al., 1986), and protein C activation (Jakubowski et al., 1986). Thus, at physiological ionic strength, F2 binds thrombin with a K_d of about 5 μM. In contrast, F2 has been reported to bind prethrombin 2 with a Kd of 33 nM (Krishnaswamy and Walker, 1997). These differences could reflect structural diversity between prethrombin 2 and thrombin.

The K_d values were determined by monitoring the changes in ANS-FPR-thrombin fluorescence intensity during peptide titration. Although both F2 and sF2(63-116) elicit changes in the thrombin active site environment upon binding, the changes in fluorescence signal are diametric (Fig. 4). The diametric changes may reflect differences in the conformation of the C-terminal connecting peptide of sF2(63-116) relative to full-length F2. However, three lines of evidence suggest that the structural integrity of the C-terminal connecting peptide is maintained in sF2(63-116). First, the C-terminal connecting peptide alone does not bind to thrombin; only when this peptide is contiguous with the kringle inner loop of F2 does binding occur (Table 2). Second, like F2, sF2(63-116) loses its ability to bind thrombin-agarose upon reduction and alkylation of disulfide bonds (data not shown). Third, rF2(1-93), which contains all but the C-terminal connecting peptide of F2, fails to bind to thrombin-agarose (Fig. 3).

The diametric changes in the thrombin active site environment elicited by the binding of F2 and sF2(63-116) may instead arise from fewer sites of contact with thrombin for the

truncated peptide relative to full-length F2. This is supported by the weaker affinity of sF2(63-116) for thrombin compared with F2. The lower affinity of sF2(63-116) may reflect the absence of residues in loop C (Leu^{47}, His^{50}, and Gln^{51}) that form interdomain contacts of less than 4 D with thrombin (Arni et al., 1993; Arni et al., 1994). However, because rF2(1-55) and rF2(1-93), both of which contain loop C, fail to bind to thrombin-agarose, the N-terminal half of F2 likely plays only an indirect role in thrombin binding, either by contributing to the stability of F2 or by providing secondary contacts with thrombin The distinct structural changes that occur in thrombin when it associates with F2 and sF2(63-116) are similar to unique binding interactions of thrombin with different forms of thrombomodulin. Although thrombin binds to the fifth and sixth epidermal growth factor-like domains of thrombomodulin with high affinity (Kurosawa et al., 1988), the fourth through sixth epidermal growth factor-like domains are needed for optimal protein C activation by thrombin (Zushi et al., 1989; Hayashi et al., 1990) and for induction of structural changes in the active site of thrombin (Ye et al., 1991).

In an attempt to modify ionic interactions between the kringle inner loop of F2 and exosite II of thrombin, five separate amino acid substitutions were introduced into sF2(63-116) and the affinities of the resultant peptides for thrombin were determined. Substitution of Val^{78} with Glu increased the affinity of sF2(63-116) for ANS-FPR-thrombin approximately 5-fold (K_d decreased from 51.3 to 10.0 μM) giving it an affinity similar to that of F2 (K_d= 4.1 μM) whereas substitution of Tyr^{87} with Glu produced only a modest increase in affinity (K_d decreased from 51.3 to 40.7 μM). Both of these mutations likely increase the number of ionic interactions formed between F2 and thrombin. In contrast, substitution of

Lys^{81} with Gln decreased the affinity of sF2(63-116) for thrombin approximately 2-fold and substitution of Lys^{31} with Glu abolished the binding of sF2(63-116) to ANS-thrombin. Since Lys^{81} was not observed to make ionic contact with thrombin (Arni et al., 1993; Arni et al., 1994), one possible explanation for the decreases in thrombin affinity is that mutations in the second inner loop of the kringle distort the hairpin β-turn which has been shown to pivot at Val^{78} and Asp^{84} (Arni et al., 1993; Arni et al., 1994). The stability of F2 appears to be enhanced by aromatic stacking components since substitution of Phe^{85} with Glu decreases the affinity of sF2(63-116) for thrombin approximately 2-fold (K_d increased from 51.3 to 99.8 μM).

Functional comparison of F2 with sF2(63-116), both of which contain the kringle inner loop (loops D and E) as well as the C-terminal connecting peptide (loop F) , indicates that both peptides influence the chromogenic activity of thrombin (Fig. 6), producing similar effects on four substrates and opposing effects on two others. Although these results illustrate changes in the active site environment of thrombin due to ligand binding at exosite II, they also suggest that there may be additional or altered contacts of F2 compared to sF2(63-116). Both F2 and sF2(63-116) displace fluorescein-labelled hirugen from FPR-thrombin, indicating that binding of either ligand to exosite II affects the binding properties of exosite I, a phenomenon previously demonstrated by Fredenburgh et al.(1997). F2 and sF2(63-116) also prolong the thrombin clotting time of fibrinogen in a dose-dependent manner (Fig. 7). That F2 appears to be a more potent inhibitor of thrombin clotting activity likely reflects its higher affinity for thrombin. The effect of the peptides on thrombin clotting times may reflect conformational changes at the active site and/or allosteric changes at

exosite I that result in decreased fibrinogen binding.

Walker and Esmon (1979) reported that F2 slows the rate of thrombin inactivation by AT because it inhibits AT binding to thrombin. In this study, we demonstrated that, unlike F2, sF2(63-116) has no inhibitory effect on the rate of thrombin inhibition by AT in the absence of heparin. One possible explanation for these findings is that the N-terminal connecting peptide or possibly the outer loop of F2, both of which are missing from sF2(63-116), sterically block access of AT to the active site of thrombin. Alternatively, F2, but not sF2(63-116), may evoke conformational changes in thrombin that limit its reactivity with AT. This is supported by the distinct changes in fluorescence of ANS-FPR-thrombin induced by F2 and sF2(63-116) (Figure 3) and by the work of others who proposed that individual residues within exosite II mediate various functions of the exosite (He et al., 1997). In contrast to their different effects in the absence of heparin, both F2 and sF2(63-116) reduce the heparin-catalyzed rate of thrombin inactivation by AT about 4-fold likely by competing with heparin for binding to exosite II on thrombin.

In summary, our findings indicate that the functional changes produced by sF2(63-116) binding to thrombin are quantitatively different from those evoked by F2. Some of these differences may simply reflect the 12-fold higher affinity of F2 for thrombin (K_d values of 4.1 μM and 51.3 μM, respectively). However, it is unlikely that thrombin binding affinity alone accounts for (a) the diametric changes in fluorescence signal elicited by the two ligands when they bind to thrombin, or (b) their differential effects on the uncatalyzed rate of thrombin inhibition by AT. Rather, these observations more likely reflect additional contacts of F2 relative to the smaller sF2(63-116) peptide and suggest that ligand binding to various

subsites within exosite II may have different effects on thrombin function.

CHAPTER 4-CONFORMATIONAL ACTIVATION OF HEPARIN COFACTOR II BY MUTATIONS IN THE GLYCOSAMINOGLYCAN-BINDING DOMAIN

Preface

This manuscript has been submitted to the Journal of Biological Chemistry. The authors are: Patricia C. Y. Liaw, Richard C. Austin, and Jeffrey I Weitz. The corresponding author is Dr. Weitz. The experiments were performed by me.

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[1]The abbreviations used are: Serpin, serine protease inhibitor; HCII, heparin cofactor II; AT, antithrombin; wt rHCII, wildtype recombinant HCII; pHCII, human plasma-derived HCII; GAG, glycosaminoglycan; RA-thrombin, recombinant thrombin mutant with Arg^{93}, Arg^{97}, and Arg^{101} changed to Ala; γ-thrombin, a proteolytic derivative of thrombin lacking exosite I; DS, dermatan sulfate; hirudin-(54-65), a synthetic analogue of the carboxyl-terminus of hirudin that interacts with thrombin exosite I;

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4.1 Summary

Heparin cofactor II (HCII) selectively inhibits thrombin in a reaction that is accelerated ≥ 1000-fold by glycosaminoglycans (GAGs) such as heparin or dermatan sulfate. Current thinking is that GAG binding to HCII disrupts salt bridges between the amino-terminal acidic domain and the GAG-binding domain of HCII, thereby promoting the interaction of the amino-terminal acidic domain with exosite I of thrombin. Based on this allosteric activation model, we predicted that substitution of basic residues in the GAG-binding domain of HCII with neutral ones would mimic the catalytic effect of GAGs. Compared with wild-type recombinant HCII expressed in BHK cells (wt rHCII), mutation of Arg^{184}, Lys^{185}, Arg^{192}, Arg^{193} (Mut C) or Arg^{184}, Lys^{185}, Arg^{189}, Arg^{192}, Arg^{193} (Mut D) reduced the affinity for heparin-Sepharose and increased the uncatalyzed rate of thrombin inactivation ~130-fold (from 4.6×10^4 M^{-1} min^{-1} to 6.2×10^6 and 6.0×10^6 M^{-1} min^{-1}, respectively). Furthermore, unlike wt rHCII or plasma-derived HCII (pHCII), neither heparin nor dermatan sulfate increased the rate of thrombin inhibition by Mut C or Mut D. The increased basal rate of thrombin inhibition by these mutants reflects displacement of their amino-terminal acidic domains because (a) they inhibit γ-thrombin at a 65-fold slower rate than α-thrombin, (b) the exosite 1-binding fragment hirudin-(54-65) decreases the rate of thrombin inhibition, and (c) deletion of the amino-terminal acidic domain (-del74) of Mut D reduces the rate of thrombin inhibition ~100-fold. To determine whether GAG-mediated bridging of thrombin to HCII contributes to accelerated thrombin inhibition, we compared the catalytic effects of longer heparin or dermatan sulfate chains with those of shorter chains. Heparin chains comprised of 30 or more saccharide units produced an ~5-fold greater

increase in the rate of thrombin inhibition by pHCII, wt rHCII, and wt-del74 than heparin chains comprised of 20 or fewer saccharide units. In contrast, dermatan sulfate and a low molecular weight fragment of dermatan sulfate stimulated thrombin inhibition by pHCII and wt rHCII to the same extent, and neither agent affected the rate of thrombin inhibition by wt-del74. Our findings support the concept that heparin and dermatan sulfate activate HCII by releasing the acidic amino-terminal domain from intramolecular connections with the GAG-binding domain. Since both GAGs produce ≥1000-fold increase in the rate of thrombin inhibition by HCII, our observation that only heparin serves as a template raises the possibility that dermatan sulfate induces more extensive allosteric changes than heparin.

4.2 Introduction

Serpins[1] are a family of structurally related proteins, many of which inhibit serine proteases involved in diverse biological processes such as coagulation, fibrinolysis, complement activation, inflammation, and angiogenesis (Church et al., 1997). A characteristic feature of protease inhibition by serpins is the formation of an inhibitory complex that is resistant to heat and denaturation (Church et al., 1997). Covalent complex formation arises from protease cleavage of the P1-P1' bond in the reactive centre loop of the serpin, a process followed by conformational reorganization wherein the solvent-exposed reactive centre loop is inserted into the body of the serpin (Stratikos and Gettins, 1997; Wilczynska et al., 1997).

Heparin cofactor II (HCII), a serpin found in human plasma at a concentration of 1.2 μM, selectively inhibits thrombin in a reaction that is accelerated ≥1000-fold by glycosaminoglycans (GAGs) such as heparin, dermatan sulfate, and heparan sulfate (Tollefsen et al., 1993). A second serpin, antithrombin (AT), also inhibits thrombin but differs from HCII in three important ways. First, whereas HCII only inhibits thrombin, AT inactivates other coagulation enzymes including factors Xa and IXa (Rosenberg, 1987). Second, the high affinity interaction of heparin with AT is mediated by a unique pentasaccharide sequence found only in a subpopulation of heparin molecules (Lindahl et al., 1980; Casu et al., 1981; Choay et al., 1983). In contrast, heparin does not possess a high affinity sequence for HCII (Sie et al., 1988). Furthermore, dermatan sulfate, a GAG found in the extracellular matrix of connective tissue (Rosenberg et al., 1985; Choi et al., 1989), catalyzes HCII, but has no effect on AT (Tollefsen et al., 1983). Third, the uncatalyzed rate

of thrombin inactivation by AT is about 10-fold faster than that for HCII, probably reflecting differences in the amino acid residue at their P1 position, with AT containing an Arg residue and HCII a Leu (Griffith et al., 1989).

Although the uncatalyzed rate of thrombin inhibition by HCII is lower than that for AT, in the presence of heparin or dermatan sulfate, HCII inactivates thrombin at a rate similar to that at which AT inhibits thrombin when heparin is present (Tollefsen et al., 1983; Olson and Shore, 1981). The current model to explain GAG-mediated catalysis of HCII suggests that binding of polyanionic GAGs to the electropositive GAG-binding domain on HCII disrupts ionic interactions between the amino-terminal acidic domain of HCII and basic residues in the GAG-binding domain (Ragg et al., 1990a; van Deerlin and Tollefsen, 1991; Sheehan et al., 1994). Once the amino terminus of HCII is no longer conformationally restrained, the region encompassing residues 54-75 (Hortin et al., 1989) interacts with exosite I on thrombin, thereby promoting enzyme-inhibitor complex formation. Two lines of evidence support this concept. First, deletion of the amino-terminal acidic domain of HCII markedly decreases the rate at which HCII inhibits thrombin in the presence of heparin or dermatan sulfate (van Deerlin and Tollefsen, 1991). Second, qualitative studies by Ragg et al. (Ragg et al., 1990a) demonstrated that two GAG-binding domain mutants (R184Q, K185Q and R192Q, R193N) form SDS-stable complexes with thrombin in the absence of GAGs, a finding that was postulated to reflect increased mobility of the amino-terminal acidic domain through disruption of ionic interactions. The allosteric changes induced by GAG binding are believed to account for most, but not all, of the stimulatory effect because studies with exosite II variants of thrombin suggest that some acceleration in the rate of

thrombin inactivation results from simultaneous binding of GAGs to exosite II of thrombin and the GAG-binding domain of HCII; an interaction that promotes ternary complex formation (Sheehan et al., 1994; Rogers et al., 1992).

Since the allosteric model predicts that ion pairs restrict the mobility of the amino terminal acidic domain of HCII, we investigated the extent to which substitution of basic residues in the GAG-binding domain with neutral amino acids mimics the catalytic effect of GAGs. We targeted the substitutions to Arg^{184}, Lys^{185}, Arg^{189}, Arg^{192}, and Arg^{193}, because (a) these residues have been implicated in GAG binding based on analysis of naturally occurring and recombinant HCII variants (Ragg et al., 1990a, Ragg et al., 1990b; Blinder et al., 1989; Blinder and Tollefsen, 1990) and (b) we wished to explore the possibility that more extensive mutations within this domain would further enhance the uncatalyzed rates of thrombin inactivation by HCII. To accomplish this, we quantified the rates of thrombin inhibition by mutants R184Q, K185Q and R192Q, R193N as well as additional variants containing all four of these mutations (Mut C) or these mutations together with R189Q (Mut D). Herein we report that substitution of 4 or 5 of the basic residues that mediate GAG binding to HCII with neutral amino acids produces a ~130-fold increase in the uncatalyzed rate of thrombin inhibition by HCII. Furthermore, we provide evidence that the increased thrombin inhibitory activities of these mutants reflect conformational changes in the amino terminal acidic domain that facilitates its interaction with exosite I of thrombin. Finally, we compared the extent to which the template mechanism contributes to the heparin- and dermatan sulfate-mediated acceleration of thrombin inhibition by HCII.

4.3 Experimental Procedures

Materials: Oligonucleotides were synthesized by the Institute for Molecular Biology and Biotechnology at McMaster University, Hamilton, ON. Human HCII and AT, isolated from plasma by affinity chromatography, and monospecific polyclonal IgG against human HCII and human AT were from Affinity Biologicals Inc. (Hamilton, ON). Polybrene was obtained from Aldrich Chemical Company (Milwaukee, WI). Heparin, hirudin-(54-65), anti-sheep IgG alkaline phosphatase, and the thrombin-directed substrate, N-p-tosyl-Gly-Pro-Arg-p-nitroanilide, were from Sigma Chemical Co. (St. Louis, MO). Heparin-Sepharose CL-6B resin, deoxynucleotides (dNTPs), and RNAguard ribonuclease inhibitor were from Pharmacia Biotech (Uppsala, Sweden). Human α- and γ-thrombin, factor Xa, factor IXa, factor XIa, and size-restricted heparin fractions of 18 kDa, 9 kDa, 6 kDa, and 4 kDa were from Enzyme Research Laboratories (South Bend, IN). The recombinant thrombin mutant with Arg^{93}, Arg^{97}, and Arg^{101} substituted with Ala (RA-thrombin) (Ye et al., 1994) was generously provided by Dr. Charles Esmon (Howard Hughes Medical Institute, Oklahoma City, OK). Dulbecco's modified Eagle's medium, Geneticin, and Superscript RNase H reverse transcriptase were from Gibco BRL (Gaithersburg, MD). Fetal bovine serum was obtained from HyClone Laboratories Inc. (Logan, UT). Dermatan sulfate was obtained from Mediolanum Farmaceutici (Milan, Italy). Desmin, a 5.6 kDa low molecular weight dermatan sulfate fraction obtained by limited depolymerization (Harenberg et al., 1996), was generously provided by Dr. Giancarlo Agnelli (Universita di Perugia, Perugia, Italy). A Baby Hamster Kidney (BHK) cell line was generously provided by Dr. William Sheffield (McMaster University, Hamilton). All other chemicals were of the highest grade

commercially available.

