STRUCTURAL DETERMINANTS FOR HEPARIN BINDING IN HUMAN
COAGULATION FACTOR XI

A Dissertation

Submitted to

The Temple University Graduate Board

in Partial Fulfillment

of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

by

Sergei Shikov

August, 2008

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ABSTRACT

Title: Structural Determinants for Heparin Binding in Human Coagulation Factor XI.

Candidate's Name: Sergei Shikov

Degree: Doctor of Philosophy

Temple University, 2008

Thesis Advisor: Peter N. Walsh, M.D., Ph.D.

Coagulation factor XI plays an important role in the consolidation phase of blood coagulation. Previous studies from our laboratory and others have demonstrated that zymogen factor XI (FXI) binds to heparin with moderate ($K_D \sim 110$ nM) affinity via residues (K252, K253 and K255) located in the apple 3 (A3) domain of the molecule. In contrast, the enzyme, factor XIa (FXIa), was shown to bind to heparin with significantly higher affinity (~ 1.5 nM by ELISA) via residues (K529, R530 and R532) within the catalytic domain (CD). The interaction between heparin and FXIa potentiates the inhibition of FXIa by protease nexin-2 by 10-fold. In addition, related polyanions heparin and dextran sulfate inhibit the catalytic activity of FXIa. The present study was designed to determine the relative contributions of positively charged residues as well as the dimeric structure of FXI in heparin binding.

During this project, wtFXI, FXI_{R504A}, FXI_{K505A}, FXI_{R507A}, FXI_{R529A}, FXI_{R530A}, FXI_{R532A}, and FXI_{R586A} have been expressed and purified. All mutants were homogenous and identical to wtFXI on SDS-PAGE, clotting assays and 1G5 monoclonal

antibody binding studied by SPR. In addition, monomeric FXI_{C321S/K331A} was expressed and purified.

Utilizing an ELISA assay, no significant difference in the affinity for heparin between FXIa and FXI was found. Surface plasmon resonance (SPR) data collected for FXI clearly indicate a complex interaction which does not conform to a simple 1:1 Langmuir binding model making it difficult to obtain quantitative information. The complexity of FXI interactions with heparin is likely to arise from the multivalent nature of the binding, in which both protein and heparin have multiple binding sites.

Two positively charged residues in the FXI catalytic domain, FXI_{R507A} and FXI_{R532A}, were found to be particularly important for interaction with heparin. The FXI_{R507A} and FXI_{R532A} mutants demonstrated ~ 65% and ~50% decreases respectively in total number of heparin binding sites based on ELISA. Also, the apparent dissociation constants for FXI_{R507A} ($K_{Dapp} \sim 13$ nM) and FXI_{R532A} ($K_{Dapp} \sim 21$ nM) were 6 and 10-fold increased respectively compared with 2.1 nM for the wtFXI. Mutant FXI_{R586A} also demonstrated a defect in affinity ($K_{Dapp} \sim 13$ nM) without an effect on the B_{max} . The monomeric FXI_{C321S/R331A} was also characterized for its ability to bind heparin compared with wtFXI. Surprisingly, the monomeric FXI displayed defective binding to heparin according to ELISA ($K_{Dapp} \sim 30$ nM) and SPR methods.

Thus, the unique homodimeric structure of FXI in addition to the residues both in its catalytic and A3 domain chains are necessary for high-affinity heparin binding.

ACKNOWLEDGMENTS

I would not have been able to complete this arduous journey without the help of God and many wonderful people I have been blessed to know.

I am grateful to my dear parents Marina and Alexander Shikov for their love support, encouragement and everything they have done for me. I thank my partner Pum Tiyanont for his incredible commitment and love that helps me to get through the most difficult moments. Thank you, Pum, for your patience during the years of our long-distance relationship. I also thank my dear sister Yana for her support.

I would like to thank my advisor Dr. Peter Walsh for his support, guidance and patience. I thank Dr. Charles Grubmeyer who saw a potential in me to succeed as a scientist. I am grateful to my committee members Drs. David Ash, Scott Shore, Robert Suhadolnik and Salim Merali for their service on my committee and their input throughout my scientific development. I thank Dr. Jimmy Collins for graciously agreeing to serve on my dissertation committee shortly before the defense. I thank Dr. Gerald Soslau for serving on my defense committee and proofreading my thesis.

I am grateful to all the people of the Walsh lab who have aided me in many ways: Fredda London, Ahmad Syed, Duraiswamy Navaneetham, Dipali Sinha, Wenman Wu, Xia Yang, Tara Miller, Paul Riley, Ya-Chi Su, Paz Vega and Mariola Marcinkiewicz.

I thank Dr. Thomas Walz for his contribution to our collaboration. I thank Dr. Calvin Yip for the beautiful electron microscopy work that he has done.

My education has been marked with many great teachers along the way and I am thankful to all of them both in Russia and the United States.

I thank Dr. Qinjian Zhao and Joan Hampton from Merck for generously donating lab equipment. I thank Debbie Miller for providing many labware samples so graciously and expeditiously.

Many thanks to Nancy Reichenbach, Shirley Burton, Pat Pileggi, Sandy McKinnon, Tina Young and Derrick Russell for their kindness and encouragement. I am thankful to the great staff of the Temple University Hospital cafeteria for their work and the laughs we shared.

I am grateful to my long time friends Jim Brown and Pavel Tsitovitch for their love and support.

Finally, I would like to thank my Philadelphia friends Naota Tanaka, Tom Dykes and Denise Ryks with whom I have shared many joyful moments outside of the academic environment.

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CHAPTER 1

INTRODUCTION

Overview of hemostasis

Blood is a fluid composed of living cells, proteins, inorganic molecules, and water. Blood transports respiratory gases and nutrients in vertebrate organisms. In addition, blood helps the entire body to maintain constant temperature and pH. Blood circulates in a closed vascular system composed of heart, arteries, veins and capillaries. The pulsatile laminar blood flow is supported by the pressure provided by the heart (Davies 2007). With the exception of the pulmonary artery, blood circulating in arteries, transports oxygen and nutrients to the tissues, while venous blood flows back to the heart and transports carbon dioxide and other waste products for excretion. Because of its paramount importance, the cardiovascular system is the first organ to develop in a vertebrate embryo (Farrell 2001).

Blood consists of cellular components and liquid medium known as plasma. The cellular components are the red blood cells, white blood cells and platelets. The function of the red blood cells or erythrocytes is to transport oxygen to the cells and tissues of the organism. The white blood cells, or leukocytes, continuously survey and protect the organism from invading and potentially pathogenic microorganisms. Platelets, also known as thrombocytes, play an imperative role in maintaining the integrity of the circulatory system.

Under normal physiological conditions, blood remains fluid contained in the closed vascular system. If the damage of the vessel occurs, the circulatory system has the

capacity to rapidly clot to prevent blood loss. The natural balance between procoagulant and anticoagulant mechanisms is referred to as hemostasis. Whenever this balance is perturbed, either bleeding (hemorrhage) or clotting (thrombosis) occurs.

The main components of the hemostatic system can be divided into vasculature, platelets and coagulation cascade. When vessel injury occurs, all of these components come into the concerted play in order to prevent blood loss. Immediately following injury, platelets become attached to the site of damage and form a primary hemostatic plug. Damaged vasculature promotes a series of biochemical and morphological changes in platelets leading to their activation and aggregation. Activated platelets provide binding sites for the coagulation enzymes which catalyze formation of fibrin- a central structural component of the blood clot (Mann, Nesheim et al. 1990; Walsh 2006). In all vertebrate organisms, formation of fibrin is achieved by an enzymatic action of thrombin-an ultimate enzyme in the blood coagulation cascade (Sherry, Troll et al. 1954; Jiang and Doolittle 2003; Bode 2006). Finally, the fibrinolytic components of the hemostatic system dissolve the clot and initiate repair of the damaged vasculature to facilitate restoration of normal blood flow (Sherry 1968).