DNA construction and mutagenesis: Human HCII cDNA was cloned from HepG2 cells by reverse transcription- polymerase chain reaction. Briefly, total RNA was isolated from HepG2 liver cells using the RNeasy total RNA kit (Qiagen Inc., Chatsworth, CA) and reverse transcribed using primer A (5' AAG GCA CTT CAG ACA CCT AGA CCT CCA 3') which hybridizes to the 3' untranslated region of HCII cDNA (Blinder et al., 1988). Reverse transcription was done by first heating 1 μg of total RNA and 50 ng of primer A for 10 min at 70°C and then placing the mixture on ice. The volume was brought to 20 μl by adding 4 μl of 5X reverse transcriptase buffer (Gibco BRL), 2 μl 0.1 M dithiothreitol, 0.5 mM of each dNTP, 37.5 U RNAguard ribonuclease inhibitor, and 200 U Superscript RNase H reverse transcriptase. cDNA synthesis was performed at 42°C for 60 min. The reaction mixture was then heated to 75°C for 10 min and chilled on ice. HCII cDNA was PCR amplified using primer A and primer B (5' AGC TCC GCC AAA ATG AAA CAC TCA TTA AAC GCA 3') which hybridizes to the 5' untranslated region of HCII cDNA (Blinder et al., 1988). The PCR product was purified on a 1% agarose gel, digested with *Eco*RV, and initially subcloned into the *Eco*RV site of pBluescript (KS) (Stratagene Ltd., La Jolla, CA). HCII cDNA was then cloned in the forward orientation into the *Eco*RI site of the phagemid vector pALTER-1 (Promega, Madison, WI). *In vitro* mutagenesis to generate and select oligonucleotide-directed mutants was performed using single-stranded phagemid DNA as described by the supplier. The sequence of the HCII cDNA and the authenticity of the mutations were confirmed by double-stranded sequencing using dideoxy chain termination (Sambrook et al., 1989) and Sequenase 2.0 (United States Biochemical, Cleveland, OH).

Stable expression of wild type and variant forms of HCII in BHK cells: cDNAs encoding the wildtype and variant forms of HCII were cloned into the *Eco*RI site of the eukaryotic expression vector pcDNA3.1(+) (Invitrogen, San Diego, CA). In the resulting plasmid, the expression of HCII cDNA is under the control of the human cytomegalovirus immediate-early promoter. Transfection of BHK cells was performed in Dulbecco's Modified Eagle Medium using Qiagen-purified pcDNA3.1 constructs employing the SuperFect transfection reagent for 3 h as described by the supplier (Qiagen). The medium was then changed to DMEM containing 10% fetal bovine serum and 1 mg/ml Geneticin. After 2 weeks of selection, in which the medium was changed every 3 days, drug-resistant colonies were isolated and levels of recombinant protein expression were determined by immunoblotting with sheep anti-HCII antibody. Clones secreting the highest level of recombinant protein were seeded into roller bottles and cultured in serum-free DMEM.

Recombinant protein purification: Sheep anti-HCII antibody was coupled to CNBr-activated Sepharose 4B matrix as described by Cuatrecasas (1970). All subsequent steps were done at room temperature. The conditioned medium of the transfected BHK cells was applied to anti-HCII resin pre-equilibrated in 20 mM Tris-HCl, 0.15 M NaCl, pH 7.4 (TBS). The column was washed with 5 column volumes of 20 mM Tris-HCl, 0.8 M NaCl, pH 7.4 followed by 5 column volumes of 20 mM Tris-HCl, 0.05 M NaCl, pH 7.4. Bound protein was eluted with Gentle Ag/Ab Elution Buffer (Pierce, Rockford, IL), dialyzed at 4°C overnight against two changes of 500 ml TBS, and then concentrated using a Centriprep-30 ultrafiltration apparatus (Amicon, Inc., Beverly MA). Protein concentration was measured using $\epsilon_{280}^{0.1\%}$ =9.1 (Enzyme Research Labs) and protein purity was determined by SDS-PAGE

analysis (Laemmli, 1970).

Rates of thrombin inhibition by wildtype and variant HCIIs: The second-order rate constants (k_2) for inhibition of thrombin by the various HCII variants were determined under pseudo first-order conditions (Olson et al., 1993) in the absence or presence of 3.3 μM GAGs. In a multiwell plate, 10 μl aliquots of thrombin (final concentration 2 nM) were incubated for varying intervals with 40 nM HCII suspended in 10μl TBS containing 0.6 % polyethylene glycol-8000 (TBSP). All reactions were terminated by the addition of 200 μM chromogenic substrate (N-p-tosyl-Gly-Pro-Arg-p-nitroanilide) in 200 μl TBSP containing 10 mg/ml polybrene. Residual thrombin activity was calculated by measuring absorbance at 405 nm for 5 min using a Molecular Devices plate reader. The pseudo-first-order rate constants (k_1) for thrombin inhibition were determined by fitting the data to the equation $k_1 \bullet \ln([P_o]/[P]_t)$, where $[P]_o$ is initial thrombin activity and $[P]_t$ is thrombin activity at time t (Olson et al., 1993). The second-order rate constant, k_2, was then calculated by dividing k_1 by the HCII concentration.

Heparin-Sepharose affinity chromatography: Heparin-Sepharose affinity chromatography was used to compare the affinities of HCII variants for heparin. 0.2 ml of purified HCII, at a concentration of 10 μg/ml, was batch adsorbed with 0.2 ml of heparin-Sepharose resin for 1 h at 4°C. Adsorbed proteins were eluted with 1 ml of HP buffer (20 mM HEPES, pH 7.4, 0.1 % polyethylene glycol-8000) containing NaCl in concentrations ranging from 30 mM to 1M. Aliquots from the flowthrough and eluates were analyzed by SDS-PAGE followed by immunoblotting with sheep anti-HCII antibody. Protein elution profiles were obtained by laser densitometry scans of immunoblots using the UltroScanTM XL laser densitometer

(Pharmacia LKB Biotechnology). The density of HCII in each fraction was expressed as a percentage of the total HCII density in the complete elution profile.

4.4 Results

Purification of HCII variants: Human HCII is a 480 amino acid, single-chain glycoprotein with a molecular mass of ~ 66 kDa (Tollefsen et al., 1982). The functional domains of HCII and the amino acid sequence of its GAG-binding domain are shown schematically in Figure 1. The GAG-binding domain of HCII has been identified by sequence homology with AT and by analysis of natural (Blinder et al., 1989) and recombinant (Ragg et al., 1990a; Ragg et al., 1990b; Blinder and Tollefsen, 1990) variants of HCII. In this study, human HCII cDNA was cloned from HepG2 cells by reverse transcription-polymerase chain reaction and site-directed mutagenesis was used to generate recombinant HCII molecules. cDNAs encoding the wildtype and variant forms of HCII were then cloned into the eukaryotic expression vector pcDNA3.1(+) and expressed in BHK cells. The apparent molecular masses of the recombinant proteins that we generated (listed in Table 1), as determined by SDS-PAGE and immunoblot analysis, are consistent with their predicted molecular masses (data not shown).

Heparin-sepharose affinity chromatography of GAG-binding domain variants: To test the hypothesis that release of the acidic amino-terminus from intramolecular interactions mimics, at least in part, the catalytic effect of heparin, we systematically replaced Arg^{184}, Lys^{185}, Arg^{189}, and Arg^{192} with Gln, and Arg^{193} with Asn (Fig. 1, Table 1), neutral residues previously shown to reduce the affinity of HCII for heparin-Sepharose when introduced as individual or double point mutations within the GAG-binding domain (Ragg et al., 1990a). The affinities of the HCII variants for heparin were determined by subjecting the proteins to heparin-Sepharose affinity chromatography and monitoring elution at increasing

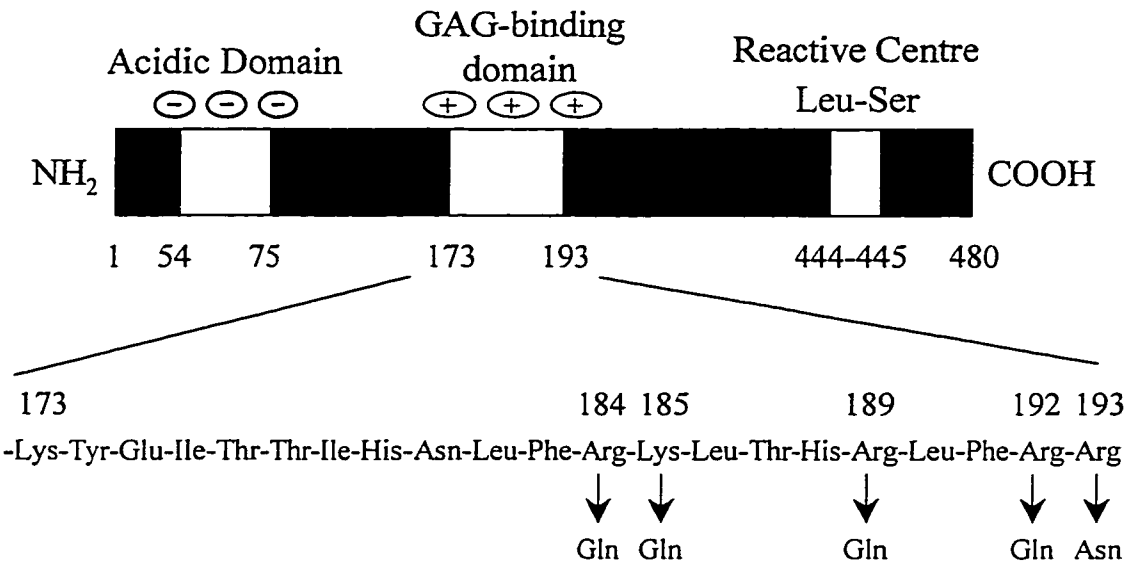

Fig. 1. **Schematic diagram of the functional domains of HCII.** The relative positions of the acidic domain (residues 54-75), the GAG-binding domain (residues 173-193), and the reactive centre (Leu^{444}-Ser^{445}) are shown in the top portion of the diagram. The amino acid residues in the GAG-binding domain are shown in the bottom portion. The arrows identify the 5 basic residues within the GAG-binding domain that have been mutated to Gln or Asn in various combinations.

TABLE 1

Summary of the HCII variants used in this study.

HCII variant	Mutation
pHCII	plasma-derived HCII
wt rHCII	wildtype recombinant HCII
Mut A	R184Q, K185Q
Mut B	R192Q, R193N
Mut C	R184Q, K185Q, R192Q, R193N
Mut D	R184Q, K185Q, R198Q, R192Q, R193N
wt-del74	residues 1-74 deleted
Mut D-del74	R184Q, K185Q, R198Q, R192Q, R193N, and residues 1-74 deleted

concentrations of NaCl. As shown in Figure 2, both pHCII and wt rHCII are retained on the heparin-Sepharose column at NaCl concentrations up to 180 mM. Mutation of positively charged residues at positions 184 and 185 (Mut A) or at positions 192 and 193 (Mut B) produces a modest reduction in affinity for heparin-Sepharose. Mutation of positively charged residues at positions 184, 185, 192, 193 (Mut C) or at positions 184, 185, 189, 192, 193 (Mut D) in the GAG-binding domain further reduces the affinity of HCII for heparin-Sepharose.

Thrombin inhibition by HCII GAG-binding domain variants: The effect of mutations in the GAG-binding domain on the ability of HCII to form complexes with thrombin was first qualitatively assessed using SDS-PAGE and immunoblot analysis to examine the extent of enzyme-inhibitor complex formation. As shown in Figure 3, incubation of pHCII or wt rHCII with thrombin for 1 min at 23 °C results in the formation of an SDS-stable complex with an apparent molecular mass of ~100 kDa in the presence, but not in the absence, of either heparin or dermatan sulfate. In contrast, Mut D forms covalent complexes with thrombin both in the absence and presence of these GAGs, as does Mut C (data not shown). Furthermore, like pHCII and wt rHCII, Mut C and Mut D retain their selectivity for thrombin and do not form SDS-stable complexes with factors Xa, IXa, or XIa (data not shown).

For quantitative comparisons, we determined the second-order rate constants for the inhibition of thrombin by the affinity-purified HCII mutants in the absence or presence of either 3.3 μM heparin or dermatan sulfate under pseudo first-order conditions (Figure 4, Table 2). This concentration of heparin and dermatan sulfate was chosen because in preliminary studies it produced maximal stimulation of thrombin inhibition by pHCII (data

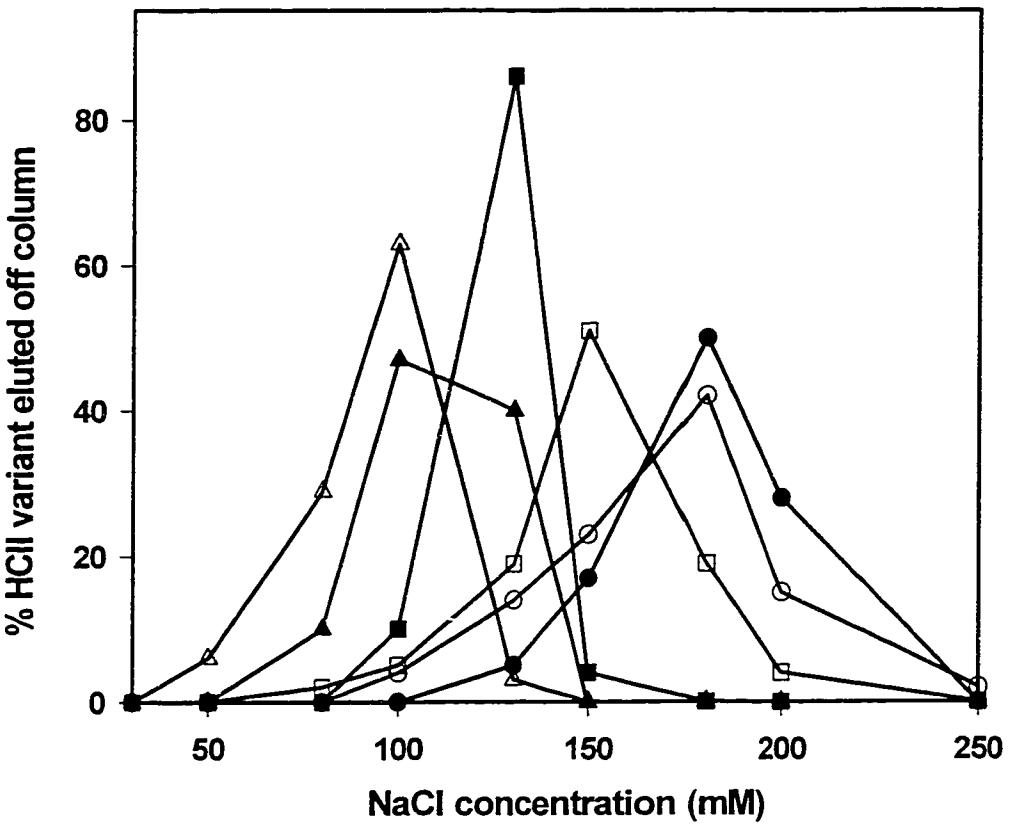

Fig. 2. **Elution of GAG-binding domain mutants of HCII from heparin-Sepharose.** HCII GAG-binding domain mutants were subjected to heparin-Sepharose affinity chromatography and eluted with an NaCl gradient (from 30 mM to 500 mM). Aliquots from the eluates were analyzed by SDS-PAGE and immunoblotting. The protein elution profiles were obtained by laser densitometry where the density of each lane was expressed as a percentage of the combined density of all the lanes in the elution. Symbols: pHCII (●), wt rHCII (○), Mut A (■), Mut B (□), and Mut C (▲), Mut D (△).

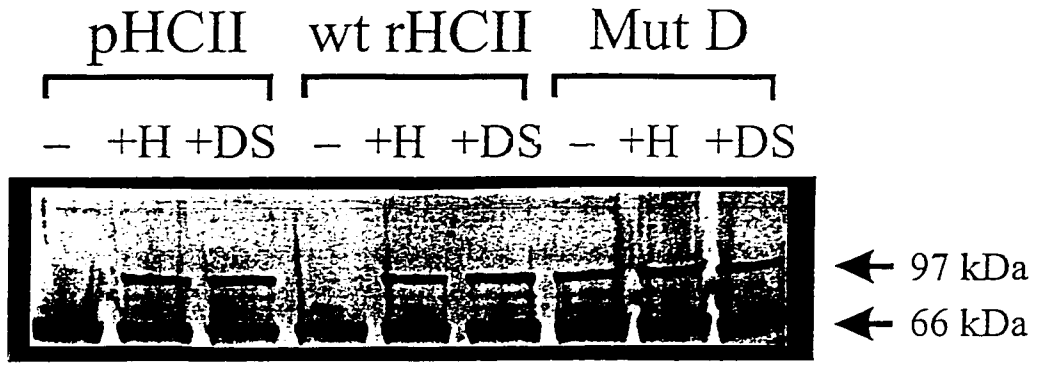

Fig. 3. **Immunoblot analysis of complex formation between thrombin and HCII variants.** Thrombin and HCII, at final concentrations of 10 nM and 100 nM, respectively, were incubated for 1 min at room temperature either in the absence (-) or presence of 3.3 μM heparin (H) or dermatan sulfate (DS). The samples were subjected to electrophoresis in 4-20 % SDS-polyacrylamide gels under reducing conditions, transferred to nitrocellulose, and probed with an antibody against HCII.

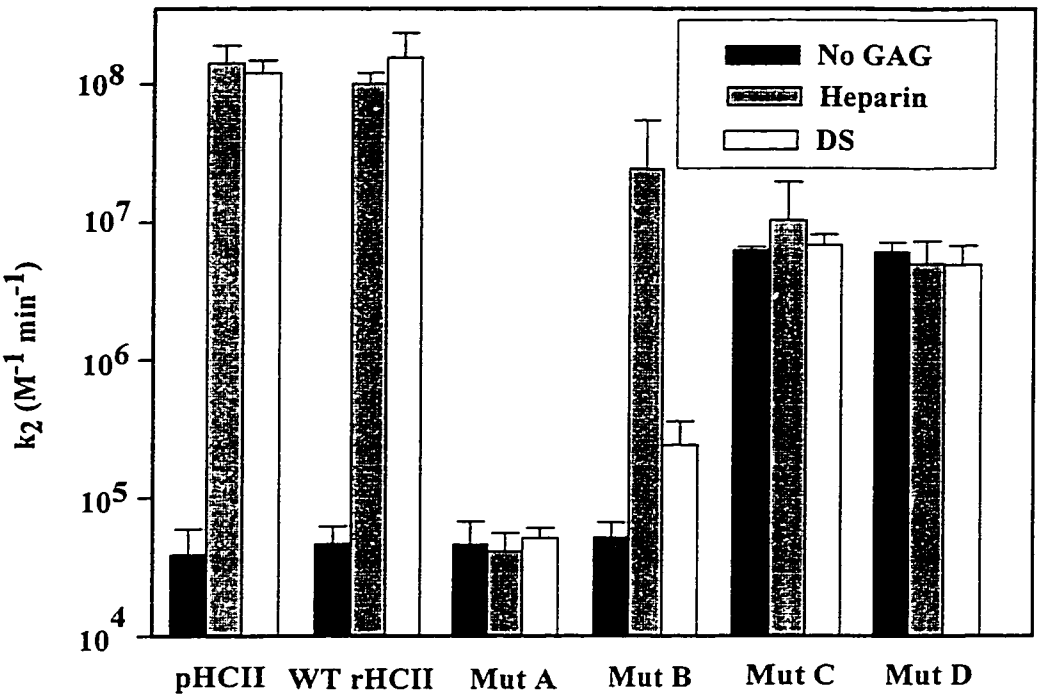

Fig. 4. **Second-order rate constants for the inhibition of thrombin by HCII GAG-binding domain mutants.** The second-order rate constants for the inhibition of 2 nM thrombin by 40 nM HCII were determined under pseudo first-order conditions in the absence or presence of 3.3 μM heparin or dermatan sulfate (DS). The bars represent the mean, while the lines above the bars reflect the standard error of the mean of three determinations.