The disorders of hemostasis can be either hereditary in nature or acquired in the course of various diseases, such as atherosclerosis or cancer. Many of the congenital disorders of hemostasis arise from quantitative or qualitative deficiencies in coagulation proteins. Hemophilia A and B, as well as von Willebrand disease are the most common hereditary bleeding disorders resulting from deficiencies of factors VIII and IX, and von Willebrand factor (vWF) respectively (Roberts 1993; Hoffbrand, Moss et al. 2006). Hemophilia symptoms range from mild to severe with spontaneous and life-threatening

bleeding into the joints or the central nervous system (Bishop and Lawson 2004). In severe cases, patients have to undergo a lifetime of clotting factor infusions to control bleeding.

A group of congenital disorders of hemostasis that is opposite to hemophilia, thrombophilia, is characterized by susceptibility to excessive thrombus formation. The most common defects comprise mutations in the genes for coagulation factor V, prothrombin, and protein C, as well as deficiencies in protein S, antithrombin, and the antiphospholipid syndrome (Boekholdt and Kramer 2007).

Acute arterial thrombosis, responsible for myocardial infarction and the majority of strokes, remains the leading cause of death in the industrialized countries. In the USA alone, acute deep vein thrombosis (DVT) and pulmonary embolism (PE) result in 300,000 deaths per year making it the third cause of mortality after myocardial infarction and stroke (Mackman 2008; Tapson 2008). The risk factors for thromboembolism are both genetic and environmental as well as a combination of the two.

Function of endothelium

Most blood vessels are composed of three layers: the tunica intima, tunica media, and tunica adventitia (**Figure 1**) (Karsan 2000). The tunica intima consists of a monolayer of endothelial cells lining the lumen of the vessel and a subendothelial layer. The tunica media is composed of circumferentially arranged smooth muscle cells and a matrix of proteoglycans. The tunica adventitia is primarily composed of fibroblasts, collagen fibers, nerve cells and nutrient capillaries.

Figure 1. Schematic structure of the blood vessel.

The illustration is adapted from www.wikipedia.com. This is a simplified scheme of the blood vessel, it is largely the same for both arteries and veins. The blood vessel is composed of three layers: tunica intima, tunica media, and tunica adventitia. The lumen of the vessels is lined with a monolayer layer of endothelial cells. Collagen, located in the extracellular matrix of adventitia, provides adhesion sites for platelets upon injury of the vessel.

Being in direct contact with the flowing blood, endothelial cells serve as a protective barrier preventing blood from coming into contact with sub-endothelial tissue which is highly thrombogenic. The thrombogenicity of subendothelial matrix is conferred by the proteins expressed in the media and adventitia. Tissue factor (TF) and collagen are the most important molecules conferring the thrombogenic character to the adventitia of the vessel wall (Wilcox, Smith et al. 1989). TF is normally absent from the endothelium but it can be induced by certain cytokines, tumor necrosis factor (TNF) and endotoxin (Rauch and Nemerson 2000). Tissue factor is present at elevated concentrations in atherosclerotic plaques and contributes to a high risk of thrombosis in cardiovascular disease (Wilcox, Smith et al. 1989; Mackman 2008).

Under normal conditions, the endothelial cell (EC) monolayer resists blood clotting by both preventative and inhibitory mechanisms (Gross and Aird 2000). EC's synthesize and secrete several vasoprotective and thromboresistant molecules (Wu and Thiagarajan 1996). Endothelial cells synthesize and release powerful suppressors of platelet activation, secretion and aggregation, prostacyclin (PGI₂), prostaglandin D₂ and nitric oxide (NO) (Moncada, Herman et al. 1977; Marcus, Safier et al. 1991; Moncada, Palmer et al. 1991; Freedman and Loscalzo 2003). Endothelium also expresses ecto-ADPase/CD39, which is instrumental in degrading ADP, another potent platelet agonist (Marcus, Safier et al. 1991).

A single endothelial cell contains up to 100,000 molecules of thrombomodulin on its surface (Maruyama and Majerus 1985). Thrombomodulin plays an important role in neutralizing thrombin activity by converting this enzyme from a procoagulant to an anticoagulant molecule (Walker and Fay 1992). Endothelial cells also express heparan

sulfate-containing proteoglycans (Marcum, Atha et al. 1986). Heparan sulfate assists inhibition of several coagulation enzymes by the natural protease inhibitors, antithrombin III and heparin cofactor II (de Agostini, Watkins et al. 1990). In addition to inhibiting coagulation, endothelial cells assist in the dissolution of fibrin by expressing tissue-type plasminogen activator (Wu and Thiagarajan 1996).

The dysfunction of endothelium has been implicated in a number of disorders such as atherosclerosis, diabetes and hypertension (Winn and Harlan 2005). Activation of endothelial cells under these conditions results in changes in surface protein expression. Upon stimulation by cytokines, endothelial cells can acquire procoagulant properties via expression of tissue factor, plasminogen activator inhibitor-1 (PAI-1) and release of vWF (Stern, Nawroth et al. 1985; Schleef, Bevilacqua et al. 1988). These changes lead to increased recruitment and activation of platelets and leukocytes which can result in inflammation, increased apoptosis and acute thrombosis (Libby 2001).

Role of platelets

Platelets are the smallest cells (2-4 μm in diameter) in the bloodstream derived from megakaryocytes by a complex fragmentation mechanism, referred to as thrombopoiesis (Patel, Hartwig et al. 2005; Battinelli, Hartwig et al. 2007; Junt, Schulze et al. 2007). Platelets are discoid annucleate cells which circulate in blood with a half-life of about 110 hours (Hartwig 2006). The normal average platelet count in humans ranges from 150,000 to 300,000 platelets per microliter (Harker and Finch 1969). A decrease in platelet count below 150,000/ μl results in thrombocytopenia associated with increased

risk of bleeding. Platelet counts greater than 600,000/ μ l can lead to life-threatening thrombotic events (Patel, Hartwig et al. 2005).

Platelets contain two types of specialized organelles called α -granules and dense granules. The alpha granules contain platelet-specific proteins that originate from megakaryocytes as well as proteins absorbed from plasma such as fibrinogen (Louache, Debili et al. 1991). Dense granules store ADP, ATP, serotonin and calcium. Upon platelet activation, the contents of the dense and α -granules are secreted by mechanisms of exocytosis (Holmsen 1994). Alpha granules release fibrinogen, coagulation factor V, vWF, and several growth factors including platelet-derived growth factor. Dense granules release ADP and serotonin (Weiss 1975).

Normally, platelets do not adhere to each other or to the intact endothelium due to its non-thrombogenic properties described earlier. The damaged vasculature recruits and localizes platelets to the site of injury (Coughlin 2005). Recruitment of platelets is accomplished by a number of specific interactions between platelet membrane receptors and the proteins of the subendothelial matrix. Platelets attach to the exposed subendothelium by complex mechanisms involving different receptors and their signaling pathways. Platelet adhesion is affected by shear stress, composition of the endothelial tissue and the extent of the vessel wall damage (Furie and Furie 2005). Cellular adhesion molecules providing binding sites for platelets include collagen, vWF, fibronectin, thrombospondin, laminin and vitronectin (Barnes, Bailey et al. 1980; Turitto, Weiss et al. 1985; Pytela, Pierschbacher et al. 1986; Lawler and Hynes 1989; Andrews, Lopez et al. 1997).

A major platelet receptor complex GPIb-IX-V (25, 000 copies per platelet) mediates adhesion of platelets to the subendothelium through vWF, a multimeric adhesive protein present both in plasma and released from activated platelets (Lopez, Andrews et al. 1998). vWF contains binding sites for collagen as well as for the two major platelet receptors, GPIb and integrin GPIIb/IIIa, also referred to as $\alpha_{IIb}\beta_3$ (Varga-Szabo, Pleines et al. 2008). This interaction is critical for the initial adhesion of platelets to the subendothelium, particularly under conditions of high shear, such as found in atherosclerotic vessels (Jackson 2007). The importance of the GPIb-IX-V complex is underscored by the serious hemostatic defect observed in the Bernard-Soulier syndrome (BSS), a rare inherited platelet disorder, in which patients lack GPIb-IX-V or have dysfunctional receptor (Nurden, Butcher et al. 1974; Phillips and Agin 1977; Nurden and Nurden 2008). Patients with BSS have prolonged bleeding times with symptoms ranging from mild to life-threatening hemorrhagic complications (Pham and Wang 2007). Defects in vWF function are associated with von Willebrand's disease, the most common inherited disorder of hemostasis with highly variable bleeding symptoms (Ewenstein 1997).