TABLE 2

Second-order rate constants for thrombin inhibition by HCII variants in the absence or presence of 3.3 μM heparin or dermatan sulfate

	No GAG	Heparin	Dermatan sulfate
	(M^{-1} min^{-1})	(M^{-1} min^{-1})	(M^{-1} min^{-1})
pHCII	$3.9 \pm 1.8 \times 10^4$	$1.4 \pm 0.4 \times 10^8$	$1.2 \pm 0.2 \times 10^8$
WT rHCII	$4.6 \pm 1.3 \times 10^4$	$1.0 \pm 0.2 \times 10^8$	$1.6 \pm 0.6 \times 10^8$
Mut A	$4.6 \pm 1.8 \times 10^4$	$4.0 \pm 1.2 \times 10^4$	$5.1 \pm 0.8 \times 10^4$
Mut B	$5.1 \pm 1.2 \times 10^4$	$2.4 \pm 2.5 \times 10^7$	$2.4 \pm 0.9 \times 10^5$
Mut C	$6.2 \pm 0.3 \times 10^6$	$1.0 \pm 0.8 \times 10^7$	$6.8 \pm 1.1 \times 10^6$
Mut D	$6.0 \pm 1.8 \times 10^6$	$5.0 \pm 1.0 \times 10^6$	$4.9 \pm 1.5 \times 10^6$
wt-del74	$8.8 \pm 6.2 \times 10^4$	$4.7 \pm 1.5 \times 10^5$	$7.6 \pm 1.7 \times 10^4$
Mut D-del74	$5.5 \pm 1.1 \times 10^4$	$4.9 \pm 1.7 \times 10^4$	$4.4 \pm 1.2 \times 10^4$

not shown). The uncatalyzed rates of thrombin inhibition by Mut C and Mut D are 6.2 x 10^6 M^{-1} min^{-1} and 6.0 x 10^6 M^{-1} min^{-1}, respectively, values that are ~2-orders of magnitude higher than the uncatalyzed rates of thrombin inhibition by pHCII and wt rHCII (3.9 x 10^4 M^{-1} min^{-1} and 4.6 x 10^4 M^{-1} min^{-1}, respectively). Neither heparin nor dermatan sulfate significantly increases the basal rates of thrombin inhibition by Mut C or Mut D. By contrast, the rates of thrombin inhibition by pHCII and wt rHCII increase 2000 to 4000-fold in the presence of these GAGs.

Neither heparin nor dermatan sulfate significantly increases the basal rate of thrombin inhibition by Mut A (4.6 x 10^4 M^{-1} min^{-1}). In contrast, the rate of thrombin inhibition by Mut B increases ~ 470-fold (from 5.1 x 10^4 M^{-1} min^{-1} to 2.4 x 10^7 M^{-1} min^{-1}) in the presence of heparin, but only 5-fold in the presence of dermatan sulfate (from 5.1 x 10^4 M^{-1} min^{-1} to 2.4 x 10^5 M^{-1} min^{-1}).

Inhibition of thrombin variants by Mut C and Mut D: To confirm that the increased thrombin inhibitory activity of Mut C and Mut D reflects interaction of their amino-terminal acidic domains with exosite I on thrombin, two sets of experiments were performed. First, we compared the rates at which Mut D inhibits γ-thrombin, a proteolytic derivative of thrombin lacking exosite I, and RA-thrombin, a recombinant thrombin variant containing three point mutations in exosite II that result in a 20-fold decrease in heparin affinity (Ye et al., 1994). As shown in Figure 5, Mut D inhibits thrombin and RA-thrombin at similar rates (6.0 x 10^6 M^{-1} min^{-1} and 7.6 x 10^6 M^{-1} min^{-1}, respectively), but inhibits γ-thrombin at a much slower rate (9.09 x 10^4 M^{-1} min^{-1}). Second, we examined the effect of hirudin-(54-65), a

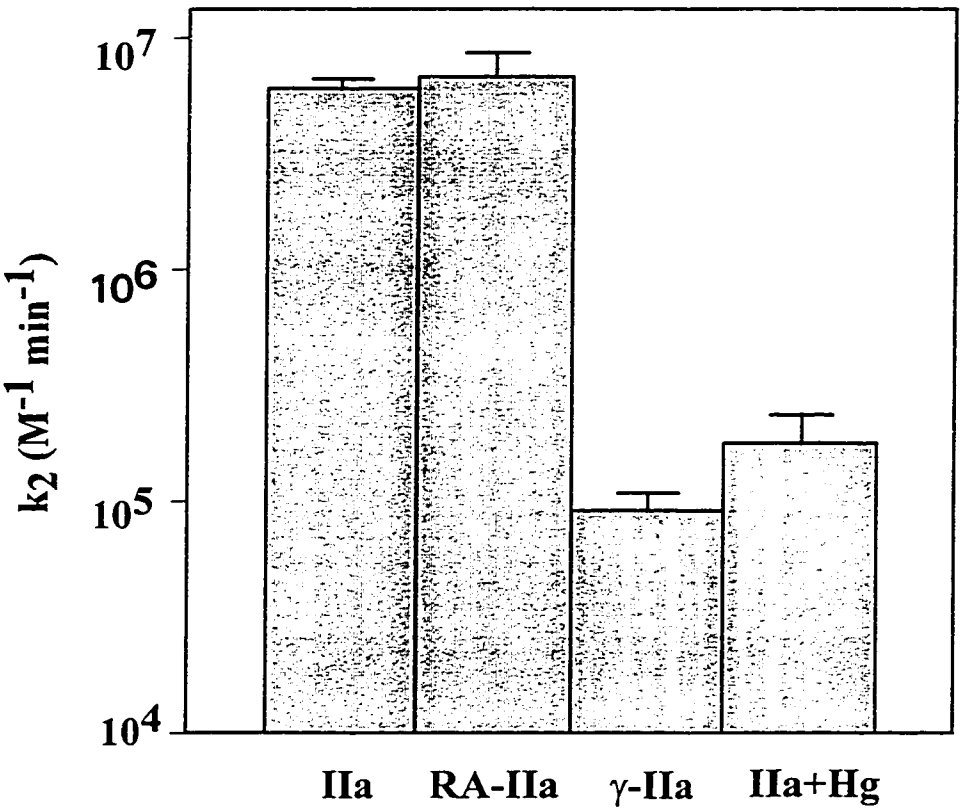

Fig. 5. **Second-order rate constants for the inhibition of thrombin variants by Mut D.** The rate constants for the inhibition of 2 nM α-thrombin (IIa), RA-thrombin (RA-IIa), γ-thrombin (γ-IIa) or IIa in the presence of 20 μM hirudin-(54-64) (IIa+Hg) by 40 nM Mut D were determined under pseudo first-order conditions. The bars represent the mean, while the lines above the bars reflect the standard error of the mean of three determinations.

synthetic analogue of the carboxyl terminus of hirudin that interacts with exosite I of thrombin (Rydel et al., 1991), on the rate of thrombin inhibition by the HCII variant. Mut D inhibits thrombin at a decreased rate (1.78×10^5 M^{-1} min^{-1}) in the presence of 20 μM hirudin-(54-64). Likewise, Mut C also inhibits γ-thrombin at a slower rate than thrombin (1.10×10^5 M^{-1} min^{-1} and 6.2×10^6 M^{-1} min^{-1}, respectively) and the rate at which it inhibits thrombin is reduced by ~ 40-fold in the presence of 20 μM hirudin-(54-65). These results highlight the importance of thrombin exosite I in mediating the increased thrombin inhibitory activity of Mut C and Mut D.

Functional analysis of HCII amino-terminal domain deletion variants: To demonstrate that the increased basal rate of thrombin inhibition by Mut D reflects release of its amino-terminal acidic domain from intramolecular interactions, we examined the ability of Mut D-del74, a variant of Mut D that lacks the amino-terminal acidic domain, to inhibit thrombin in the absence and presence of GAGs. As shown in Figure 6 and in Table 2, the rate of thrombin inhibition by Mut D-del74 is 109-fold lower than that for Mut D (5.5×10^4 M^{-1} min^{-1} and 6.0×10^6 M^{-1} min^{-1}, respectively). Like Mut D, neither heparin nor dermatan sulfate increases the uncatalyzed rate of thrombin inhibition by Mut D-del74. Furthermore, whereas heparin produces an ~5-fold increase in the rate of thrombin inhibition by wt-del74 rHCII (from 8.8×10^4 M^{-1} min^{-1} to 4.7×10^5 M^{-1} min^{-1}), dermatan sulfate has no effect on this reaction (Table 2, Fig. 6), even when present at a 10-fold higher concentration (data not shown).

To determine the importance of GAG-mediated bridging of HCII to thrombin, we

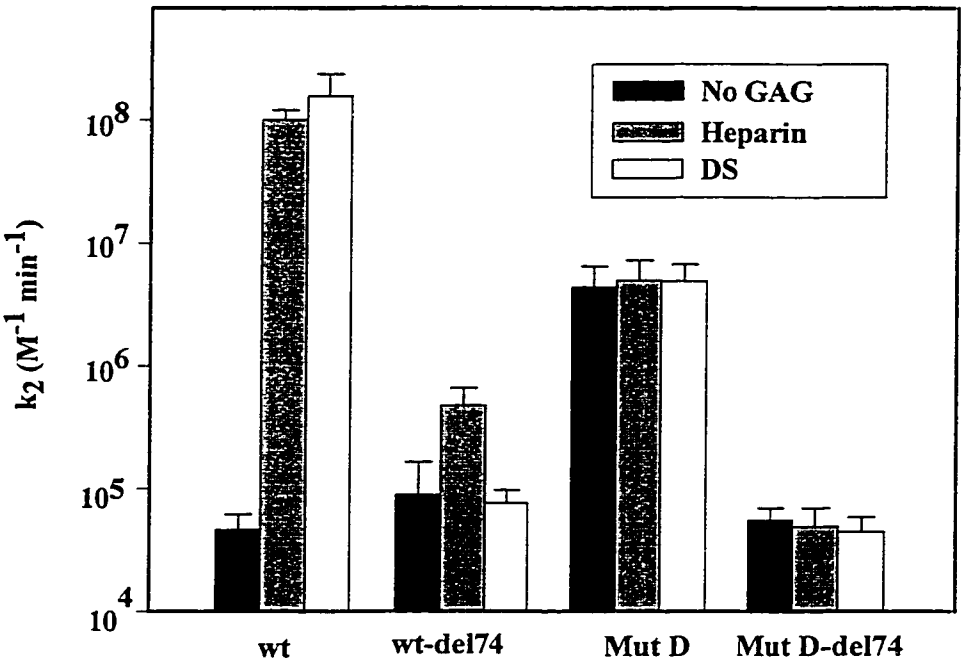

Fig. 6. **Second-order rate constants for the inhibition of thrombin by HCII amino-terminal deletion mutants.** The second-order rate constants for the inhibition of 2 nM thrombin by 40 nM HCII were determined under pseudo first-order conditions in the absence or presence of 3.3 μM heparin or dermatan sulfate (DS). The bars represent the mean, and the lines above the bars reflect the standard error of the mean of three determinations.

TABLE 3

Effect of heparin fractions of varying molecular weight on second-order rate constants for thrombin inhibition by HCII variants

	4 kDa	6 kDa	9 kDa	18 kDa
	(M^{-1} min^{-1})	(M^{-1} min^{-1})	(M^{-1} min^{-1})	(M^{-1} min^{-1})
pHCII	$2.4 \pm 0.6 \times 10^7$	$3.5 \pm 1.0 \times 10^7$	$1.2 \pm 0.1 \times 10^8$	$1.4 \pm 0.4 \times 10^8$
wt rHCII	$2.0 \pm 0.8 \times 10^7$	$1.7 \pm 0.2 \times 10^7$	$1.2 \pm 0.2 \times 10^8$	$1.0 \pm 0.2 \times 10^8$
wt-del74	$4.5 \pm 0.5 \times 10^4$	$5.6 \pm 0.8 \times 10^4$	$6.9 \pm 1.7 \times 10^5$	$4.7 \pm 1.5 \times 10^5$

compared the rates of thrombin inhibition in the presence of longer heparin or dermatan sulfate chains with those in the presence of smaller chains. As shown in Table 3, heparin fractions of 9 and 18 kDa (which correspond to 30 and 60 saccharide units, respectively) increase the rate of thrombin inhibition by pHCII, wt rHCII, and wt-del74 to a greater extent than heparin fractions of 6 kDa or less (i.e., 20 saccharides units or fewer). In contrast, both dermatan sulfate, which has a mean molecular weight of 20 kDa, and desmin, a 5.6 kDa low molecular weight fraction of dermatan sulfate (Harenberg et al., 1996), increase the rate of thrombin inhibition by pHCII to the same extent (from 3.9×10^4 M^{-1} min^{-1} to 1.2×10^8 M^{-1} min^{-1} and 1.0×10^8 M^{-1} min^{-1}, respectively). Furthermore, neither dermatan sulfate nor desmin increases the rate of thrombin inhibition by wt-del74 rHCII. Thus, while the minimum molecular weight of heparin required for catalysis via the template mechanism is between 6 and 9 kDa, bridging does not appear to play a role in dermatan sulfate-mediated catalysis of thrombin inhibition by HCII.

Since the amino-terminal acidic domain of HCII is believed to bind intramolecularly to the basic GAG-binding domain, deletion of the amino-terminal acidic domain should unmask the GAG-binding domain, thereby increasing the affinity of HCII for heparin. To explore this possibility, the affinities of the amino-terminal deletion mutants for heparin were determined by subjecting the proteins to heparin-Sepharose affinity chromatography (Fig. 7). wt rHCII-del74 was retained on the column at higher NaCl concentrations than wt rHCII (700 mM and 180 mM NaCl, respectively). In contrast, Mut D-del74 was retained on the

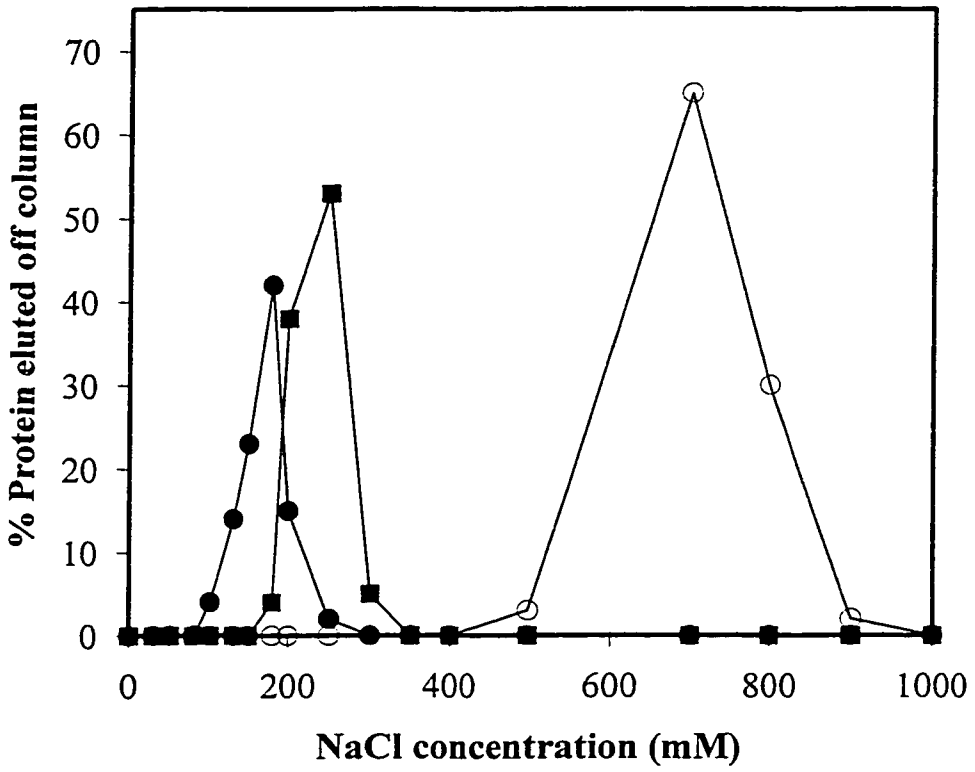

Fig. 7. **Elution of amino-terminal deletion mutants from heparin-Sepharose.** HCII amino-terminal deletion mutants were subjected to heparin-Sepharose affinity chromatography and eluted with an NaCl gradient (from 30 mM to 1 M). Aliquots from the eluates were analyzed by SDS-PAGE and immunoblotting. The protein elution profiles were obtained by laser densitometry where the density of each lane was expressed as a percentage of the combined density of all the lanes in the elution. Symbols: wt rHCII (●), wt-del74 (○), Mut D-del74 (■).

column at NaCl concentrations as high as 250 mM.