The binding of the platelet GPIb to vWF has a fast off-rate and is insufficient to stabilize platelet adhesion (Huizinga, Tsuji et al. 2002). During this "rolling" process, platelets establish contacts with collagen through another receptor, GPVI (Nieswandt and Watson 2003). Platelet adhesion and aggregation is an integrated process involving a number of surface receptors, including integrins, immunoglobulin (Ig)-like receptors, and G-protein-coupled receptors.

Platelet aggregation immediately follows platelet adhesion to the damaged vasculature. The process of platelet aggregation requires agonists such as thrombin or ADP to stimulate changes in platelet integrins (Dorsam and Kunapuli 2004). It is believed that the small amount of thrombin generated during early stages of blood coagulation is sufficient to stimulate platelets (Majerus and Miletich 1978; Monroe, Hoffman et al. 2002).

The most abundant platelet receptor, integrin GPIIb/IIIa (~ 80,000 copies per platelet), exists in a low-affinity state in unstimulated platelets (K_D in micromolar range) (Shattil, Ginsberg et al. 1994). Within seconds after platelet activation, a conformational change in GPIIb/IIIa enables it to bind to a specific amino acid sequence RGD (Arg-Gly-Asp) found in fibrinogen (Pytela, Pierschbacher et al. 1986; Phillips, Charo et al. 1988; Thiagarajan and Kelly 1988; Calvete 1999). The fibrinogen molecule, having multiple binding sites, cross-links adjacent platelets to form aggregates. Aggregation further activates outside-in signaling pathways in platelets (Weiss, Turitto et al. 1991; Schwartz, Schaller et al. 1995). The importance of GPIIb/IIIa interaction with fibrinogen is underscored by Glanzmann thrombasthenia, a rare, but serious bleeding disorder resulting from impaired platelet aggregation (Phillips and Agin 1977; Coller, Seligsohn et al. 1994; Woodside, Liu et al. 2001).

Platelets can be activated by thrombin, ADP, collagen, epinephrine, thromboxane A_2 , serotonin and platelet activating factor 4 (PAF4) (Born 1962; Holmsen 1994). These molecules act in autocrine and paracrine fashion to amplify the procoagulant response to the vascular damage. Thrombin is the most potent platelet agonist (Coughlin, Vu et al. 1992). Thrombin acts upon protease-activated receptors (PARs). PAR1 is the most

important thrombin receptor in human platelets (Sambrano, Weiss et al. 2001). Action of thrombin, ADP and TXA₂ upon the PAR1, P2Y₁₂, and thromboxane receptors respectively, leads to an increased cytoplasmic Ca²⁺ concentration (Vu, Hung et al. 1991; Hirata, Ushikubi et al. 1996; Hollopeter, Jantzen et al. 2001). Once activated, platelets undergo a dramatic change in shape from discoid to spherical, accompanied by formation of multiple protruding filopodia (Woodside, Liu et al. 2001). Platelet shape changes are accompanied by dramatic rearrangements in cytoskeleton assembly, actin polymerization and membrane reorganization (Phillips, Charo et al. 1991). Upon activation, the anionic lipid phosphatidyl serine shifts from the inner to the outer leaflet of the platelet membrane (Zwaal, Comfurius et al. 1998). This mechanism exposes the binding sites for several coagulation proteins and cofactors which dramatically increase the efficiency of the reactions leading to thrombin generation via the coagulation cascade (reviewed below) (Heemskerk, Bevers et al. 2002).

Increased platelet activation such as that which occurs in atherosclerosis is associated with the risk of developing cerebral stroke or myocardial infarction (Ruggeri 2002; Jorgensen 2006). Several anti-platelet drugs are currently used for prevention and treatment of arterial thrombosis. Aspirin, targeting TXA₂ production by cyclooxygenase-1, has been effective for prevention of thrombosis in coronary, cerebrovascular and peripheral arterial disease (Bhatt and Topol 2003). ADP-induced platelet activation is another target for pharmacological agents. Two of the currently FDA-approved drugs, ticlopidine and clopidogrel are irreversible inhibitors of ADP binding to the P2Y₁₂ platelet receptor (Michelson 2008). PAR1 antagonists comprise the novel class of drugs currently in phase II clinical trials (Mackman 2008).

Coagulation proteins and the coagulation cascade

Centuries before the modern research tools became available to scientists, it was known that blood congeals upon contact with glass. It was hemophiliacs and the patients whose blood had abnormally long clotting times in clinical tests, who have paved the way to the discovery of the coagulation proteins or factors. Historically, all of the coagulation proteins have been named factors. All of these proteins are synthesized and circulate in bloodstream as inactive precursors. They become activated by a limited proteolysis of one or two peptide bonds (Furie and Furie 1988). In their activated form, some of them act as enzymes (FIIa, FVIIa, FIXa, FXa, FXIa, FXIIa) while some act as cofactors, like FVa, FVIIIa, and high molecular weight kininogen (HK). As shown in **Figure 2**, all coagulation enzymes are multidomain proteins with significant variability in the N-terminal domain structure, but having a C-terminal region highly homologous to trypsin (Patthy 1985). With the exception of FXIII, all of the coagulation enzymes are serine proteases that catalyze peptide bond hydrolysis employing exactly the same chemistry as their closest homologues trypsin and chymotrypsin (Furie and Furie 1988). Unlike the digestive proteases, which are relatively non-specific, the coagulation enzymes are highly specific towards their substrates (Page, Macgillivray et al. 2005). The specificity arises from the variability in protein architecture surrounding the active site as well as from additional regions (exosites), located remote from the serine protease active site (Patthy 1985; Hedstrom 2002; Krishnaswamy 2005). Cofactor proteins FVIIIa, FVa, TF, HK, and protein S dramatically enhance the activity of coagulation proteases via elaborate allosteric mechanisms.

Figure 2. Domain organization in coagulation proteins.

All coagulation factors are synthesized as inactive precursors. Site of removal of the signal peptide is indicated. Sites of proteolytic activation by respective enzymes are shown with arrows. Triangles indicate the sites of removal of the proleader sequence. Figure taken from <http://tollefsen.wustl.edu/projects/coagulation/coagulation.html>

The group of vitamin K-dependent coagulation factors, which includes prothrombin, factors VII, IX, FX, protein C, and a cofactor protein S, plays a central role in hemostasis. The N-terminal region of these proteins is involved in a Ca^{2+} -mediated binding to the negatively charged phospholipids on the cell surface membrane (Sunnarhagen, Drakenberg et al. 1996; Flaumenhaft and Furie 2000). This critical function is dependent on the GLA domain, an entity that contains multiple (10-12) γ -carboxy-glutamic acid residues whose synthesis depends on vitamin K (Stenflo, Fernlund et al. 1974). Vitamin K is required for this post-translational modification by a vitamin K-dependent carboxylase (Burnier, Borowski et al. 1981).

Upon the damage of the vessel, the primary platelet plug formation is usually insufficient to resist the blood flow. Coagulation proteins engage in a series of enzymatic reactions leading to a formation of an insoluble fibrin clot, which further stabilizes the platelet plug (Furie and Furie 2000). The series of enzymatic reactions leading to conversion of fibrinogen to fibrin is referred to as the coagulation cascade (Davie, Fujikawa et al. 1991).

In 1964, two groups independently of one another described the waterfall or cascade concepts of coagulation reactions (Davie and Ratnoff 1964; MacFarlane 1964). According to these concepts, the activation of clotting factors proceeds via sequential activation of downstream substrates which serve as the enzymes for the succeeding reactions (**Figure 3**). This was a key discovery explaining the amplification resulting in a burst of thrombin generation. Following the discovery of the tissue factor (TF), the cascade has undergone several revisions.

Figure 3. The blood coagulation cascade.

The coagulation proteins or factors are labeled with Roman numerals. Activated forms of coagulation factors designated by adding a subscript “a”. The arrows indicate a conversion of zymogens to their respective active serine proteases. PL indicates platelets. The enzymes of blood coagulation include thrombin, FVIIa, FIXa, FXa, FXIa, and FXIIa. The cofactors are FV, FVIIIa and TF. FXIIIa is the only non-protease enzyme in the cascade. The illustration is taken from (Davie 2003).

Historically, the coagulation cascade has been divided into two major parts- extrinsic and intrinsic. The term “extrinsic” means that the principal components of this pathway are not found in blood; while the term “intrinsic” indicates that all the necessary components are already present in blood (Furie and Furie 1988). The extrinsic pathway is initiated when blood comes into contact with tissue factor expressing cells.

As indicated in **Figure 3**, these pathways are intertwined by complex feedback mechanisms which have direct impact on the final stages of coagulation process, i.e. thrombin generation and fibrin formation (Davie 2003). It is now generally accepted that the extrinsic pathway plays the major role in the initiation of blood coagulation following vascular injury and primary platelet plug formation (Davie 2003).

Tissue factor is an integral membrane glycoprotein located in the adventitia and functions as a receptor for factor VII (or VIIa) circulating in blood. In the next step, it appears likely that factor Xa activates some of the factor V to factor Va and forms a complex with this activated protein in the presence of calcium and cell membrane phospholipid (PL). This complex then converts some prothrombin to thrombin. These reactions are referred to as "the initiation and priming phase" in the cell-based model of coagulation (Roberts, Monroe et al. 1998). Initiation of the intrinsic pathway leads to an increase in the concentration of factor IXa, factor Xa, and a burst in thrombin generation. This is followed by an increase in the conversion of fibrinogen to fibrin and factor XIII to factor XIIIa. Factor XIIIa then cross-links the fibrin monomers forming a tough insoluble clot that stabilizes the platelet plug. The activation of the intrinsic pathway occurs when blood comes into contact with glass or other anion surfaces. Both extrinsic and intrinsic pathways converge at the so-called common pathway.

The central enzyme of coagulation cascade is thrombin, also termed FIIa. Activation of thrombin occurs in two steps, in which its precursor prothrombin is first cleaved at Arg320 to produce meizothrombin followed by the cleavage at Arg271 to generate the thrombin molecule (Lane, Philippou et al. 2005; Crawley, Zanardelli et al. 2007). In addition to catalyzing the fibrin formation, thrombin also functions in dissolving excess fibrin and in inflammation.

The ultimate target of thrombin is fibrinogen, a 340 kDa glycoprotein circulating at the highest molar ($\sim 9 \mu\text{M}$) concentration among all coagulation proteins (Doolittle 1984; Lord 2007). Fibrinogen is an extended dimeric molecule (450Å long) in which three polypeptides, i.e. α -, β and γ chains are arranged in antiparallel orientation (Clegg and Bailey 1962; Hoeprich and Doolittle 1983; Doolittle 1984). Thrombin converts fibrinogen to fibrin by a sequential hydrolysis of the α -chain and the β -chain, releasing fibrinopeptides A and B respectively, while the γ -chains remain unchanged (Litvinov, Gorkun et al. 2005). The exposure of the new sites in fibrin monomer triggers non-covalent association into dimers, and then, into protofibrils (Blomback, Hessel et al. 1978). Protofibrils associate laterally forming thick fibers which form insoluble aggregates constituting the fibrin clot (Weisel 2008). Once fibrin starts to form, it accelerates the conversion of FXIII to FXIIIa by thrombin. FXIIIa (320 kDa) is a transglutaminase which catalyzes covalent cross-linking of adjacent fibrin monomers via formation of γ -glutamyl- ϵ -lysyl amide bonds between them (Folk and Finlayson 1977).

Tissue Factor Pathway

The tissue factor pathway plays a key role in initiation of blood coagulation.

Tissue factor (thromboplastin, CD142, formerly coagulation factor III) is an integral membrane glycoprotein constitutively expressed by the fibroblasts and smooth muscle cells (Maynard, Heckman et al. 1975; Weiss, Turitto et al. 1989; Rauch and Nemerson 2000). Normally absent from unstimulated endothelium, tissue factor becomes exposed to the circulating blood during vascular injury. Exposed TF rapidly forms a complex with FVII or FVIIa. The TF/FVIIa complex can then activate FX to FXa and FIX to FIXa (Osterud and Rapaport 1977; Nemerson and Repke 1985; Rao and Rapaport 1988; Rauch and Nemerson 2000). TF and calcium ions act as cofactors in the TF/FVIIa complex, strongly enhancing the activity of FVIIa toward its substrates via an allosteric mechanism (Nemerson and Gentry 1986; Bom and Bertina 1990; Banner, D'Arcy et al. 1996).

Activation of FX to Xa occurs by a proteolytic cleavage of an Arg52-Ile53 peptide bond with a release of a 52 aa peptide (Di Scipio, Hermodson et al. 1977). In addition to the TF pathway, FXa is also generated by the so-called tenase complex (described below).

Factor Xa forms 1:1 complex with a cofactor molecule, factor Va, in the presence of calcium and phospholipids. This complex converts prothrombin to thrombin by hydrolyzing the bonds Arg323-Ile324 and Arg274-Thr275 in a sequential manner. The complex formation between FXa and FVa increases the $V_{\max}$ of the reaction by about 1000-fold (Krishnaswamy, Church et al. 1987). Importantly, the generated thrombin is devoid of the membrane binding domain and can diffuse away from the surface to participate in the intrinsic pathway and platelet activation.

Contact factor or intrinsic pathway

When blood comes in contact with glass or other negatively charged materials such as kaolin, asbestos or celite, it clots within a few minutes. If a glass tube is precoated with paraffin or silicone, plasma does not congeal for more than 24 hours. Upon contact with glass, FXII becomes activated, and the resulting enzyme, FXIIa initiates the intrinsic pathway of coagulation (Ratnoff and Rosenblum 1958). The contact activation pathway also includes high molecular weight kininogen (HK), plasma kallikrein (PK) and FXI (Waldmann, Abraham et al. 1975; Colman 1984). The lack of abnormal bleeding in patients deficient in HK, PK, and FXII has led to the conclusion that these proteins are not required for physiological coagulation (Cochrane and Griffin 1982). Originally viewed as a principal initiating event in coagulation, the intrinsic pathway has undergone a significant revision. In 1991, it was demonstrated that FXI can be more effectively activated by thrombin than by FXIIa in the presence of the negatively charged polysaccharide dextran sulfate (Gailani and Broze 1991). **Figure 3** reflects the current model of the blood coagulation cascade. The revised model of the coagulation cascade states that FXI in the intrinsic pathway serves to propagate and consolidate the procoagulant response via a feedback mechanism. The modern view of coagulation postulates that the initially small amount of thrombin generated by the TF pathway is rapidly and effectively inactivated by TFPI synthesized by the apical granules in endothelial cells (Wu and Thiagarajan 1996; Walsh and Ahmad 2002). TFPI forms a quaternary complex consisting of TFPI, FXa, FVIIIa and TF which turns off FXa generation (Broze, Warren et al. 1988; Girard, MacPhail et al. 1990). The nature of molecular events leading to activation of the intrinsic pathway *in vivo* is still not clear.

The fact that the genes for factor XI, prekallikrein and FXII are not found in fish, suggests that they have evolved very recently (Jiang and Doolittle 2003). The question remains: what is the evolutionary advantage of these proteins in mammals? Recent studies with FXII- and FXI-deficient mice have demonstrated that these animals have defective arterial thrombus formation (Renne, Pozgajova et al. 2005). These observations raise a possibility that the contact factor pathway does play a significant role in thrombosis but not in normal hemostasis.

The modern concept of blood coagulation

Although the cascade model successfully explained the observations from the *in vitro* laboratory coagulation tests, major flaws remained in explaining hemostatic defects *in vivo*. Prolongation in APTT-clotting times resulting from deficiencies in FXII or HK did not correlate with the lack of bleeding problems in the patients deficient in these proteins. At the same time, patients with FIX or FVIII deficiency have severe bleeding complications, while their extrinsic pathway is completely functional. The modern concept of blood coagulation describes this process as a more complex and unified scheme consisting of three stages: initiation (or priming), propagation (or amplification), and consolidation (Roberts, Monroe et al. 1998).

In the initiation phase, the TF-FVIIa complex generates FXa and FIXa which play distinct roles in clotting. FXa remains on the surface of the TF-bearing cell and forms a complex with FVa which generates picomolar amounts of thrombin. At such a low concentration, thrombin is not sufficient to generate a fibrin clot, but is enough to activate platelets, dissociate FVIII from vWF and activate cofactors FV and FVIII (Roberts,

Monroe et al. 1998). FXI is important in the consolidation phase of the procoagulant response (Gailani and Broze 1991).