4.5 Discussion

The current model of HCII structure suggests that in the absence of GAGs the amino-terminal acidic domain is bound to the electropositive GAG-binding domain, thereby limiting its interaction with thrombin. The major stimulatory effect of GAGs is believed to involve conformational changes that prime the amino-terminal acidic domain of HCII for interactions with thrombin (Ragg et al., 1990a; van Deerlin et al., 1991; Sheehan et al., 1994). In this study, we explored the extent to which mutations in the GAG-binding domain of HCII obviates the GAG dependence for thrombin inhibition. Our study extends qualitative studies by Ragg et al. (Ragg et al., 1990a) which demonstrated that GAG-binding domain mutants R184Q, K185Q and R192Q, R193N exhibited a slightly enhanced level of thrombin inhibitory activity in the absence of GAGs as analyzed by covalent complex formation on SDS-polyacrylamide gels.

Our kinetic studies support the current allosteric model because conversion of four (Mut C) or five (Mut D) basic residues within the GAG-binding domain to neutral ones increases the basal rate of thrombin inhibition ~130-fold over that of wt rHCII or pHCII (Fig. 6; Table 2). In contrast, the uncatalyzed rate of thrombin inhibition by Mut A (R184Q, K185Q) and Mut B (R192Q, R193N) are similar to those of pHCII and wt rHCII (Fig. 6, Table 2). Our results with R184Q, K185Q (Mut A) and R192Q, R193N (Mut B) differ from those of Ragg et al. (Ragg et al., 1990a). This may reflect differences in the expression systems because we expressed the proteins in BHK cells, whereas Ragg and colleagues used COS cells. Alternatively, endogenous GAGs may account for the increased activities

reported by Ragg et al. because their recombinant HCII variants were obtained directly from culture supernatants. In contrast, our mutants were isolated by affinity chromatography using a high NaCl concentration. When this step was omitted, increased thrombin inhibitory activity was detected in some instances.

Three lines of evidence suggest that the increased thrombin inhibitory activity of Mut C and Mut D reflects the release of the amino-terminal acidic domain from intramolecular interactions. First, γ-thrombin, a trypsinized derivative of thrombin lacking exosite I, is inhibited by Mut D at a rate that is approximately 2-orders of magnitude slower than that observed for thrombin and RA-thrombin, a thrombin variant with three point mutations in exosite II that lower its affinity for heparin 20-fold (Ye et al., 1994) (Fig. 5). Second, the rate of thrombin inhibition by Mut D is decreased in the presence of hirudin-(54-65), an analogue of the carboxy-terminus of hirudin that competes with the amino-terminal acidic domain of Mut D for exosite I of thrombin (Fig. 5). Third, as illustrated in Figure 6 and Table 2, deletion of the amino-terminal acidic domain of Mut D decreases the basal rate of thrombin inhibition 120-fold (from 6.0×10^6 M^{-1} min^{-1} to 5.5×10^4 M^{-1} min^{-1}).

It was important to exclude the possibility that the increase in the uncatalyzed rate of thrombin inhibition by Mut C and Mut D reflects conformational changes at the reactive centre that promote proteolytic attack by thrombin. This is relevant because Derechin et al. (1990) have shown that substitution of Leu^{444} with Arg in the P1 position of HCII produces a 100-fold increase in the uncatalyzed rate of thrombin inhibition and broadens the repertoire of coagulation enzymes inhibited by HCII to include factor Xa, in addition to thrombin (Bray

et al., 1989). The observation that none of the mutations that we introduced into the GAG-binding domain of HCII affected the target specificity of the proteins for thrombin makes it unlikely that the increased thrombin inhibitory activities of Mut C and Mut D reflect conformational changes at the reactive centre of HCII (data not shown).

The rates of thrombin inhibition by Mut C and Mut D are over an order of magnitude lower than the heparin- and dermatan sulfate-catalyzed rates of native HCII. Based on our data, we propose two different models to account for the maximal stimulatory effect achieved by heparin and by dermatan sulfate. Heparin accelerates thrombin inhibition by HCII by a combination of allosteric changes induced in the amino-terminus of HCII as well as by serving as a template. The template effect likely explains why longer heparin chains, but not shorter ones, produce a 5-fold increase in the rate of thrombin inhibition by wt-del74 rHCII (Table 2), an HCII deletion variant that lacks the amino-terminal acidic domain. Our observation that longer heparin chains produce a ~5-fold increase in the rate of thrombin inhibition by this mutant is in agreement with the results of other investigators who obtained similar results when the same mutant was expressed in *E. coli* (van Deerlin and Tollefsen, 1991). The minimal heparin chain length required for catalysis via the template mechanism is between 20 and 30 saccharide units (which corresponds to a molecular weight of between 6 and 9 kDa) because only these longer heparin chains accelerate the rate of thrombin inactivation by wt-del74 rHCII (Table 3). These findings are consistent with the results of other investigators who demonstrated that only heparin chains comprised of 24 or more saccharide units produce maximal catalysis of HCII (Sie 1988; Bray et al., 1989). The

mutations introduced into the GAG-binding domain of Mut C and Mut D reduce their affinity for heparin so that templating cannot occur. Consequently, we postulate that the 130-fold increase in the basal rate at which Mut C and Mut D inactivate thrombin reflects allosteric changes in the amino terminus of HCII similar to those induced by shorter heparin chains. In support of this concept, the uncatalyzed rate of thrombin inhibition by Mut D is only 2.8-fold slower than the rate at which wt rHCII inactivates thrombin in the presence of a 6 kDa heparin fraction (6.0×10^6 M^{-1} min^{-1} and 1.7×10^7 M^{-1} min^{-1}, respectively), a heparin chain that is too short to bridge HCII to thrombin.

In contrast to heparin, dermatan sulfate appears to accelerate thrombin inhibition by HCII exclusively through induction of allosteric changes in the amino-terminal acidic domain because (a) desmin, a 5.6 kDa low molecular weight fraction of dermatan sulfate, increases the rate of thrombin inhibition by pHCII to the same extent as dermatan sulfate, and (b) dermatan sulfate, a GAG comprised of more than 30 saccharide units, does not increase the rate of thrombin inhibition by wt-del74 rHCII (Fig. 6, Table 2). Whereas dermatan sulfate and desmin increase the rate of thrombin inhibition by pHCII $\geq$ 1000-fold, Mut C and Mut D inhibit thrombin at a rate only 130-fold greater than the uncatalyzed rate of thrombin inhibition by pHCII. These observations suggest that the allosteric changes induced by dermatan sulfate and desmin binding to HCII are more extensive than those produced by heparin or by the mutations that we introduced into the GAG-binding domains of Mut C and Mut D. This concept is supported by our findings that the heparin and dermatan sulfate binding sites on HCII are not identical. We have shown that mutation of Arg^{192} and Arg^{193}

to Gln and Asn, respectively (Mut B), decreases the ability of dermatan sulfate to catalyze thrombin inhibition over two orders of magnitude, but has little effect on heparin's ability to accelerate thrombin inhibition. In contrast, substitution of Arg^{184} and Lys^{185} with Gln residues (Mut A) abolishes the ability of both GAGs to enhance thrombin inhibition.

Our kinetic results can be explained by studies examining heparin and dermatan sulfate binding to natural (Blinder et al., 1989) and recombinant (Ragg et al., 1990a; Ragg et al., 1990b; Blinder and Tollefsen, 1990) HCII variants. These studies demonstrated that Lys^{173}, Arg^{184}, and Lys^{185} are important determinants of heparin binding, whereas Arg^{184}, Lys^{185}, Arg^{189}, Arg^{192}, and Arg^{193} are important for dermatan sulfate binding. The overlap in these binding domains at Arg^{184} and Lys^{185} explains why neither heparin nor dermatan sulfate catalyzes Mut A. In addition to Arg^{184} and Lys^{185}, the dermatan sulfate binding site on HCII also encompasses Arg residues at positions 189, 192, and 193, thereby explaining why Mut B is stimulated by heparin, but not by dermatan sulfate. Differences in the allosteric changes in the amino-terminal acidic domain induced by dermatan sulfate or desmin relative to heparin may reflect the more extensive contacts that the former GAGs make with HCII. The fact that neither dermatan sulfate nor desmin accelerates thrombin inhibition by wt-del74 rHCII makes it unlikely that these GAGs induce conformational changes at the reactive centre of HCII that render the Leu^{444}-Ser^{445} peptide bond a more favorable site for thrombin cleavage.

Although weakening of salt bridges between the amino-terminal acidic domain and the GAG-binding domain of HCII promotes the interaction between the amino-terminal

acidic domain and exosite I of thrombin, the conformational constraint of the amino terminus in native HCII may involve residues in addition to those found in the GAG-binding domain. Ciaccia et al. (1997) have recently shown that mutagenesis of Arg^{200} of HCII to Glu increases the basal thrombin inhibitory rate ~5-fold, even though this residue is not part of the GAG-binding domain. The authors propose that Arg^{200} either helps to maintain ionic interactions between the acidic amino-terminus and the GAG-binding domain, or directly binds the acidic domain. Even within the GAG-binding domain, residues other than the ones mutated in this study may mediate GAG binding to HCII. As shown in Figure 6, removal of the amino terminal acidic domain of HCII (wt-del74) increases the affinity of HCII for heparin-Sepharose. This finding is consistent with the results of van Deerlin et al. (van Deerlin and Tollefsen, 1991), and suggests that the heparin-binding domain is unmasked upon deletion of the amino terminal 74 residues. However, Mut D-del74 is retained on the heparin-Sepharose column at slightly higher concentrations of NaCl than is wt rHCII (230 versus 180 mM NaCl, Fig. 7), raising the possibility that additional residues may be involved in heparin binding. One potential residue is Lys^{173} which has been shown to bind heparin, but not dermatan sulfate (Whinna et al., 1991).

In summary, our studies demonstrate that the GAG-induced conformational changes that accelerate thrombin inhibition by HCII can be mimicked, at least in part, by substitution of basic residues in the GAG-binding domain of HCII with neutral ones. These findings support the concept that the major stimulatory effect of GAGs reflect allosteric changes in the amino-terminal acidic domain of HCII that facilitate its interaction with exosite I of

thrombin; an interaction that influences both target protease specificity and the rate of protease inhibition. In addition, our studies suggest that heparin utilizes both allosteric and template mechanisms to accelerate thrombin inhibition by HCII, whereas acceleration by dermatan sulfate is mediated exclusively through an allosteric mechanism.

CHAPTER 5- DERMATAN SULFATE, UNLIKE HEPARIN DOES NOT PROMOTE TERNARY THROMBIN-FIBRIN-GLYCOSAMINOGLYCAN COMPLEX FORMATION: MOLECULAR BASIS FOR HEPARIN COFACTOR II-MEDIATED INACTIVATION OF FIBRIN-BOUND THROMBIN

Preface

This manuscript is in preparation. It will be submitted to the Journal of Clinical Investigation. The authors are: Patricia C. Y. Liaw, Debra L. Becker, Alan R. Stafford, James C. Fredenburgh, and Jeffrey I. Weitz. The corresponding author is Dr. Weitz. Most of the experiments were performed by me. Debra Becker determined the rates of thrombin inhibition of thrombin by HCII +/- GAGs, +/- Fm (Fig. 1). Alan Stafford and I determined the dissociation constants (Table 1).

This work was supported in part by grants-in-aid from the Medical Research Council of Canada and the Heart and Stroke Foundation of Canada. P. C. Y. Liaw is the recipient of a Research Traineeship from the Heart and Stroke Foundation of Canada. Dr. J. I. Weitz is the recipient of a Career Investigator Award from the Heart and Stroke Foundation of Canada.

5.1 Introduction

Heparin, a sulfated polysaccharide, acts as an anticoagulant by accelerating the inhibition of coagulation enzymes, especially thrombin and factor Xa by antithrombin (AT) (Rosenberg, 1987). Although heparin is a cornerstone of treatment for acute coronary syndromes, it is of only limited value as an adjunct to thrombolytic therapy (Gold et al., 1986; Galvani et al., 1994; Merlini et al., 1995) or to percutaneous coronary revascularization procedures (Popma et al., 1995). The limitations of heparin in these settings have been attributed to the inability of the AT/heparin complex to inactivate clotting enzymes bound to components of the thrombus, particularly thrombin bound to fibrin (Hogg and Jackson, 1989; Weitz et al., 1990).

The resistance of fibrin-bound thrombin to inactivation by the AT/heparin complex reflects its incorporation into a ternary thrombin-fibrin-heparin complex (Hogg and Jackson, 1990a, 1990b; Hogg et al., 1996). To form this complex, it is hypothesized that heparin interacts with exosite II on thrombin (Church et al., 1989; Gan et al., 1994; Sheehan and Sadler, 1994) as well as the D domain of fibrin (Odrljin et al., 1996), thereby bridging thrombin to fibrin (Hogg and Jackson, 1990a). By performing this bridging function, heparin enhances the interaction between fibrin and exosite I on thrombin (Hogg and Jackson, 1990a; Hogg et al., 1996). The protection of thrombin within the ternary thrombin-fibrin-heparin complex from inactivation by AT requires ligation of both thrombin's exosites (Becker et al., 1997a) and may reflect spatial constraints and/or allosteric changes in the active site of thrombin induced by ternary complex formation. (Becker et al., 1997b).

HCII is a plasma serpin that selectively inhibits thrombin in a reaction that is accelerated >1000-fold by dermatan sulfate (DS) or heparin (Tollefsen, 1983). DS activates HCII by binding to an electropositive region on HCII, thereby releasing the amino-terminal acidic domain of HCII from intramolecular interactions with its glycosaminoglycan (GAG)-binding domain (Ragg et al., 1990a, van Deerlin et al., 1991; Sheehan et al., 1994; Liaw et al., 1998). The conformational change evoked by DS permits association of the amino-terminal acidic domain of HCII with exosite I of thrombin, an interaction that promotes enzyme-inhibitor complex formation. Although the bulk of the stimulatory effect of heparin is mediated by allosteric changes in the amino-terminal domain of HCII, similar to that observed with DS, heparin may also provide a modest stimulatory effect by simultaneously binding to the GAG-binding domain of HCII and exosite II on thrombin (van Deerlin et al., 1991; Sheehan et al., 1994; Liaw et al., 1998).

Studies in plasma systems suggest that thrombin bound to fibrin monomer (Fm)or to plasma clots is more susceptible to inactivation by the HCII/DS complex than by the AT/heparin complex (Okwusidi et al., 1990; Bendayan et al., 1994). These observations may explain why DS is more effective than heparin at inhibiting fibrin accretion onto preformed venous thrombi in rabbits when the two agents were given at doses that produced the same inhibition of thrombin *in vitro* (Boneu et al., 1985; van Ryn-McKenna et al., 1989; Carrie et al., 1992). Because heparin enhances fibrin/thrombin interactions by bridging thrombin to fibrin, we hypothesized that the susceptibility of fibrin-bound thrombin to inactivation by the HCII/DS complex but not by the AT/heparin complex reflects the fact that, unlike

heparin, DS does not promote productive ternary complex formation. To explore this possibility, we compared (a) the effect of Fm on the rates of thrombin inhibition by the HCII/DS complex and by the HCII/heparin complex, (b) the abilities of DS and heparin to promote the binding of ^{125}I-labelled active site-blocked thrombin to fibrin clots, and (c) the effects of ternary thrombin-fibrin-GAG complex formation on the thrombin-mediated rates of hydrolysis of chromogenic substrates. We also determined the dissociation constants for the relevant binary interactions involved in ternary thrombin-fibrin-DS complex formation. Our results indicate that, unlike heparin, DS does not promote the formation of a productive ternary complex formation, thereby explaining why fibrin-bound thrombin is susceptible to inactivation by the HCII/DS complex but not by the AT/heparin or HCII/heparin complexes.

5.2 Experimental Procedures

Materials: Human HCII (pHCII), isolated from plasma by affinity chromatography, was from Affinity Biologicals Inc. (Hamilton, ON). Human α-thrombin and fibrinogen were from Enzyme Research Laboratories (South Bend, IN). Dermatan sulfate (DS) was from Mediolanum Farmaceutici (Milan, Italy). Heparin, the thrombin-directed substrate, N-p-tosyl-Gly-Pro-Arg-p-nitroanilide (Chz-Th), and Gly-Pro-Arg-Pro-amide (GPRP-NH_2) were from Sigma Chemical Co. (St. Louis, MO). Fluorescein-5-isothiocyanate (FITC) was from Molecular Probes Inc. (Eugene, OR). D-Phenyl-alanyl-L-propyl-L-arginine chloromethyl ketone (FPR) was from Novabiochem Intl. (San Diego, CA). FITC-FPR was from Hematologic Technologies, Inc. (Essex Junction, VT). *N*-Methylsulfonyl-D-Phe-Gly-Arg *p*-nitroanilide (Chz-tPA) and N-Methoxycarbonyl-Nle-Gly-Arg-4-nitroanilide acetate (Chz-Xa) were from Boehringer Mannheim Canada (Laval, PQ). Benzyloxycarbonyl-Ile-Glu-(OR)-Gly-Arg *p*-nitroanilide (S2222), H-D-Phe-Pip-Arg *p*-nitroanilide (S2238), and L-pyroglutamyl-L-Pro-L-Arg *p*-nitroanilide hydrochloride (S2366) were from Chromogenix (Helena Laboratories, Missisauga, ON). Hexadimethrine bromide (polybrene) was obtained from Aldrich Chemical Company (Milwaukee, WI). CNBr-activated Sepharose 4B was from Pharmacia Biotech (Dorval, Quebec). The recombinant thrombin mutant with Arg^{93}, Arg^{97}, Arg^{101} substituted with Ala (RA-thrombin) (Ye et al., 1994) was generously provided by Dr. Charles Esmon (Howard Hughes Medical Institute, Oklahoma city, OK). Mut D, a recombinant HCII variant containing five amino acid substitutions in its GAG-binding domain (R184Q, K185Q, R198Q, R192Q, R193N), was expressed in BHK cells and purified

by affinity chromatography as previously described (Liaw et al., 1998). The uncatalyzed rate of thrombin inhibition by Mut D (6.0×10^6 M^{-1} min^{-1}) is ~130-fold higher than that of plasma-derived HCII. Because the amino acids mutated in Mut D are essential for GAG-binding (Ragg et al., 1990a; Ragg et al., 1990b; Blinder et al., 1989; Blinder and Tollefsen, 1990), neither heparin nor DS enhances its activity.