The propagation phase of blood coagulation takes place on the surface of the activated platelets (Walsh 2004). FIXa, generated by TF pathway, binds to the activated platelet surface, associates with a cofactor FVIIIa and forms the so-called “intrinsic” FX activating complex, also known as tenase (Walsh and Biggs 1972; Mann, Krishnaswamy et al. 1992). Binding of FX to activated platelets, ($B_{\max} \sim 16,000$, $K_D = 320 \text{ nM}$) along with FIXa and FVIIIa allows optimal co-localization of the proteins leading to a dramatic increase in the catalytic efficiency of the reaction (Scandura, Ahmad et al. 1995). This assembly leads to a 1000-fold increase in K_m and an $\sim 10,000$ -fold increase in k_{cat} (Hultin 1982; Rawala-Sheikh, Ahmad et al. 1990; Gilbert and Arena 1996). Factor Xa, generated by the tenase complex, remains associated with the platelet surface and binds its cofactor FVa in a Ca^{2+} dependent manner (Miletich, Jackson et al. 1977; Bouchard, Catcher et al. 1997). The FVa/FXa complex bound to the phospholipid membrane, also known as prothrombinase, generates a sufficient amount of thrombin required for fibrin formation (Tracy, Eide et al. 1985; Krishnaswamy, Church et al. 1987). The reaction proceeds via two cleavages in the prothrombin molecule. The first cleavage takes place at Arg320-Ile321, and is followed by subsequent cleavage at Arg271-Thr272. Factor Va increases the $V_{\max}$ of this reaction by 1000-fold, whereas the phospholipid surface decreases the K_m for prothrombin about 1400-fold (Krishnaswamy, Church et al. 1987). The phospholipid membrane can be provided not only by the activated platelets, but also by monocytes or neutrophils (Tracy, Eide et al. 1985).

The assembly of the coagulation enzyme-cofactor complexes on the phospholipid membrane leads to a reduction in the apparent K_m for the substrate and an increase in the k_{cat} of the reaction (Mann, Nesheim et al. 1990). Activated platelets assist the coagulation process by providing a phospholipid membrane surface for assembly of coagulation factor complexes (Heemskerk, Bevers et al. 2002; Walsh 2006). The initial formation of thrombin is followed by a second explosive phases of thrombin generation. This is accomplished both by the feedback mechanism of FXI activation and activation of platelets (Kahn, Zheng et al. 1998). This provides a potential explanation for why deficiency in PK, FXII or HK does not lead to bleeding complications. Thus, cellular components play critical roles in both initiation and propagation phases of blood coagulation in which formation of the intrinsic FX activating complex is required for the sufficient amount of thrombin to be generated. This unified model of coagulation cascade better explains why patients deficient in FIX or FVIII have severe hemophilia.

For the treatment of the hemophilia patients in the USA, frequent infusions of plasma derived or recombinant FVIII or IX are administered. As a more advantageous alternative, gene therapy is an actively pursued strategy for treatment of both hemophilias A and B (Kaufman 1999; High 2007). In order to control bleeding during surgery in non-hemophiliac patients, recombinant factor VIIa is now successfully used (Aldouri 2002).

Control of coagulation

Under normal physiological conditions, generation of thrombin is tightly regulated by several mechanisms. Plasma contains natural inhibitors of coagulation in a large molar excess compared to coagulation factors (Mann, Nesheim et al. 1990). Three

inhibitory proteins play particularly important roles in coagulation, antithrombin III, protein C and tissue factor pathway inhibitor or TFPI (Esmon 2000).

Tissue factor pathway inhibitor (TFPI), formerly known as LACI (lipoprotein associated coagulation inhibitor) is critically important for the inhibition of the TF/VIIa complex at the earliest stages of coagulation (Wun, Kretzmer et al. 1988; Novotny, Girard et al. 1989; Girard, MacPhail et al. 1990). TFPI (Mr~43 kDa) belongs to a family of kunitz protease inhibitors and contains three kunitz domains (Girard, Warren et al. 1989). TFPI is secreted by the endothelium and circulates in blood at a relatively low concentration of 2.5 nM (Novotny, Girard et al. 1989; Bajaj, Kuppuswamy et al. 1990). TFPI is also found in resting platelets and is released upon simultaneous stimulation by collagen and thrombin (Bajaj, Kuppuswamy et al. 1990; Maroney, Haberichter et al. 2007). Only about 10% of TFPI is present as a full length protein in plasma, while the rest of the protein is present in a truncated form, which has a weaker inhibitory activity and binds to glycosaminoglycans with lower affinity (Crawley and Lane 2008). TFPI forms a complex with FXa, which results in a conformational change in the inhibitor molecule making it a more potent inhibitor of TF/VIIa. Thus, TFPI directly regulates two major proteases in the initiation phase of coagulation.

The most physiologically important inhibitor of coagulation is antithrombin III (ATIII) which inhibits all coagulation proteases, except FVIIa (Pike, Buckle et al. 2005). Deficiencies or detrimental mutations in this protein have been linked to an increased risk of thrombosis (Beauchamp, Pike et al. 1998; Raja, Chhablani et al. 2003). ATIII (58 kDa) is synthesized by the liver and circulates in plasma at concentration of 2.4 μ M (150 μ g/ml) (Rau, Beaulieu et al. 2007). In the organism 40% of antithrombin III circulates in plasma,

while the rest is associated with the vasculature (10%) and extravascular pool (50%) (Mammen 1998). Antithrombin belongs to a class of inhibitors referred to as serpins (serine protease inhibitors) which act as suicide substrates for the proteolytic enzymes (Gettins 2002). The primary targets of ATIII are thrombin and FXa. ATIII also inhibits factors IXa, XIa, and XIIa. Antithrombin by itself, however, is a poor inhibitor of any of these enzymes. ATIII circulates in an inactive conformation, which is converted to the activated form upon binding to heparin or heparan sulfate. Binding of ATIII to heparin leads to a 1000-fold enhancement in its inhibitory activity towards proteases (Craig, Olson et al. 1989). Inactivation of proteases by ATIII involves formation of a covalent bond between the P1 residue and the active site serine residue (Bjork and Olson 1997; Rau, Beaulieu et al. 2007). The acyl-enzyme becomes trapped and deacylation is impaired because the oxyanion hole of the enzyme is deformed (Huntington 2006).

In addition to ATIII, α_2 -macroglobulin, protein C inhibitor, heparin cofactor II, protein Z dependent inhibitor, and C1 inhibitor also contribute to anticoagulant mechanisms (Tollefsen, Majerus et al. 1982; Olson, Bock et al. 1995; Han, Fiehler et al. 2000).

Finally, the function of thrombin can be transformed from a procoagulant into an anticoagulant. This completely opposite functionality is achieved via an intricate molecular mechanism by which thrombin alters its substrate specificity (Di Cera 2007). Once thrombin forms a complex with thrombomodulin (TM) expressed by the endothelium, thrombin can no longer cleave fibrinogen or activate FV, FXIII and platelets (Esmon 2006). TM binding to thrombin blocks access of procoagulant substrates while leaving the active site available (Fuentes-Prior, Iwanaga et al. 2000). Thrombin

binding to TM stimulates activation of anticoagulant protein C (Stenflo 1976; Owen and Esmon 1981). Activated protein C (APC), in turn, forms a complex with protein S, which degrades the activated FVIII and FV thereby inhibiting blood coagulation (DiScipio and Davie 1979; Hackeng, Hessing et al. 1993). Thrombomodulin contains the sulfated glycosaminoglycan, chondroitin sulfate, which accelerates thrombin inhibition by ATIII and heparin cofactor II (Bourin, Ohlin et al. 1988; Bourin and Lindahl 1990). Interestingly, thrombin bound to a fibrin clot is protected from inhibition by the heparin-ATIII complex (Hogg and Jackson 1989; Weitz, Hudoba et al. 1990).