Preparation of soluble fibrin monomer (Fm) and fibrin monomer(Fm)-Sepharose: Fm was prepared by clotting fibronectin-free human fibrinogen with thrombin as described by Becker et al. (1998). Fibrin monomer polymerization was blocked by addition of GPRP-NH_2. Fm-Sepharose was prepared by coupling fibronectin-free fibrinogen with CNBr-activated Sepharose as described by Becker et al. (1998).

Rates of thrombin inhibition by HCII and Mut D in the absence or presence of Fm, or GAGs, or both: The second-order rate constants (k_2) for inhibition of thrombin by HCII variants were determined under pseudo first-order conditions in the absence or presence of 3.3 μM heparin or DS, or 4 μM Fm, or both. Thrombin (10 nM) was incubated for 5 minutes at room temperature in TSP (20 mM Tris-HCl, 150 mM NaCl, 0.6% PEG-8000, pH 7.4) containing various concentrations of heparin or DS (0 to 100 μM) and Fm (0 to 12 μM), 10 mM GRRP-NH_2, and 15 mM Tris-HCl, pH 7.5. Reaction mixtures (10 μl) were aliquotted to 96-well round bottom microtitre plates and an equal volume of either HCII or MutD (in a concentration at least 10-fold higher than that of thrombin) was added to each well at various time intervals (2 seconds to 5 minutes). All reactions were terminated by the addition of 200 μM chromogenic substrate (N-p-tosyl-Gly-Pro-Arg-p-nitroanilide) in 200 μl

TBSP containing 10 mg/ml polybrene. Residual thrombin activity was calculated by measuring absorbance at 405 nm for 5 min using a Molecular Devices plate reader. The pseudo-first-order rate constants (k_1) for thrombin inhibition were determined by fitting the data to the equation $k_1 \bullet \ln([P_o]/[P]_t)$, where $[P]_o$ is initial thrombin activity and $[P]_t$ is thrombin activity at time t. The second-order rate constant, k_2, was then determined by dividing k_1 by the HCII concentration (Olson, 1993).

Effect of heparin and DS on the binding of ^{125}I-FPR-thrombin to fibrin clots: Active site-blocked thrombin (FPR-thrombin) was prepared as described by Fredenburgh et al. (1997). Iodination of FPR-thrombin was performed using Iodo-beads iodination reagent as described by the supplier (Pierce Co., Rockford, IL). The binding of ^{125}I-FPR-thrombin to fibrin clots in the absence or presence of either heparin or DS was studied in 20 mM Tris-HCl, pH. 7.4, 150 mM NaCl, containing 0.6% PEG-8000 and 0.01% Tween-20 at room temperature. Fibrinogen (7.5 μM) was incubated with increasing concentrations of either heparin or DS (0 to 2.5 μM) in a total volume of 40 μl in a series of 400 μl nipple tubes. Clotting was initiated by addition of 10 μl of stock A containing 10 mM $CaCl_2$, 500 nM ^{125}I-FPR-thrombin, and 10 nM thrombin. After 45 min incubation at room temperature, fibrin was pelleted by centrifugation for 5 min at 15, 000 x g, and aliquots of supernatant were removed for gamma counting. The fraction of thrombin bound to fibrin was calculated as the change in ^{125}I-FPR-thrombin binding compared with controls lacking GAGs.

Effect of ternary thrombin-fibrin-GAG complexes on thrombin-mediated hydrolysis of chromogenic substrates: The chromogenic activity of 10 nM thrombin in TBS was

determined with 500 μM of various thrombin substrates in the presence of 4 μM Fm and either 250 nM heparin, or 250 nM DS. The rates of thrombin-mediated hydrolysis were monitored at 405 nm in a plate reader and the percentage changes from control rates were determined.

Determination of the affinities of FITC-heparin, FITC-DS, and FITC-FPR-thrombin for fibrin clots: Heparin and DS were labelled with FITC as described by Nagasawa and Uchiyama (1978). FITC-labelled active site-blocked thrombin (FITC-FPR-thrombin) was prepared as described by Fredenburgh et al. (1997). The affinity of FITC-labelled ligands for fibrin was determined by measuring unbound ligand in supernatants of clots prepared by clotting varying concentrations of fibrinogen clotted with 1 nM thrombin. Briefly, 200 μl of 2 mM $CaCl_2$, 100 nM FITC-labelled ligand, various concentrations of fibrinogen (30 to 3000 nM), and 1 nM thrombin were mixed in a series of eppendorf tubes. After 1 hour incubation at 23 °C, fibrin was pelleted by centrifugation for 5 minutes at 10,000 x g. 100 μl of supernatant was removed and added to 300 μl of TS buffer. The fluorescence intensity of the samples was measured using a Perkin-Elmer LS50B luminescence spectrometer with excitation and emission wavelengths set to 492 and 522 nm, respectively, and excitation and emission slit widths both set to 15 nm. The K_d values were calculated by plotting I/Io versus fibrinogen concentration, where I and Io represent the fluorescence intensities before and after adding a fixed quantity of fibrinogen, respectively. The parameters K_d and α were calculated by nonlinear regression ("Tablecurve", Jandel Scientific, San Rafael, CA) using the equation $I/Io=(1+(K_d+[Y])/[X])-((1+((K_d+[Y])/[X]))^2-(4\times[Y]/([X]))^{0.5})\times(\alpha/2)+1$ where

α is the maximum fluorescence change, Y is FITC-heparin, FITC-DS, or FITC-FPR-thrombin, X is fibrinogen, and assuming a stoichiometry of 1 (Fredenburgh et al., 1997).

Determination of the affinities of thrombin for heparin and DS: The affinities of heparin or DS for thrombin were determined by monitoring GAG-dependent intrinsic protein fluorescence change of thrombin. 2 ml of 100 nM thrombin in TBS (20 mM Tris-HCl, pF 7.4, 150 mM NaCl) was added to a semi-micro quartz cuvette. Using a Perkin-Elmer LS50B luminescence spectrometer with excitation and emission wavelengths set to 280 and 340 nm, respectively, and excitation and emission slit widths set to 6 nm, readings were taken of thrombin alone (Io). Known quantities of either heparin or DS were then added to the cuvette and, after mixing, the change in fluorescence was monitored (I). The Kd values were calculated by plotting I/Io versus GAG concentration and the data were fit by nonlinear regression to the equation given above.

5.3 Results

Comparison of the effect of Fm on the Heparin- and DS-catalyzed rates of thrombin inhibition by HCII: To quantify the effects of Fm on the rates of thrombin inhibition by serpin/GAG complexes, the second-order rate constants for the inhibition of Fm-bound thrombin by either heparin/HCII or DS/HCII were measured. As shown in Fig. 1 (panel A), at concentrations up to 4 μM, Fm causes only a modest decrease in the DS-catalyzed rate of thrombin inhibition by DS. In contrast, both 0.25 and 4 μM Fm cause a dose-dependent decrease in the heparin-catalyzed rate of thrombin inhibition by HCII (panel B). At 1 μM heparin and 4 μM Fm, a maximal decrease of 240-fold in the rate is observed, a value consistent with that reported by Becker et al. (1997). These results indicate that Fm-bound thrombin is not protected from inhibition by DS-catalyzed HCII.

Effect of Heparin and DS on the binding of ^{125}I-FPR-thrombin to fibrin: Hogg and Jackson (1990a) have previously shown that heparin enhances the binding of thrombin to fibrin, an effect that occurs regardless of whether the heparin has high or low affinity for AT. Only heparin chains of 11,200 Da or more are able to promote thrombin binding to fibrin (Hogg and Jackson, 1990a). In this study, we compared the ability of DS and an 18,000 Da heparin fraction to promote thrombin binding to fibrin. As shown in Figure 2, DS has no effect on ^{125}I-FPR-thrombin binding to fibrin clots. In contrast, at concentrations up to 250 nM, heparin enhances ^{125}I-FPR-thrombin binding to fibrin clots in a dose-dependent manner. At heparin concentrations above 250 nM, ^{125}I-FPR-thrombin binding to clots decreases, likely reflecting the formation of nonproductive heparin/fibrin and heparin/thrombin complexes.

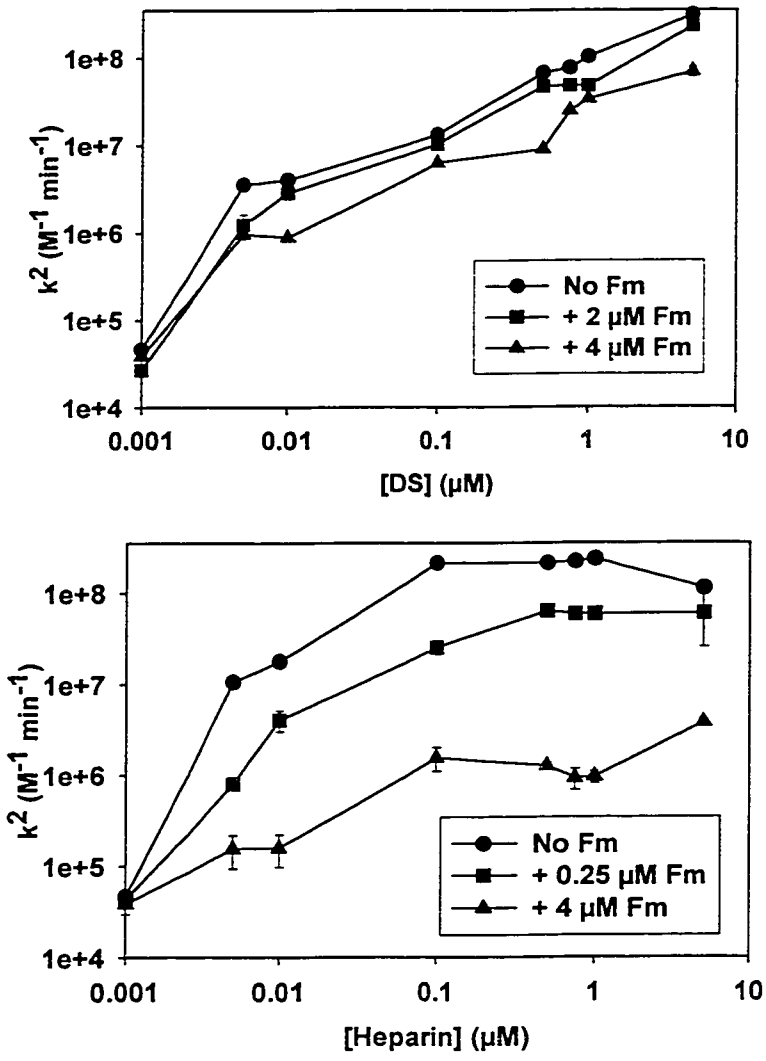

Fig. 1. **Effects of Fm on the second-order rate constants for the inhibition of thrombin by DS-catalyzed (top panel) or heparin-catalyzed (bottom panel) HCII.** The second-order rate constants for the inhibition of thrombin (10 nM) by 100 nM DS- or heparin-catalyzed pHCII in the absence or presence of Fm were determined under pseudo first-order conditions. The values represent the mean and the standard error of the mean of three determinations.

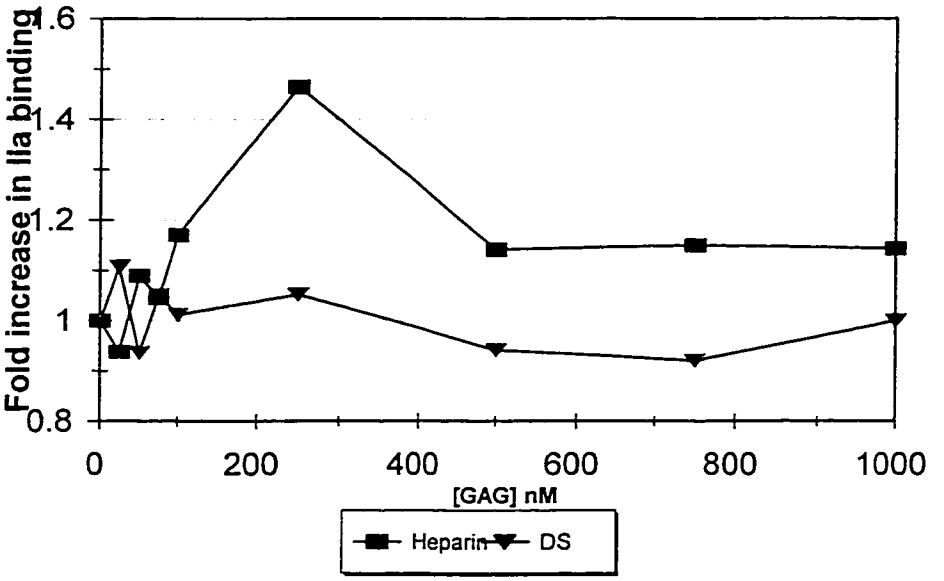

Fig. 2. **Effect of heparin and DS on the binding of ^{125}I-FPR-thrombin to fibrin.** The binding of ^{125}I-FPR-thrombin to fibrin clots was determined in the presence of increasing concentrations of either heparin (■) or DS (▼). The values represent the mean of two determinations.

Effect of ternary thrombin-fibrin-GAG complex formation on thrombin-mediated hydrolysis of chromogenic substrates: Previous studies have shown that the incorporation of thrombin into a productive ternary thrombin-fibrin-heparin complex results in alterations in the rates of thrombin-mediated hydrolysis of chromogenic substrates (Hogg et al., 1996). Changes in the active site environment of thrombin upon interaction with 4 μM Fm and either 250 nM DS or heparin were analyzed by measuring changes in the thrombin-mediated rates of hydrolysis of six chromogenic substrates. This concentration of GAG was chosed because 250 nM of heparin promotes ^{125}I-FPR-thrombin binding to fibrin clots (Fig. 1). As shown in Figure 3, Fm and DS have little effect on the chromogenic activity of thrombin with all six substrates tested. In contrast, the combination of Fm and heparin increases the chromogenic activity of thrombin with three substrates (Chz-Xa, S2238, and Chz-IIa), reduce the activity with one substrate (S2222), and has little effect on two substrates (Chz-tPA and S2366).

Quantification of binary interactions that comprise ternary thrombin-fibrin-GAG complex formation: The assembly of the ternary thrombin-fibrin-heparin complex is postulated to occur through a series of binary interactions between thrombin-fibrin, thrombin-heparin, and fibrin-heparin (Hogg and Jackson, 1990a). In this study, we determined the dissociation constants for the following binary complexes: thrombin-heparin, thrombin-DS, thrombin-fibrin, fibrin-heparin, and fibrin-DS. The affinities of DS and heparin for thrombin were determined by monitoring changes in intrinsic protein fluorescence of thrombin when titrated with DS or heparin. After titration, the I/Io values were plotted against GAG concentration

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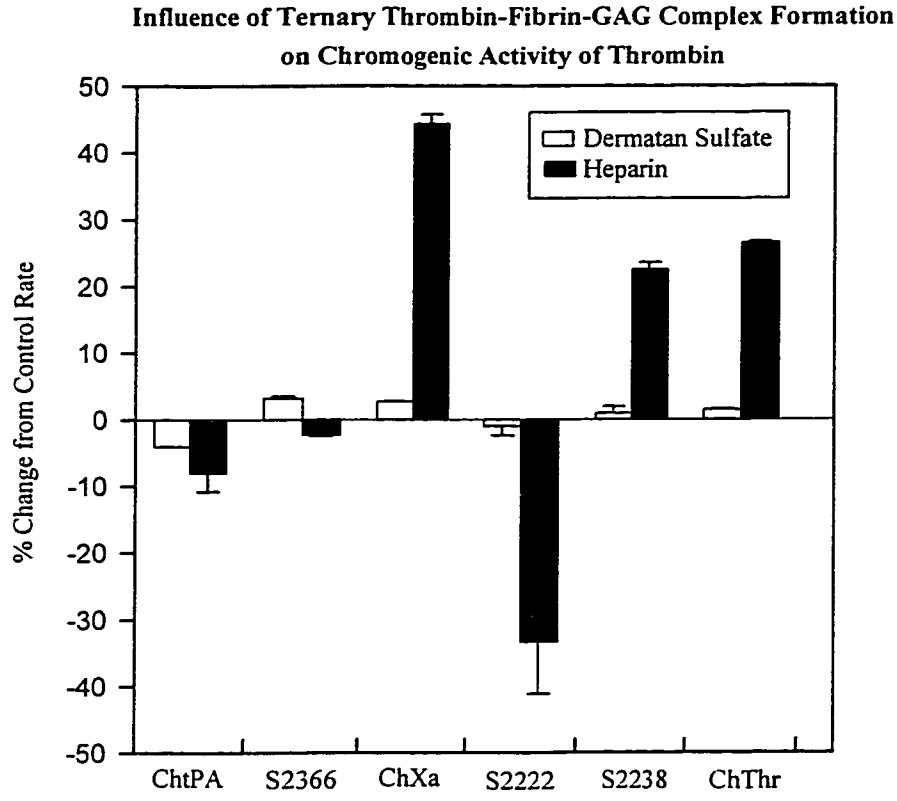

Fig. 3. **Influence of DS and heparin on the chromogenic activity of Fm-bound thrombin.** The rates of hydrolysis of chromogenic substrates by Fm-bound thrombin were determined in the absence and presence of 250 nM DS or heparin and the percentage changes from the control rate were calculated.

Dissociation constants for the interaction of components of the ternary thrombin-fibrin-GAG complexes

The affinities of individual components of the ternary complexes for each other were determined as described under "Experimental Procedures".

	Kd (nM)
Thrombin and Heparin	117
Thrombin and DS	2, 600
Fibrin and FITC-thrombin	1, 500
Fibrin and FITC-Heparin	187
Fibrin and FITC-DS	No Binding

and the K_d values were calculated by nonlinear regression. DS and heparin bind saturably to thrombin with K_d values of 2.6 μM and 116 nM, respectively (Table 1). The K_d value for the thrombin-heparin interaction is in agreement with that determined by titration of ANS-thrombin with increasing concentrations of heparin (K_d= 59 nM) (Hogg et al., 1996).

Although the heparin-binding site on thrombin has been mapped to exosite II (Church et al., 1989; Gan et al., 1994; Sheehan and Sadler, 1994), the location of the DS-binding site on thrombin is unclear. In this study, we observed that DS binds to α-thrombin and γ-thrombin with similar affinities (K_d values of 2.6 μM and 6.8 μM, respectively). In contrast, DS binds RA-thrombin, a thrombin variant with three point mutations in exosite II that lowers its affinity for heparin 20-fold (Ye et al., 1994), with a 7-fold lower affinity (K_d=17 μM). These studies suggest that, like heparin, DS also binds to exosite II on thrombin, albeit with a 22-fold lower affinity than heparin.

The affinities of FITC-FPR-thrombin, FITC-heparin, and FITC-DS for fibrin were monitored by clotting varying concentrations of fibrinogen with a catalytic amount of thrombin in the presence of the fluorescently-labelled ligand of interest, and quantifying unbound ligand in the clot supernatant. The K_d values were determined by nonlinear regression of binding curves. As shown in Table 1, FITC-FPR-thrombin and FITC-heparin bind to fibrin with K_d values of 1.5 μM and 187 nM, respectively. These values are consistent with published values (Hogg and Jackson, 1990; Hogg et al., 1994). In contrast, FITC-DS does not bind to fibrin.

Elucidation of the protective effect of heparin on the inhibition of fibrin-bound thrombin by HCII: There are two possible mechanisms by which fibrin-bound thrombin may be protected from inactivation by HCII in the presence of heparin. First, the incorporation of thrombin in a productive thrombin-fibrin-heparin complex can impair access of the HCII/heparin complex to exosites I and II on thrombin. Alternatively, binding of heparin to fibrin or to the fibrin-thrombin complex, may reduce the amount of heparin available to activate HCII. Heparin may preferentially bind to fibrin because its affinity for fibrin (K_d=187 nM) is ~100-fold higher than its affinity for HCII (K_d=13 μM) (Weitz et al., 1998).

To distinguish between these two possibilities, two sets of experiments were performed. First, we examined the inhibitory effects of Mut D, a GAG-independent recombinant HCII (Liaw et al., 1998). The uncatalyzed rate of thrombin inactivation by Mut is ~ 130-fold faster than that of native HCII, presumably reflecting expulsion of its amino-terminal acidic domain, which promotes its interaction with exosite I of thrombin. Because Mut D contains mutations within its GAG-binding domain, neither heparin nor DS enhances its activity. As shown in Figure 4, the uncatalyzed rate of thrombin inhibition by Mut D is decreased ~ 3-fold in the presence of 4 μM Fm. (from 6.0 x 10^6 M^{-1} min^{-1} to 2.1 x 10^6 M^{-1} min^{-1}) presumably because heparin bridges thrombin to fibrin, thereby enhancing the interaction between fibrin and exosite I on thrombin. Addition of 3.3 μM heparin produces a further 10-fold reduction in inhibition (from 2.1 x 10^6 M^{-1} min^{-1} to 2.7 x 10^6 M^{-1} min^{-1}). In contrast, a combination of DS and Fm has no effect on the rate of thrombin inhibition by Mut D.

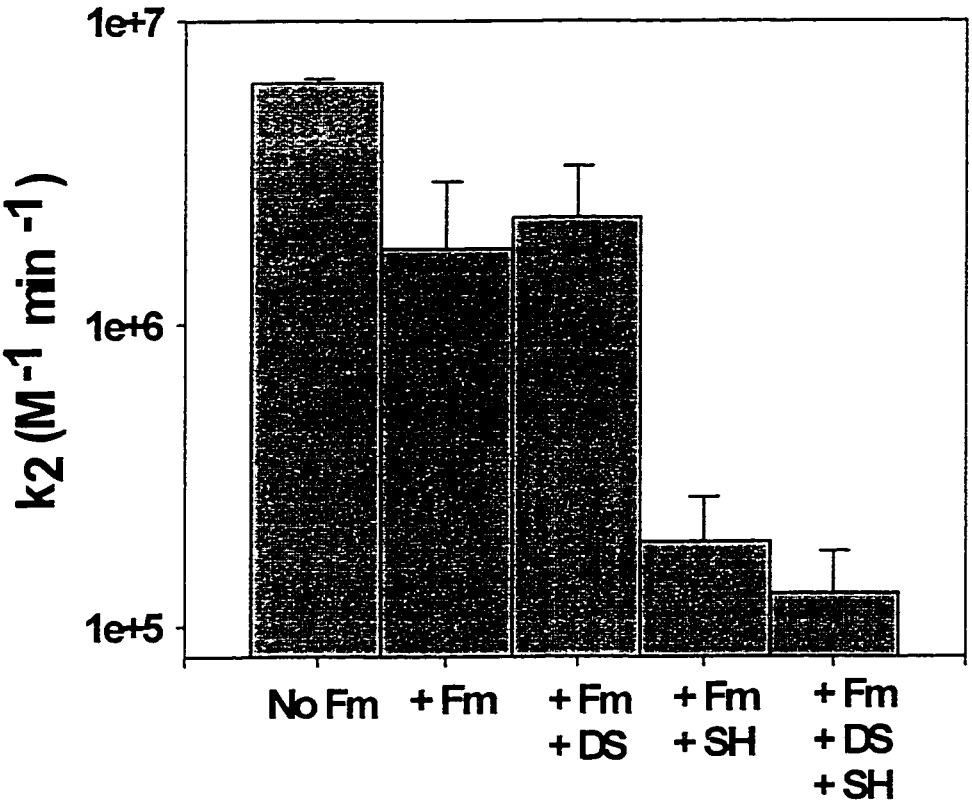

Fig. 4. **Effect of Fm and GAGs on thrombin inhibition by Mut D.** The second-order rate constants for the inhibition of thrombin (4 nM) by 40 nM Mut D in the absence or presence of 4 μM Fm, 3.3 μM DS, and/or 3 μM heparin were determined under pseudo first-order conditions. The values represent the mean and standard error of the mean of three determinations.

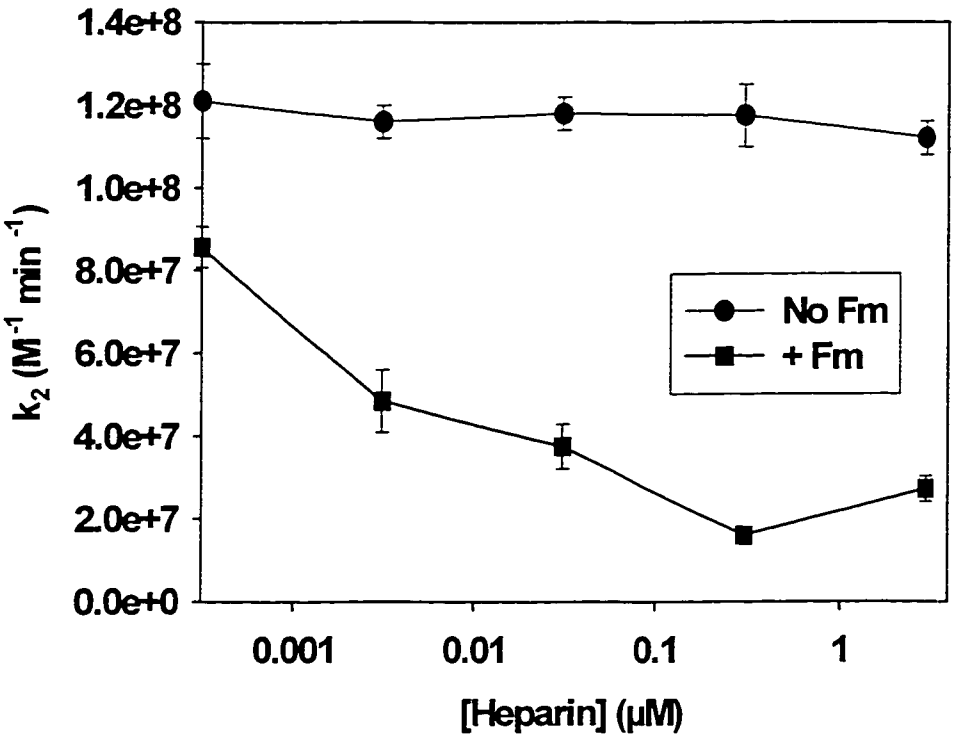

Fig. 5. **Effect of heparin on thrombin inhibition by DS-catalyzed pHCII either in the absence (●) or presence (■)of 4 μM Fm.** The second-order rate constants for the inhibition of thrombin (10 nM) by 100 nM DS-catalyzed pHCII in the absence or presence of Fm were determined under pseudo first-order conditions. The values represent the mean and standard error of the mean of three determinations.

Second, we examined the effect of heparin on the rate of thrombin inhibition by DS-catalyzed HCII either in the absence or presence of Fm (Fig. 5). In the absence of Fm, increasing concentrations of heparin have no effect on the DS-catalyzed rate of thrombin inhibition by HCII (Fig. 5). In contrast, in the presence of 4 μM Fm, heparin causes a dose-dependent decrease in the DS-catalyzed rate of thrombin inhibition by HCII. These results support the concept that heparin protects thrombin from inhibition by HCII by reducing the access of the amino-terminus of HCII to exosite I on thrombin.

5.4 Discussion

The aim of this study was to determine the molecular basis for the ability of the HCII/DS complex to inactivate fibrin-bound thrombin. The results of this study indicate that, unlike heparin, DS does not promote the formation of a productive ternary thrombin-fibrin-DS complex. Three lines of evidence support this concept. First, in the presence of fibrin monomer, thrombin is protected from inhibition by heparin/HCII but not by DS/HCII as quantified by protease inhibition assays under pseudo-first-order conditions. (Fig.1). Second, DS does not promote the binding of ^{125}I-FPR-thrombin to fibrin. In contrast, heparin augments ^{125}I-FPR-thrombin binding to fibrin in a concentration-dependent fashion, findings that are in agreements with those of Hogg and Jackson (1990a). Third, DS does not interact with fibrin (Table 1). Furthermore, DS binds to thrombin with a 22-fold lower affinity than heparin (K_d values of 2.6 μM and 117 nM, respectively).

These results reveal that, although exosite I and exosite II of thrombin can be ligated by fibrin and DS, respectively, productive ternary complex formation does not occur because DS does not bridge thrombin to fibrin. These findings indicate that all three binary interactions are essential for productive ternary complex formation. Further evidence that ligation of exosite I and exosite II of thrombin by fibrin and DS, respectively, does not result in productive ternary complex formation comes from the observation that the reactivity of thrombin toward chromogenic substrates is unaltered in the presence of fibrin and DS (Fig. 3).

Soluble fibrin has previously been shown to produce an ~250-fold reduction in the

rate of thrombin inhibition by heparin/HCII, presumably because access to exosite I on thrombin is impaired when thrombin is anchored in a productive ternary complex (Becker et al., 1998). In this study, we have more thoroughly investigated the mechanism by which the protective effect occurs. Heparin-mediated catalysis of thrombin inhibition by HCII involves both exosite I and II of thrombin. Specifically, heparin accelerates enzyme-inhibitor complex formation not only by promoting the interaction of the amino-terminal acidic domain of HCII with exosite I of thrombin, but also by serving as a template by binding simultaneously to the GAG-binding domain of HCII and exosite II on thrombin. There are two possible mechanisms by which heparin protects fibrin-bound thrombin from inactivation by HCII. First, ternary thrombin-fibrin-heparin complex formation may impair access of the heparin/HCII complex to thrombin's exosites. Alternatively, since the affinity of heparin for fibrin is ~100-fold greater than the reported affinity of heparin for HCII (Weitz et al., 1998), it is possible that heparin is sequestered by fibrin or by the fibrin-thrombin complex, and thus is unavailable or unable to activate HCII.

To address this issue, two experiments were performed in which the requirement for heparin in the activation of HCII was bypassed. First, we examined the protective effect of the thrombin-fibrin-heparin complex on thrombin inhibition by MUT D, a conformationally activated HCII variant due to mutations introduced into its GAG-binding domain (Liaw et al., 1998). Although the mutations in MUT D facilitate the release of the amino-terminal acidic domain from intramolecular interactions, they abolish the ability of heparin and DS to further enhance the activity of MUT D. Whereas Fm alone has little effect on the

uncatalyzed rate of thrombin inhibition by MUT D, addition of heparin decreases the rate of thrombin inhibition by Mut D ~30-fold (from 6.0 x 10^6 M^{-1} min^{-1} to 2.1 x 10^5 M^{-1} min^{-1}). This finding reflects impaired access of thrombin exosite I due to the anchoring of thrombin in a productive thrombin-fibrin-heparin complex. Second, we observed that, in the presence of Fm, heparin causes a dose-dependent decrease in the DS-catalyzed rate of thrombin inhibition by HCII. Like Mut D, the amino-terminal acidic domain of DS-catalyzed HCII is primed for interactions with exosite I of thrombin. These results reveal that the protective effect of heparin results from the incorporation of thrombin into a productive thrombin-fibrin-heparin complex in which exosite I is inaccessible to the amino-terminal acidic domain of HCII. Since ternary complex formation presumably strengthens binary interactions between all components of the thrombin-fibrin-heparin complex, exosite II of thrombin is likely also inaccessible to exogenous heparin bound to the GAG-binding domain of HCII.

In summary, our results indicate that DS, unlike heparin, does not promote the formation of a productive thrombin-fibrin-GAG ternary complex. The ineffectiveness of DS in facilitating ternary complex formation reflects its inability to form an intermediate binary complex with fibrin, a prerequisite for the assembly of the ternary complex. Furthermore, we have elucidated the mechanism by which heparin protects fibrin-bound thrombin from inactivation by HCII. Although *in vitro* and *in vivo* studies in animal models suggest that DS is an effective antithrombotic agent, the clinical efficacy of DS is unclear. However, these finding can be used to rationalize the development of thrombin inhibitors that are more effective in the management of acute coronary syndromes in which fibrin-bound thrombin

contributes to the prothrombotic nature of existing thrombi.

CHAPTER 6- SUMMARY AND PERSPECTIVES

These studies were undertaken to explore different aspects of the regulation of thrombin, a multifunctional enzyme central to hemostasis. Unlike related serine proteases of the coagulation and fibrinolytic pathways, thrombin is unique in that it serves multiple functions, which in some instances are antagonistic. For example, as a procoagulant enzyme, thrombin clots circulating fibrinogen, activates platelets and factor XIII, and amplifies the coagulation cascade by activating factors V and VIII (Mann, 1994). In contrast, when complexed with TM on the endothelial cell surface, thrombin functions as an anticoagulant by activating the protein C pathway (Esmon, 1991). Thrombin can also inhibit fibrinolysis by activating TAFI, an enzyme that suppresses plasminogen activation (Bajzar et al., 1996; Bajzar et al., 1998). Structural features defined by X-ray crystallographic studies of thrombin provide a molecular basis for the enzyme's specificity. These features include the active site cleft and two anion-binding exosites located on opposite poles of the thrombin moleculae. What is less evident from crystallographic studies is thrombin's capacity to undergo allosteric changes upon ligand binding to the exosites.

This thesis has three main goals: (a) to determine how F2, a prothrombin activation peptide, binds and modulates thrombin function, (b) to examine the molecular mechanism by which thrombin is inactivated by HCII, and (c) to elucidate why thrombin bound to fibrin is protected from inactivation by heparin but not dermatan sulfate. The current chapter emphasizes the biochemical and physiological relevance of these studies in the context of

hemostasis, outlines how these apparently diverse topics are interrelated, and describes future studies that extend the work already done.

6.1 Modulation of thrombin function by F2

Ligand binding to thrombin's exosites offers a potential mechanism for directing thrombin's specificity for a diverse range of natural substrates, inhibitors, and cofactors. F2, a prothrombin activation fragment that interacts with exosite II on thrombin, has been shown to evoke allosteric changes in thrombin's active site that modulate thrombin function (Myrmel, 1976; Walker and Esmon, 1979; Jakubowski et al., 1986; Bock, 1992; Liu et al., 1994). In Chapter 3, the structural domains that mediate the interaction of F2 with thrombin were identified. Using plasma, recombinant, and synthetic F2 peptides, the thrombin binding domain on F2 was localized to the kringle inner loop (residues 64-93) as well as the carboxy-terminal connecting peptide (residues 94-116). Although previous co-crystallization studies revealed that positively charged residues within exosite II on thrombin form salt bridges with the kringle inner loop of F2 (Arni et al., 1993; Arni et al., 1994), the observation that the carboxy-terminal peptide of F2 also is required for F2/thrombin interactions would not have been predicted from the F2/thrombin cocrystal structure because the peptide was disordered in the crystal.

The studies outlined in Chapter 3 also suggest the presence of discrete subdomains within exosite II of thrombin. This concept is based on the observation that the functional changes evoked by F2 and sF2(63-116) are quantitatively different, even though both ligands bind to exosite II. For example, the peptides influence the chromogenic activity of thrombin

to different extents, and only F2 slows the uncatalyzed rate of thrombin inactivation by AT. In contrast, both peptides prolong the thrombin clotting time of fibrinogen in a dose-dependent manner and can displace fluorescein-labelled hirugen from FPR-thrombin. These findings raise the possibility that by binding to subdomains of exosite II, ligands tightly control thrombin specificity, a concept likely to revise the hypothesis of Hortin and Trimpe (1991)- namely, that binding of any peptide to a particular exosite on thrombin exerts the same allosteric effect on thrombin's active site.