Fibrinolysis

Once the clot is formed and the injury has been localized, there is a need for subsequent removal of the fibrin clot in order to restore normal blood flow. The dissolution of fibrin, or fibrinolysis, begins within a few hours after deposition of fibrin. Fibrinolysis is catalyzed by a serine protease, plasmin (92 kDa), which is generated from plasminogen (Collen 1999). Activation of plasminogen proceeds by cleavage of the peptide bond Arg560-Val561 (Robbins, Summaria et al. 1967). Plasmin catalyzes the hydrolysis of peptide bonds in fibrin with preference for Arg and Lys residues. The main endogenous plasminogen activators are tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Under normal conditions, the endothelium secretes tissue plasminogen activator (Levin and Loskutoff 1982). The activation of plasminogen by t-PA is highly dependent on the presence of fibrin, which binds to and alters the conformation of plasminogen (Kolev and Machovich 2003). Plasma kallikrein can also activate urokinase thereby providing a link between coagulation and fibrinolysis.

Control of fibrinolysis is achieved through direct inhibition of plasmin as well as inactivation of plasminogen activators. Plasminogen activator inhibitor 1 (PAI-1) is the most important physiological inhibitor of fibrinolysis (Fay 2004). Synthesized by endothelial cells and adipocytes, PAI-1 controls fibrinolysis by inactivating both t-PA and u-PA (Kruithof, Tran-Thang et al. 1986). Importantly, platelets contain PAI-1 in their α -granules (Erickson, Ginsberg et al. 1984). Even though up to 80% of PAI-1 in platelets is present in the latent form, it can be secreted in sufficient amounts to inhibit local fibrinolysis (Fay, Eitzman et al. 1994). Plasmin is also directly inactivated by a serpin α -2-antiplasmin (Christensen, Bangert et al. 1996). Thrombin activatable fibrinolysis inhibitor (TAFI) is a basic carboxypeptidase that cleaves C-terminal lysine residues from fibrin. After being activated by thrombin, TAFI inhibits fibrinolysis by inhibiting binding of plasminogen to the fibrin surface (Nesheim, Wang et al. 1997).

Heparin as an anticoagulant

Heparin is a classical anticoagulant, widely used to reduce thrombosis in cardiovascular and orthopedic surgery as well as hemodialysis. Heparin is also used for prophylaxis and treatment of deep venous thrombosis (DVT), pulmonary embolism (PE) and acute coronary syndrome (Hirsh and Lee 2002; Warkentin and Crowther 2005; Gross and Weitz 2008). Heparin is also used for coating medical devices and blood collection tubes. Since its discovery in 1916, heparin remains a major anticoagulant drug in clinical practice.

Unfractionated heparin (UFH) is a heterogeneous mixture of highly sulfated polysaccharides ranging in molecular mass from 3 to 30 kDa (Johnson and Mulloy 1976).

Low molecular weight heparin (LMWH) obtained by chemical or enzymatic depolymerization varies in molecular mass from 2,000 to 9,000 Da.

Heparin is a highly negatively charged glycosaminoglycan composed of alternating disaccharide units of uronic acid and amino-substituted sugar such as N-acetyl-D-glucosamine (**Figure 4**). The uronic acid can be either α -L-iduronic or β -D-glucuronic acid (Rabenstein 2002). The glucosamine moiety may be either N-sulfated (GlcNS) or N-acetylated (GlcNAc), both of which may contain 6-O-sulfate.

Figure 4. Chemical structure of heparin.

Shown is the most common repeating disaccharide unit in heparin, containing 2-O-sulfo-L-iduronic acid (IdoA(2S)) and N-sulfo-D-glucosamine-6-sulfate (GlcNS(6S)) connected via α 1-4 glycosidic bond.

Heparin binding to antithrombin III (ATIII) strongly enhances anticoagulant effect of the inhibitor (Abildgaard 1968). Heparan sulfate (HS) is a less sulfated analogue of heparin that is believed to localize AT to the endothelium thereby facilitating its anticoagulant properties (de Agostini, Watkins et al. 1990). Both heparan sulfate and heparin acquire sulfate groups by the enzymatic process which replaces N-acetyl by N-

sulfate groups (Kusche, Backstrom et al. 1988). This function is carried out by a single bifunctional enzyme N-deacetylase/N-sulfotransferase (Strott 2002).

Unfractionated heparin binds to ATIII with very high affinity ($K_D \sim 6 \times 10^{-8}$ M). Heparin contains a unique pentasaccharide sequence which presents a high-affinity binding site for antithrombin III (Lindahl, Backstrom et al. 1979; Rosenberg and Lam 1979). In UFH, only about 30% of the molecules contain this unique binding site. The binding of ATIII to heparin has been extensively studied by both mutational and crystallographic analyses (Koide, Odani et al. 1984; Arocas, Bock et al. 1999). The interaction involves a two-step mechanism. Initial weak binding to the pentasaccharide sequence is followed by a transition of ATIII into a high affinity state. This induced-fit mechanism leads to a 1000-fold enhancement in affinity for heparin resulting in the expulsion of the reactive center loop (RCL) in the ATIII. The binding of heparin pentasaccharide to ATIII results in a 500-fold acceleration in inhibition of the two major coagulation proteases- FXa and FIXa. Thrombin inhibition, however, is not affected by this mechanism. The smallest heparin fragment has to contain at least 18 units (~5400 Da) in order to inhibit thrombin (Lane, Denton et al. 1984). Instead, thrombin inhibition is potentiated by formation of a ternary complex between thrombin, ATIII and heparin. This bridging mechanism leads to a reduction of dimensional freedom from three to one degree, thereby accelerating the inhibition of thrombin (Rau, Beaulieu et al. 2007).

Bleeding is the most common side effect of caused by administration of heparin. heparins have higher risk (7%) of causing heparin induced thrombocytopenia (HIT), while LMWH have lower incidence of about 3% (Meissner 2005). Heparin induced thrombocytopenia (HIT) is associated with decreased platelet count (less than

150,000/ml). Two types of thrombocytopenia occur in patients, I and II. Type I HIT is characterized by a decrease in platelet count within first three days of heparin therapy and does not require an interruption in administering of the anticoagulant as the platelet counts return to normal. HIT type II results from formation of heparin-platelet factor 4-antibody complexes. These complexes bind to the platelet Fc receptors causing platelet activation and formation of procoagulant microparticles (Meissner 2005). Type II HIT is associated with a high risk of thrombosis. Prolonged use of heparin may also induce osteoporosis (Kher, Bauersachs et al. 2007).

In addition to the complications mentioned above, potential shortage of raw materials of animal origin as well as a recent crisis resulting from heparin contamination point to a need for alternative anticoagulants (Guerrini, Beccati et al. 2008; Kishimoto, Viswanathan et al. 2008). The solution of both problems can be addressed as exemplified by a successful use of synthetic heparin pentasaccharide known as fondaparinux (Arixtra) (Petitou and van Boeckel 2004; Kemsley 2008).

Factor XI in hemostasis

Coagulation factor XI, formerly known as hemophilia C factor or plasma thromboplastin antecedent (PTA) was originally discovered in a patient with a bleeding disorder (Rosenthal, Dreskin et al. 1955; Monroe, Hoffman et al. 2007). Factor XI has been traditionally regarded as a component of the intrinsic pathway blood coagulation (Walsh 2003). As mentioned earlier, patients deficient in contact pathway proteins FXII, PK, and HK do show abnormally prolonged APTT, yet they do not have bleeding complications. About 50% of FXI deficient patients suffer from bleeding to various

extents, but only after surgery or trauma (Rosenthal, Dreskin et al. 1955; Rapaport, Proctor et al. 1961). Unlike hemophilia A and B, FXI deficiency has a much less pronounced phenotype. In contrast to hemophilia A and B patients, spontaneous bleeding into joints does not occur in FXI deficiency (Bouma and Griffin 1977). The physiological role of FXI remains a subject of ongoing investigation.

Several studies have demonstrated that the elevated levels of FXIa correlate with an increased risk of deep vein thrombosis (DVT) (Meijers, Tekelenburg et al. 2000; Berliner, Rybicki et al. 2002; Bezemer, Bare et al. 2008). Recent clinical data demonstrate that the patients with acute coronary artery disease have elevated levels of FXIa circulating in blood (Butenas, Undas et al. 2008)

The exact mechanism of FXI activation *in vivo* is still not established and is a subject of current research. It has been suggested that the more physiologically relevant FXI activation pathway in plasma might be via feedback activation by thrombin or possibly by autoactivation by FXIa (Gailani and Broze 1991; Naito and Fujikawa 1991). Recent study, however, challenges this hypothesis (Pedicord, Seiffert et al. 2007).