The functional observations of this work are complemented by recent structural data obtained from the X-ray crystal of bovine meizothrombin (desF1) in complex with D-Phe-Pro-Arg-chloromethylketone (PPACK) (Martin et al., 1998). PPACK covalently inhibits the active site of meizothrombin (desF1), thereby preventing autolysis. An important feature of the crystal structure is that the autolysis loop is rigid in meizothrombin (desF1), whereas it is disordered in thrombin. This suggests that the F2 domain of meizothrombin (desF1), despite being distant from the autolysis loop, influences the conformation of the loop through allosteric linkage (Martin et al., 1998). Furthermore, the covalently linked F2 domain in meizothrombin (desF1) differs in structure from that of F2 noncovalently complexed with thrombin (Arni et al., 1993; Arni et al., 1994). Compared with the F2/thrombin cocrystal structure, the F2 domain in meizothrombin (desF1) is more intimately associated with thrombin than F2 noncovalently complexed with thrombin. The closer association of the F2 domain in meizothrombin (desF1) reflects additional interdomain salt bridges and hydrogen bonds. It would thus be of interest to determine whether these additional contacts confer

meizothrombin (desF1) with functional properties different from those of the noncovalent F2/thrombin complex. In support of this concept, He et al. (1997) have demonstrated that individual Arg residues in exosite II of thrombin make unique contributions to exosite II-mediated functions of thrombin. Furthermore, F2 is thought to optimize the conformation of prothrombin for proteolytic cleavage by factor Xa (Krishnaswamy and Walker, 1997). To explore this phenomenon, mutagenesis techniques could be used to localize the region of F2 that alters the conformation of the scissile bond(s) of prothrombin. With this information, it may be possible to construct prothrombin variants that generate unique intermediates upon activation by the prothrombinase complex.

Although F2 was used as a tool in these studies to further our understanding of the allosteric nature of thrombin, the peptide also plays an important regulatory role *in vivo*. When compared with equimolar concentrations of thrombin, meizothrombin and meizothrombin (desF1) have only 10% and 1%, respectively, of the activity of thrombin toward factor V, fibrinogen, or platelets (Doyle and Mann, 1990). Furthermore, when TM-dependent activation of protein C is compared, meizothrombin and meizothrombin (desF1) have greater anticoagulant activity than thrombin. In contrast, whereas the rate of TM-dependent activation of TAFI by meizothrombin is only 10% that of thrombin (Cote et al., 1997). These differences likely reflect the interaction of the F2 domain within meizothrombin and meizothrombin (desF1) with exosite II of the protease domain, thereby modulating their specificities.

At physiologic concentrations of prothrombin (> 1 μM), ~ 70 % of the product

initially formed by the prothrombinase complex is meizothrombin, a product that accumulates to between 10 and 20% of total available prothrombin (Rosing et al., 1986). Since meizothrombin retains its F1 domain, it remains bound to phospholipid surfaces where it is readily converted to thrombin by factor Xa in the prothrombinase complex (Rosing and Tans, 1988). In contrast, because meizothrombin (desF1) lacks its F1 domain, it will not bind to phospholipid surfaces. Consequently, it is likely to accumulate to a greater extent than meizothrombin because meizothrombin (desF1) will only be slowly converted to thrombin by factor Xa in a phospholipid-independent manner (Rosing and Tans, 1988). Since meizothrombin (desF1) has only ~1% of the procoagulant activity of thrombin (Doyle and Mann, 1990), the accumulation of meizothrombin (desF1) during prothrombin activation may favour an anticoagulant state rather than the usual procoagulant situation.

In addition to its ability to allosterically regulate thrombin function, F2 can also block ligand interactions at exosite II. For example, unlike thrombin inhibition by AT, inhibition of meizothrombin and meizothrombin (desF1) by AT is not accelerated by heparin (Rosing et al., 1986; Schoen and Lindhout, 1987; Cote et al., 1997). The X-ray structure of meizothrombin (desF1) reveals that exosite II is masked by F2, suggesting that the covalent attachment of F2 prevents heparin from binding to exosite II on these intermediates (Martin et al., 1998). In contrast, thrombin, meizothrombin, and meizothrombin (desF1) bind hirudin with similar affinities, suggesting that exosite I of these enzymes is equally accessible (Cote et al., 1997). Consequently, these intermediates will be less readily inhibited by the AT/heparin complex than thrombin. In contrast, all three enzymes should be similarly

inhibited by hirudin (Cote et al., 1997).

The studies detailed in Chapter 3 highlight the importance of exosite II in the regulation of thrombin function. Like exosite I, exosite II does not simply function as an electropositive region that interacts nonspecifically with anionic molecules. Instead, the binding of proteins and GAGs to exosite II of thrombin can evoke conformational changes at the active site and at exosite I of thrombin, as well as restrict the approach of other exosite II ligands. The recent report of direct allosteric linkage between exosite I and II of thrombin (Fredenburgh et al., 1997), as well as previous reports for allosteric linkage between exosite I and the active site (Liu et al., 1991; Ye et al., 1991; Ye et al., 1993; Dang et al., 1995; DeCristofaro et al., 1995), reveal that the regulation of thrombin function involves complex cooperative interactions between separate functional domains.

6.2 Mechanism of thrombin inhibition by HCII

The hemostatic function of thrombin is also directed by the balance between its activation and inhibition by protease inhibitors in plasma. The second objective of this work was to gain insight into the mechanism by which thrombin is inactivated by HCII, a plasma serpin that selectively inhibits thrombin by forming a covalent, bimolecular complex in which the protease is inactive. The rate of thrombin inhibition by HCII is accelerated > 1000-fold in the presence of DS or heparin. Current thinking is that the catalytic effect of these GAGs involves GAG-induced conformational changes in HCII that facilitate covalent serpin-protease complex formation. Specifically, binding of polyanionic GAGs to the electropositive GAG-binding domain of HCII displaces the amino-terminal acidic domain

from intramolecular interactions with the GAG-binding site, thereby permitting the acidic domain to interact with exosite I of thrombin (Ragg et al., 1990; van Deerlin and Tollefsen, 1991; Sheehan et al., 1994).

The studies outlined in Chapter 4 support the current allosteric model because substitution of 4 (Mut C) or 5 (Mut D) basic residues within the GAG-binding domain with neutral amino acids increases the basal rate of thrombin inhibition by ~ 130-fold. Release of the acidic domain from intramolecular interactions appears to require the disruption of at least four ionic bonds since substitution of two residues at a time does not influence the uncatalyzed rate of thrombin inactivation. Furthermore, these studies provide a revised model of GAG-mediated catalysis of thrombin inhibition by HCII. DS appears to catalyze thrombin inhibition by HCII exclusively through induction of allosteric changes in the amino-terminus. In contrast, while the bulk of the stimulatory effect of heparin results from conformational changes in the amino terminus of HCII, similar to those observed with DS, heparin also serves a template function by interacting simultaneously with the GAG-binding domain of HCII and exosite II of thrombin. The ~130-fold increase in the basal rate at which Mut C and Mut D inactivate thrombin likely reflects conformational changes in the amino-terminus of HCII similar to those induced by heparin.

F2 can be employed as a tool to assess the relative importance of thrombin exosite II in mediating thrombin inhibition by HCII in the presence of heparin. By competing with heparin for binding to exosite II, the peptide should decrease the heparin-catalyzed rate of thrombin inhibition by HCII by an order of magnitude. Since DS-mediated catalysis of HCII

does not utilize exosite II, F2 should not influence the rate of thrombin inhibition by the HCII/DS complex. It is possible, however, that F2 binding to thrombin evokes conformational changes at the active site of thrombin that limits its reactivity with HCII, in a manner analogous to our observation that F2 slows the rate of thrombin inactivation by AT. It would thus be of interest to examine the effect of F2 on the uncatalyzed rate of thrombin inhibition by HCII.

The revised model of GAG-mediated catalysis of thrombin inhibition by HCII raises the possibility that DS evokes more extensive conformational changes in the amino-terminus of HCII than heparin, thereby optimizing the interaction of the amino-terminus with exosite I of thrombin. In order to mimic the catalytic effect of DS, it may be necessary to weaken additional salt bridges between the amino-terminus and the GAG-binding domain of HCII. Thus, it would be interesting to combine the amino acid substitutions of Mut D with substitutions at Arg^{200} and Lys^{173}, other residues implicated in the conformational constraint of the amino-terminus of HCII (Ciaccia, 1997; Whinna et al., 1991).

To explore the possibility that DS induces a larger translocation of the amino-terminus of HCII than with heparin, fluorescence resonance energy transfer studies could be employed to measure the extent of GAG-mediated expulsion of the amino-terminus of HCII. This method has been used to demonstrate that the location of the protease in a covalent serpin-protease complex is different from that in the initial Michaelis-like complex (Stratikos and Gettins, 1997). Briefly, the authors measured the separation between fluorescein attached to a single cysteine on the serpin and tetramethylrhodamine attached to the protease

in (a) a covalent serpin-protease complex, and (b) a non-covalent, Michaelis-like, serpin-protease complex. The large increase in separation between the fluorophores in the noncovalent and covalent complexes reflects the movement of the protease upon covalent complex formation (Stratikos and Gettins, 1997). These results support the concept that insertion of the cleaved reactive centre loop into the body of the serpin is accompanied by extensive movement of the covalently-linked protease (Stratikos and Gettins, 1997; Wilczynska et al., 1997). It may be possible to specifically label the amino-terminal amine group of HCII with tetramethylrhodamine isothiocyanate and an internal free cysteine of the same HCII molecule with iodoacetamideofluorescein. The consequences of DS or heparin binding could then be monitored by calculating the interfluorophore separation before and after GAG titration.

The studies proposed above may provide insight into the extent of unfolding of the amino-terminus of HCII evoked by different GAGs. However, to determine if the affinity of the amino-terminus of HCII for exosite I of thrombin is higher when HCII is activated by DS compared with heparin, additional experiments could be performed. First, the apparent affinities of the DS/HCII complex and the heparin/HCII complex for fluorescently labelled active-site blocked RA-thrombin could be compared. RA-thrombin, a recombinant thrombin variant containing three point mutations in exosite II that result in a 20-fold decrease in heparin affinity (Ye et al., 1994), will minimize the ability of heparin to bridge thrombin to HCII, thereby reducing the possibility that binding reflects interactions other than those between the amino-terminus of HCII and exosite I on thrombin. A kinetic analysis could be

done to examine the effect of hirugen, an analogue of the carboxy-terminus of hirudin that specifically binds to exosite I, on the GAG-catalyzed rate of thrombin inactivation by HCII. If the affinity of the amino-terminus of HCII for exosite I of thrombin is greater when HCII is activated by DS than it is when activated by heparin, the Ki for hirugen-mediated reduction in the rate of thrombin inactivation by DS-catalyzed HCII should be greater than with heparin-catalyzed HCII.

The studies described in Chapter 4 also highlight the diverse ways in which serpins inactivate target proteases. Covalent serpin-protease complexes arise from protease-mediated cleavage of the P1-P1' peptide bond of the serpin's solvent-exposed reactive centre loop. Consequently, the conformation of the reactive centre loop is an important determinant of the rate at which the serpin inhibits its target protease. For example, the latent form of plasminogen activator inhibitor (PAI-1) is inactive because the N-terminal side of its reactive centre loop is located in the hydrophobic interior of the protein (Mottonen et al., 1992). Upon denaturation and refolding (Hekman and Loskutoff) or upon association with vitronectin (Wiman et al., 1988; Andreasen et al., 1990), inhibitory activity of PAI-1 can be restored. Also, it has been proposed that the serpin ovalbumin lacks inhibitory activity because of the helical nature of its reactive centre loop (Steine et al., 1990). This concept is supported by the observation that heating confers ovalbumin with inhibitory activity because it alters the helical structure of the reactive centre loop. Thereby allowing it to adopt the distorted conformation observed in active serpins (Mellet et al., 1996).

HCII is an intriguing serpin because although the Leu-Arg at the P1-P1' position of

its reactive centre loop is an unfavourable thrombin recognition sequence, HCII compensates by possessing a secondary binding site, that promotes intermolecular interactions with thrombin upon catalysis by GAGs. The specificity of the amino-terminus for exosite I of thrombin imparts HCII with selectivity for thrombin. Utilization of secondary protease binding sites has also been observed in the inactivation of plasmin by alpha-2 antiplasmin (AP) (Hortin et al., 1989). The kinetics of the AP-plasmin reaction consists of two successive steps: a fast, reversible second-order reaction, followed by a slow, irreversible first-order reaction (Lijnen and Collen, 1986). The rate of the first reaction is dependent on the accessibility of both the active site and a lysine-binding kringle domain in the plasmin molecule. The lysine-binding kringle of plasmin interacts with lysine residues at the carboxy-terminus of AP (Hortin et al., 1989).

In summary, these studies demonstrate that conformational activation of HCII by GAGs can be mimicked, at least in part, by disrupting ionic interactions between the amino-terminus of HCII and its GAG-binding domain. Furthermore, these studies reveal that DS and heparin employ distinct mechanisms in the catalysis of thrombin inhibition by HCII. The conformational constraint of the amino-terminus of native HCII is physiologically important because it renders HCII relatively inactive in the circulation, but functionally active at extravascular sites where DS or heparan sulfate is found. Consequently, it is likely that HCII is a physiologically relevant thrombin inhibitor in the extravascular space.

6.3 Molecular basis for HCII-mediated inactivation of fibrin-bound thrombin

In addition to regulation by serpins, thrombin activity also is modulated by

nonenzymatic interactions with the growing fibrin network. Despite being catalytically active, fibrin-bound thrombin exhibits impaired reactivity with selected inhibitors, notably AT/heparin. The resistance of fibrin-bound thrombin to inactivation by AT/heparin reflects the anchoring of thrombin in a ternary thrombin-fibrin-heparin complex (Hogg and Jackson, 1990a; Hogg and Jackson, 1990b; Hogg et al., 1996). It has been postulated that productive ternary complex formation assembly is dependent on binary interactions between thrombin-heparin, thrombin-fibrin, and heparin-fibrin (Hogg and Jackson, 1990a; Hogg et al., 1996).

Unlike heparin, DS inhibits soluble and fibrin-bound thrombin equally well (Okwusidi et al., 1990; Bendayan et al., 1994). The third objective of this work was to determine why fibrin-bound thrombin is susceptible to inactivation by the HCII/DS complex but not by the AT/heparin complex. The studies described in Chapter 5 reveal that DS, unlike heparin, does not promote the formation of a productive thrombin-fibrin-GAG ternary complex. Although exosite I and exosite II of thrombin can be ligated by fibrin and DS,respectively, the ineffectiveness of DS in anchoring thrombin in a productive ternary complex reflects the inability of DS to bind fibrin. These findings illustrate the importance of individual binary interactions in enhancing the affinity between components of the ternary complex.

Like AT, HCII displays limited reactivity with fibrin-bound thrombin when heparin is present (Becker et al., 1998). The studies described in Chapter 5 have more thoroughly investigated the mechanism by which this protective effect occurs. Specifically, the ineffectiveness of HCII/heparin in the inactivation of fibrin-bound thrombin reflects the

inaccessibility of thrombin exosite I to the HCII/heparin complex when thrombin is anchored in a thrombin-fibrin-heparin complex. These results were obtained by utilizing Mut D, a recombinant HCII variant which bypasses the requirement for heparin in the inhibition of thrombin. To determine whether inaccessibility of exosite II to HCII also contributes to the limited reactivity of thrombin within the the ternary thrombin-fibrin-heparin complex, F2 could be employed to compete with heparin for occupancy of exosite II. Finally, to confirm that both exosites must be ligated for thrombin to be protected, thrombin could be substituted with chymotrypsin, a target protease of HCII that does not utilize surface exosites for inhibition by HCII.

Since DS is found in the extracellular matrix of connective tissue, it would also be interesting to explore how vascular wall DS facilitates thrombin inhibition by HCII. Previous studies have shown that thrombin binds to the subendothelial extracellular matrix (ECM) with an apparent K_d of 13 nM (Bar-Shavit et al., 1989). Studies using GAG-degrading enzymes have shown that thrombin binding to ECM is DS-mediated (Hatton and Moar, 1985; Bar-Shavit et al., 1989), as have competition studies using various GAGs (Bar-Ner et al., 1987; Bar-Shavit et al., 1989). The ability of ECM-bound thrombin to be inactivated by HCII can be evaluated by allowing ECM-bound radiolabelled thrombin to interact with HCII (Bar-Shavit et al., 1989). The serpin-protease reaction can then be stopped at different time intervals by the addition of SDS-PAGE sample buffer and analyzed by SDS-PAGE (Bar-Shavit et al., 1989). Whereas the endothelial cell surface is normally thromboresistant, because of anticoagulant mechanisms such as activation of protein C by

TM-bound thrombin (Esmon et al., 1991) and the presence of heparan sulfate (Shimada and Ozawa, 1985), the underlying subendothelium is thrombogenic. The subendothelium contains tissue factor that initiates the extrinsic coagulation pathway (for review, see Nemerson, 1994) as well as von Willebrand factor and collagen, which promote platelet adhesion (for review, see Preissner and Potzsch). Following endothelial injury, DS-catalyzed HCII may limit the procoagulant activities of thrombin in the extravascular space.

Although *in vitro* experiments and studies in laboratory animal models suggest that DS is an effective antithrombotic agent, the benefit-to-risk profile of DS relative to heparin has yet to be established. The studies described in Chapter 5 provide direction for development of thrombin inhibitors capable of inactivating fibrin-bound thrombin. Since productive ternary complex formation is heparin-dependent, one approach to circumvent the protection of fibrin-bound thrombin from inactivation by AT/heparin or HCII/heparin is to modify heparin in a way that destroys its ability to assemble the ternary complex. Recently, Fredenburgh et al. (1998, unpublished) have demonstrated that a 6000 Da heparin fraction is of insufficient length to support productive ternary complex formation, yet maintains the capacity to catalyze thrombin inhibition by AT because it is long enough to bridge thrombin to AT. The 6000 Da heparin fraction also catalyzes factor Xa inhibition by AT, thereby preventing thrombin generation and decreasing the amount of thrombin available to bind to fibrin. Since factor Xa bound to platelets within a thrombus also contributes to the prothrombotic nature of thrombi (Eisenberg et al., 1993), this heparin species may have a theoretical advantage over direct inhibitors of thrombin or factor Xa inhibitors by virtue of

its ability to effectively inhibit free and fibrin-bound thrombin, as well as factor Xa.

6.4 Conclusions

Control of thrombin activity is fundamental to hemostasis and represents one of the most intricate systems of enzyme regulation. The current study advances our knowledge of the regulation of thrombin in a number of important ways. First, the role of exosite II binding in modulating interactions with exosite I and the catalytic site of thrombin was investigated. This work reveals that the binding of different peptide sequences to exosite II of thrombin exerts different effects on thrombin's function. In addition, it provides further insight into the mechanism by which thrombin is inactivated by HCII in the presence of GAGs. Finally, these studies reveal why fibrin-bound thrombin can be inactivated by DS/HCII by not by heparin/HCII. These observations illustrate the multiple and interrelated levels of thrombin regulation in the context of hemostasis. Elucidation of the determinants of thrombin specificity are likely highly relevant in the development of effective strategies for the pharmacological control of thrombin activity.

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Patricia C. Y. Liaw, James C. Fredenburgh, Alan R. Stafford, Alexander Tulinsky, Richard C. Austin, and Jeffrey I. Weitz. (1998) Localization of the Thrombin-binding Domain on Prothrombin Fragment 2. *J. Biol. Chem.* **273**, 8932-8939.

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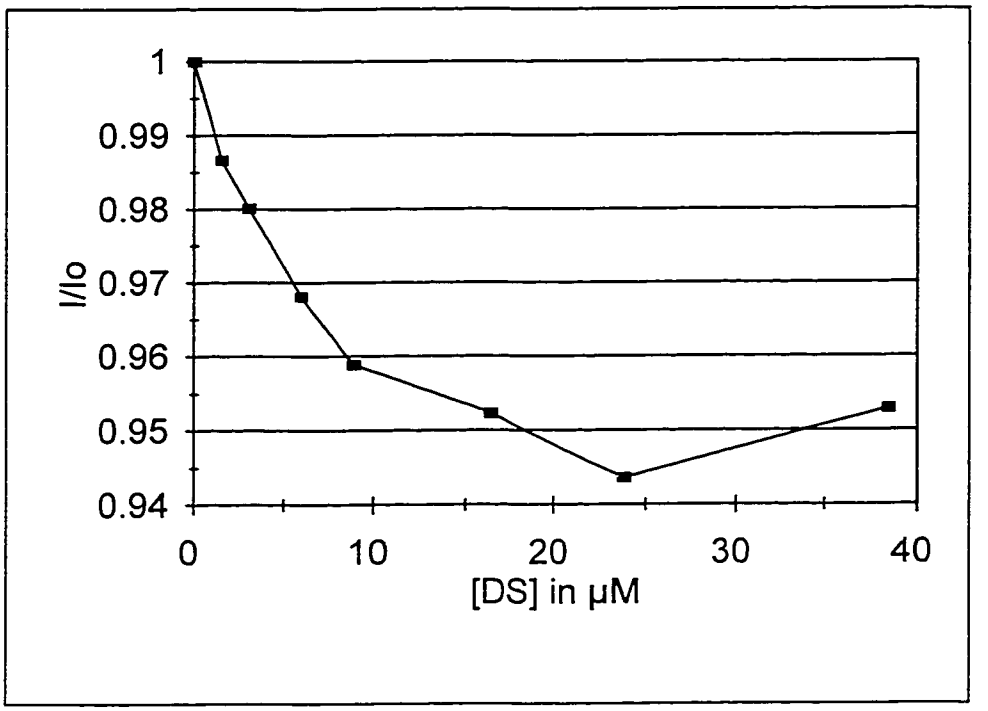
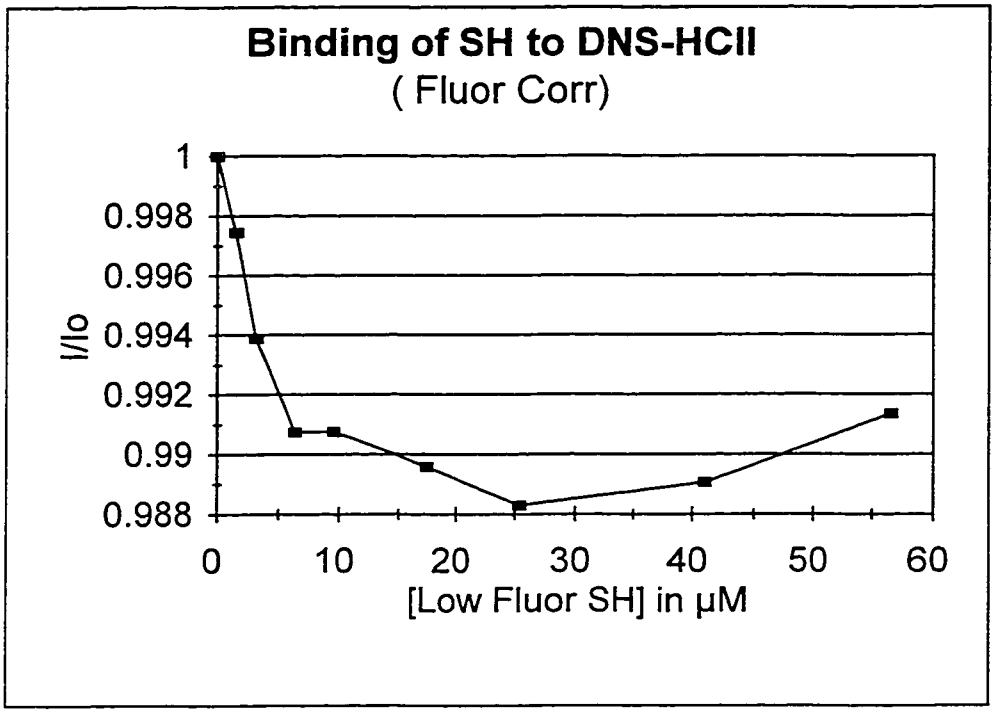
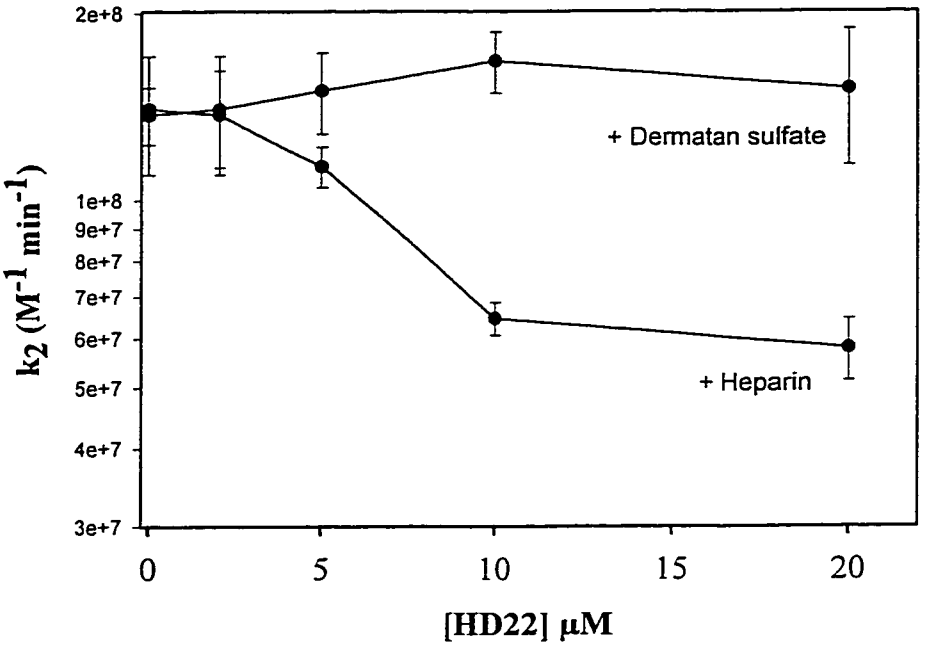


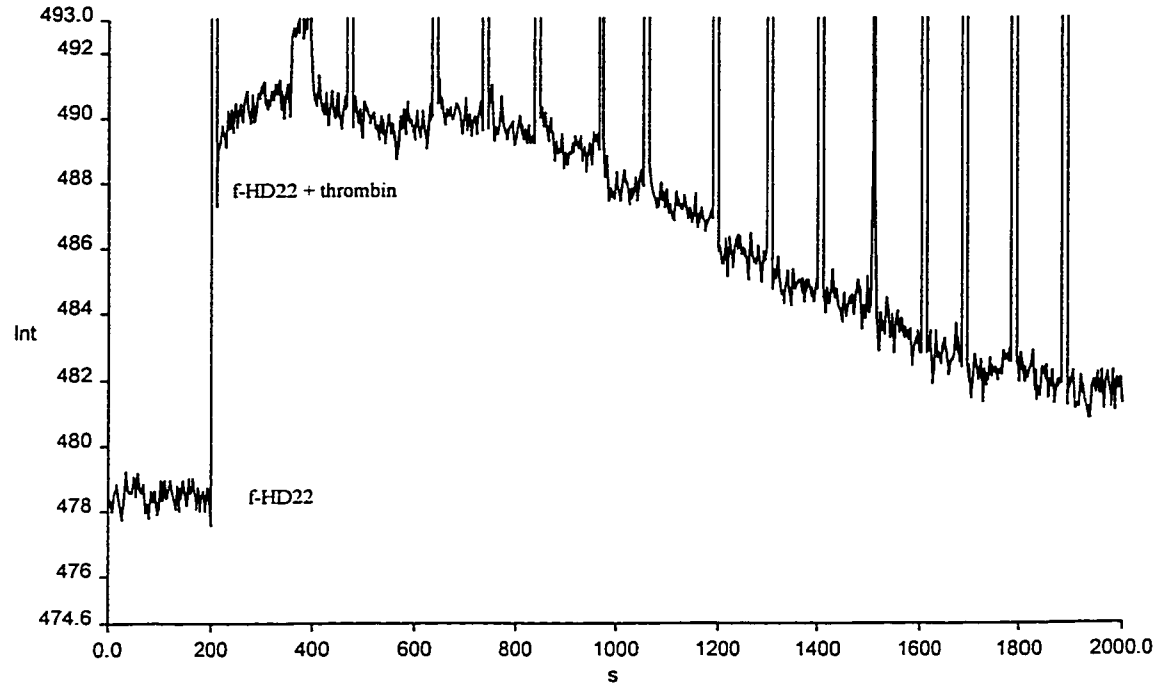
Appendix A. **Binding of dermatan sulfate to Dansyl-HCII. Increasing amounts of dermatan sulfate were mixed with 50 nM Dansyl-HCII and changes in fluorescent intensity were monitored.** I/Io is plotted versus dermatan sulfate concentration, where I is the fluorescent intensity at a given dermatan sulfate concentration and Io is the initial fluorescence intensity. (Ex 335 nm, Em 520 nm, 430 filter, slits widths= Ex 15 nm, Em 20 nm).



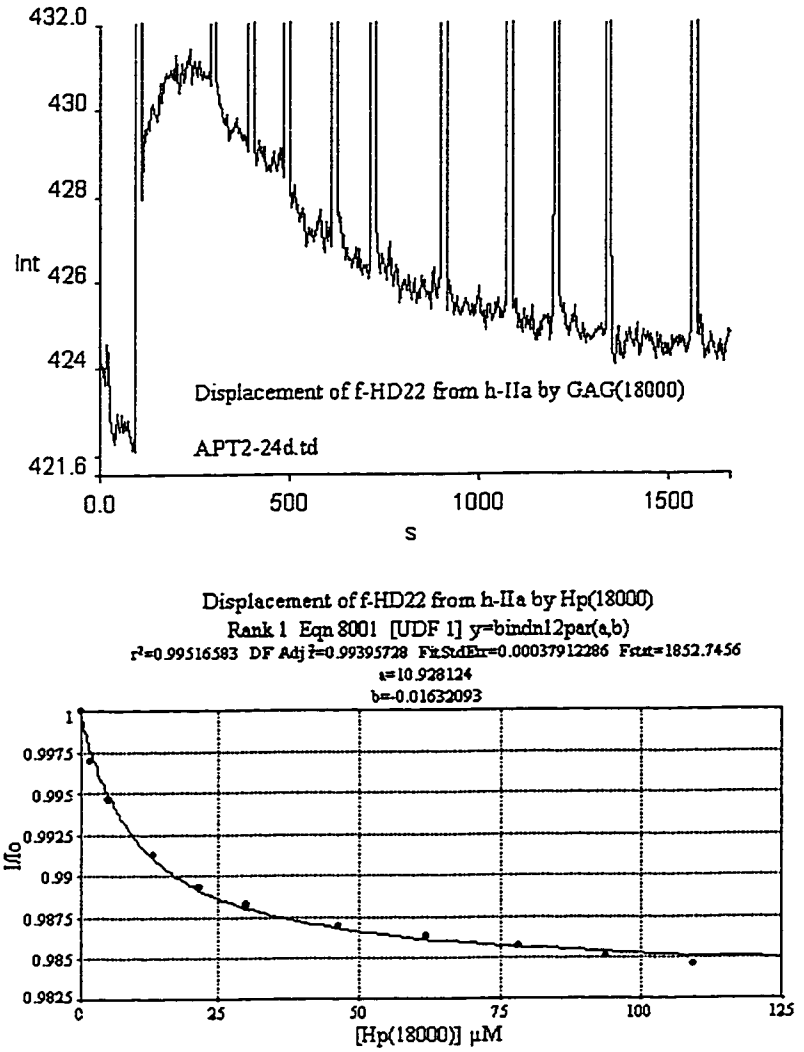
Appendix B. **Binding of heparin to Dansyl-HCII. Increasing amounts of heparin were mixed with 50 nM Dansyl-HCII and changes in fluorescent intensity were monitored.** I/Io is plotted versus heparin concentration, where I is the fluorescent intensity at a given heparin concentration and Io is the initial fluorescence intensity. (Ex 335 nm, Em 520 nm, 430 filter, slits widths= Ex 15 nm, Em 20 nm).



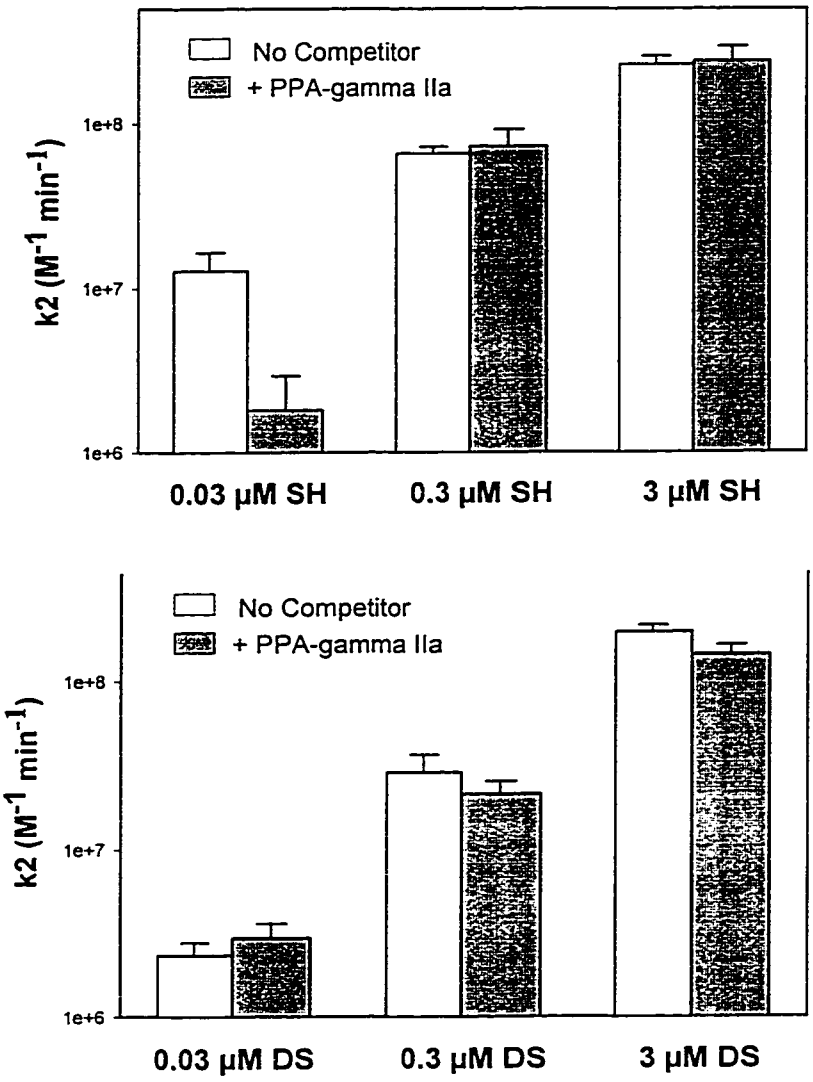
Appendix C. **Effect of HD22 exosite II aptamer on thrombin inhibition by HCII in thepresence of heparin or dermatan sulfate.** The second-order rate constants for the inhibition of 4 nM thrombin by 25 nM plasma-derived HCII were determined under pseudo first-order conditions in the presence of 3.3 μM heparin or dermatan sulfate (DS) and increasing concentrations of HD22. The points represent the mean of two determinations and the lines reflect the standard error of the mean.



Appendix D. **Displacement of f-HD22 exosite II aptamer from thrombin by dermatan sulfate.** The fluorescence of 7 nM fluorescein-HD22 DNA aptamer was monitored in time drive. Thrombin was then added to 50 nM, causing a 2.9 % increase in *I*. When aliquots of 3 μM dermatan sulfate were added, the *I* decreased to almost that obtained with free fluorescein-HD22.



Appendix E. **Displacement of f-HD22 exosite II aptamer from thrombin by heparin.** The fluorescence of 7 nM fluorescein-HD22 DNA aptamer was monitored in time drive (top panel). Thrombin was then added to 50 nM, causing a 2.9 % increase in *I*. When aliquots of 3 µM heparin were added, the *I* decreased to that obtained with free fluorescein-HD22. The *Ki* value for the f-HD22-thrombin interaction, determined by non-linear regression analysis, is 10.9 µM (bottom panel).



Appendix F. **Effect of FPR-gamma thrombin on thrombin inhibition by heparin-catalyzed HCII (top panel) or dermatan sulfate-catalyzed HCII (bottom panel).** The second-order rate constants for the inhibition of 4 nM thrombin by 25 nM GAG-catalyzed HCII in the presence of 6 µM FPR-gamma thrombin were determined under pseudo first-order conditions. The bars represent the mean, while the lines above the bars reflect the standard error of the mean of two determinations.