In vivo studies in animal models have clearly demonstrated that FXI plays an important role in augmenting pathological thrombus formation (Gruber and Hanson 2003; Kravtsov, Wu et al. 2004). Importantly, the removal of FXI activity either by gene knock-out in mice or by injecting antibody against FXI in baboons have antithrombotic effects without affecting the bleeding times (Gruber and Hanson 2003; Wang, Cheng et al. 2005; Wang, Smith et al. 2006). Taken together, these observations provide convincing evidence that coagulation factor XI/XIa represents an attractive target for antithrombotic therapy (Goto 2006; Wang, Smith et al. 2006).

Human factor XI circulates in human plasma at a concentration of approximately 30 nM (4-6 µg/mL) as an inactive zymogen in complex with the high molecular weight kininogen (HK) and has a plasma half-life of about 80 hours (Koide, Kato et al. 1977). In plasma, HK is believed to mediate the binding of FXI to the negatively-charged surfaces (Thompson, Mandle et al. 1977; Wiggins, Bouma et al. 1977). An alternatively spliced isoform of FXI is produced by platelets and megakaryocytes but absent from other cell types (Hsu, Shore et al. 1998).

The human gene for FXI is located on the long arm of chromosome 4 (4q35) and is composed of 15 exons and 14 introns (Asakai, Davie et al. 1987; Kato, Asakai et al. 1989). Exons III to X encode four apple domains, while exons XI through XV encode the serine protease catalytic domain of FXI. The precursor of FXI protein is translated with a 18 amino acid leader sequence, which is subsequently removed upon secretion resulting in mature protein of 607 amino acids. There are five potential N-glycosylation sites in FXI, namely N72, N108, N335, N432, N437. The amino acid sequence of the entire mature FXI polypeptide is shown in **Figure 5**. The light chain of FXI (aa 370-607) comprises the catalytic serine protease domain with a characteristic chymotrypsin-like fold (Jin, Pandey et al. 2005). The cDNA sequence of FXI shares 58% identity with human plasma prekallikrein (Fujikawa, Chung et al. 1986). Both PK and FXI genes are located on the long arm of chromosome 4 separated by less than ten kilobases. Such close proximity suggests that one of the genes has been duplicated approximately 124 million years ago, coincident with the age of mammals (Veloso, Shilling et al. 1986).

Figure 5. Amino acid sequence and primary structure of human coagulation factor XI.

The heavy chain of FXI contains four apple domains (90-91 a.a.). The arrow indicates the site of proteolytic cleavage at Arg369-Ile370 leading to activation of FXI. The residues of the catalytic triad residues His413, Asp462, and Ser557 are circled. The glycosylation sites N72, N108, N432 and N472 are shown as diamonds. Apple domains are 23-34% identical to one another. Figure taken from www.accessmedicine.com

Structure and function of FXI

Human FXI consists of two identical subunits, held together by a disulfide bond, and has a total molecular mass of 160 kDa (Bouma and Griffin 1977). Each FXI subunit consists of 607 amino acids, which form its light and heavy chains (Fujikawa, Chung et al. 1986). The amino acid sequence of FXI shows 58% identity to plasma prekallikrein (PK), a zymogen of another serine protease which acts on the different substrates and does not seem to play a role in coagulation (Fujikawa, Chung et al. 1986). Unlike FXIa, which activates FIX, plasma kallikrein (EC 3.4.21.34) triggers release of a vasoactive peptide from HK referred to as bradykinin (Bouma, Miles et al. 1980; Chung, Fujikawa et al. 1986). In addition to blood pressure control, plasma kallikrein participates in a number of physiological processes such as inflammation and fibrinolysis (Fujikawa, Chung et al. 1986).

The heavy chain of FXI contains four homologous repeats called apple domains (A1-A4) which are involved in the interactions with other coagulation enzymes, HK, heparin and platelets (Fujikawa, Chung et al. 1986; Walsh and Gailani 2006). The apple domains in FXI and PK demonstrate a disulfide bonding pattern similar to that of so-called PAN modules found in plasminogen, hepatocyte growth factor and several nematode proteins (Tordai, Banyai et al. 1999). The common structural motif in the PAN module is a central β -sheet sandwiched by an α -helix and loops or additional beta strands on the opposite sides (Brown, Mulvey et al. 2003). Despite structural similarities, there are significant functional differences between the proteins containing PAN modules.

Each apple domain in FXI contains 90-91 residues. Apple domains 1-3 have the same disulfide bond pattern in which the disulfide bonds are formed between the first and

sixth, the second and fifth and the third and fourth cysteine residues. The A1 domain contains an extra cysteine at position 11 that forms a disulfide bond with a free cysteine. The A4 domain has a cysteine at the position 326 which forms disulfide with Cys462 of the FXI light chain.

The apple 1 domain (Glu1-Ser90) has been shown to bind to HK and prothrombin (Baglia, Jameson et al. 1990; Seaman, Baglia et al. 1994; Baglia and Walsh 1996; Baglia, Badellino et al. 2000). Also, the monoclonal antibody 5F7 recognizes the A1 domain (Sinha, Koshy et al. 1985). The A2 domain (A95-L180) has been implicated in binding to the physiological substrate of FXIa, factor IX (Baglia, Sinha et al. 1989; Baglia, Jameson et al. 1991). A synthetic peptide corresponding to the residues Asn145-Ala176 in the A2 domain has been shown to be a competitive inhibitor of FIX activation by FXIa (Baglia, Jameson et al. 1991). The A2 domain of PK, along with A4 domain, has been shown to mediate the interaction with HK (Renne, Sugiyama et al. 2002). Both apple 2 and apple 4 domains participate in binding to HK (Renne, Gailani et al. 2002). Interestingly, A2 domain in PK contains an additional cleavage site which can be cleaved by FXIIa.

The A3 domain (A181-V271) has also been reported to contain FIX binding sites (Sun and Gailani 1996; Sun, Zhao et al. 1999). A chimeric protein with A3 domain replaced by that of plasma prekallikrein has been shown to be defective in FIX binding and activation (Sun and Gailani 1996). Apple 3 domain contains a heparin consensus binding sequence (²⁵²KKSK²⁵⁵) which has been shown to mediate binding to heparin (Ho, Badellino et al. 1998; Zhao, Abdel-Razek et al. 1998). Using mutational analysis, Gailani et. al concluded that the residue Lys253 was the most important in heparin binding in zymogen FXI. In addition, several residues in the A3 domain, namely Arg250, Lys255,

Phe260 and Gln263 were shown to mediate binding of FXI to activated platelets (Ho, Baglia et al. 2000). The proposed platelet receptor for FXI binding is GP1b α (Baglia, Badellino et al. 2002).

The A4 domain (residues 271-360) is known to be the primary determinant for homodimerization in FXI. The dimeric structure of FXI is unique among all coagulation proteins. Residue Cys321 mediates covalent homodimerization between the A4 domains of FXI. Mutational analysis of Cys321 demonstrated that FXI can exist as a non-covalent dimer indicating that there are additional residues involved in homodimerization (Meijers, Mulvihill et al. 1992). The information obtained from the crystal structure of FXI (**Figure 6**) permitted the identification of the residues in the A4 domain responsible for the non-covalent interactions necessary for dimerization (Papagrigoriou, McEwan et al. 2006; Wu, Sinha et al. 2008). Mutational analysis has confirmed that a salt bridge between Lys331 of one subunit and Glu284 on the opposite subunit is important for dimerization (Wu, Sinha et al. 2008). Residues Ile290, Leu284 and Tyr329, are also essential for homodimer formation. In addition, residue Cys326 in the A4 domain forms a covalent bond with Cys482 of the light chain of the FXI subunit. It should be noted that the dimer interface in FXI appears to have two unusual features. First, the disulfide bond Cys321 demonstrates a high degree of solvent exposure. Second, the total buried area at the dimer interface is relatively small compared with the average dimeric protein structures (Jones and Thornton 1995). The A4 domain also provides some of the binding energy for FXIIa as the activation of FXI by FXIIa was inhibited by a cyclic peptide Ala317-Gly350 (Baglia, Jameson et al. 1993).

Figure 6. X-ray crystal structure of FXI zymogen.

Secondary structure elements are represented as a ribbon diagram of the FXI dimer with the protease domain colored white and Cys321 disulfide bond in yellow. Domains A1, A2, A3, and A4 are colored gray, blue, orange and yellow respectively. Connecting loops are colored in black and the loop following A4, which leads to the C-terminal protease domain, is colored in dark blue. Figure was generated from PDB file 2F83 with modifications from (Papagrigoriou, McEwan et al. 2006).

FXI is an inactive precursor of a serine protease FXIa (EC 3.4.21.27). *In vitro*, FXI can be activated by FXIIa, FXIa or thrombin (Bouma and Griffin 1977; Kurachi and Davie 1977; Gailani and Broze 1991). All three proteases cleave each monomer of FXI at the R³⁶⁹-I³⁷⁰ bond generating the new amino terminal sequence of the catalytic domain. Upon binding to the negatively charged surfaces, a complex is formed between FIX and HK in plasma, facilitating recognition of FXI as a substrate by FXIIa (Ratnoff, Davie et al. 1961; Saito, Ratnoff et al. 1973; Schiffman and Lee 1974; Schiffman and Markland 1975; Griffin and Cochrane 1976; Liu, Scott et al. 1977; Meier, Pierce et al. 1977; Thompson, Mandle et al. 1977; Wiggins, Bouma et al. 1977). *In vitro*, the activation of FXI by thrombin as well as autoactivation requires the presence of polyanionic compounds such as dextran sulfate or heparin (Gailani and Broze 1991; Naito and Fujikawa 1991).

FXI is the only homodimeric protein in the coagulation cascade. The significance of the dimeric structure of FXI is a subject of ongoing investigation. It has been proposed to be essential for proper activation of its physiological substrate, factor IX. Recent studies, however, disproved this hypothesis. Various monomeric species of FXI have full capacity for activating FIX to FIXa (Sinha, Marcinkiewicz et al. 2005; Smith, Verhamme et al. 2008; Wu, Sinha et al. 2008). In addition, an intermediate with only one subunit being activated has been isolated and shown to be completely active with respect to FIX (Smith, Verhamme et al. 2008). Studies performed by Dr. Wenman Wu in our laboratory have demonstrated that FXI dimeric structure is important for normal proteolytic activation by FXIIa, thrombin and FXIa (Wu, Sinha et al. 2008). Activation of XI by thrombin and by autoactivation requires the presence of dextran sulfate. FXIIa activates

FXI without dextran sulfate, albeit at a slower rate. The molecular mechanism involved is not clear at the present. It should be noted, however, that the mechanism of FXI activation by FXIIa may be different from that of activation by thrombin of FXIa. Does the FXI dimeric structure form a composite site in which occupancy of one subunit enhances a productive occupancy of the other subunit? Is there a composite binding site for either dextran sulfate, FXIIa or thrombin in the FXI homodimer? The functional significance of the FXI dimeric structure in binding to heparin is partially addressed in this work.

Enzyme FXIa belongs to the trypsin family of serine proteases, a characteristic feature of which is the presence of Asp189 residue in the S1 specificity pocket of the active site (Rawlings and Barrett 1994; Perona and Craik 1995). Coagulation factor IX is a major physiological substrate for FXIa (Osterud, Bouma et al. 1978). Factor XIa cleaves two bonds in FIX molecule in a sequential manner. This reaction requires the presence of Ca^{2+} ions. Initial cleavage at Arg145-Ala146 is followed by hydrolysis of Arg180-Val181, resulting in a release of the activation peptide (Smith, Verhamme et al. 2008). The amino acid sequences of catalytic domains of human plasma kallikrein and factor XI share 64% identity. The X-ray crystal structures of the catalytic domains of both PK and FXI show a typical chymotrypsin-like fold, composed of two six-stranded beta-barrels, with the active site located in the cleft (Lesk and Fordham 1996; Jin, Pandey et al. 2005).

The zymogen factor XI binds to platelets with high affinity ($K_D \sim 10\text{nM}$, $B_{\text{max}} \sim 1500$ sites per platelet) in the presence of 45 nM HK and 25 μM Zn^{2+} ions (Greengard, Heeb et al. 1986). A number of previously published observations suggested that platelets

dramatically accelerate activation of FXI by thrombin. However, these data have not been validated by a number of researchers in our group (Baglia and Walsh 2007; Pedicord, Seiffert et al. 2007; Walsh 2007). Physiological significance of this interaction, thus, remains unclear. The enzyme FXIa also binds to platelets with high affinity ($K_D=1.7\text{nM}$, 250 ± 50 sites/platelet (Sinha, Seaman et al. 1984). The region important for the interaction of FXIa with platelets has been recently localized to the 170's loop in the serine protease domain of FXIa (loop borders ⁵²⁷Cys and ⁵⁴²Cys, FXI numbering) (Miller, Sinha et al. 2007).

Regulation of FXIa

FXIa activity is regulated by a number of protease inhibitors circulating in plasma as well as those released from the activated platelets. Among plasma inhibitors, C1 is the most potent FXIa inhibitor, followed by antithrombin III, α_1 -antitrypsin and α_2 -antiplasmin (Forbes, Pensky et al. 1970; Damus, Hicks et al. 1973; Heck and Kaplan 1974; Wuillemin, Minnema et al. 1995). Protein Z-dependent protease inhibitor (ZPI) also inhibits FXIa in plasma (Han, Fiehler et al. 2000). Factor XIa inhibition by C1 inhibitor and ATIII is potentiated by the presence of heparin. Factor XIa inhibition by protease nexin 1 is also potentiated by heparin, which results from a 20-fold increase in association rate constant (Knauer, Majumdar et al. 2000). Basic residues in the autolysis loop of FXIa have been shown to be involved in inhibition by the serpin protease inhibitors antithrombin III, ZPI and C1 inhibitor (Rezaie, Sun et al. 2006). In particular, alanine substitution of Arg 507 impaired inhibition of FXIa by AT approximately 7-fold.

Activated platelets secrete high levels of the potent physiological inhibitor of FXIa, protease nexin 2 (PN-2, $K_i \sim 0.5$ nM). PN2 is an extracellular domain of the amyloid precursor protein (APP). There are three major alternatively spliced isoforms of APP 695, 751 and 770 a.a. long (Tanzi, McClatchey et al. 1988; Smith, Higuchi et al. 1990). The full length APP is a type I membrane glycoprotein expressed in a number of alternatively spliced isoforms in various cell types including neurons and platelets (Botelho, Gralle et al. 2003). The PN2 isoform of APP is 751 amino acids long and contains a Kunitz protease inhibitory (KPI) domain of 57 aa (GLu289-Ile345) (Ponte, Gonzalez-DeWhitt et al. 1988; Tanzi, McClatchey et al. 1988; Smith, Higuchi et al. 1990; Selkoe and Schenk 2003). PN-2 is a slow tight binding inhibitor of FXIa (Van Nostrand, Wagner et al. 1989; Navaneetham, Jin et al. 2005). The function of amyloid precursor protein in the adult organism remains unclear. Platelets release significant amounts (~ 30 nM) of APP from their alpha granules upon activation thus serving as a major reservoir of FXIa inhibitor (Van Nostrand, Schmaier et al. 1990). The co-crystal structure of APP-PKI domain with FXIac has been solved (Navaneetham, Jin et al. 2005).

Enzymatic activity of factor XIa activity is modulated by the negatively charged polysaccharides. Dextran sulfate and heparin were shown to inhibit FXIa activity *in vitro*, modulating the cleavage of both the chromogenic substrate S-2366 and factor IX (Sinha, Badellino et al. 2004). This effect has been attributed to the allosteric changes which occur at the active site upon binding of polyanions (Sinha, Badellino et al. 2004). The region of FXIa involved in modulation of substrate cleavage has been proposed to be located at the loop 170 (chymotrypsin numbering) (Sinha, Badellino et al. 2004).

Factor XIa inhibition by ATIII is enhanced 40-fold by heparin. The pseudo-first order rate constants changes from 1.0 min^{-1} to 0.025 min^{-1} (Beeler, Marcum et al. 1986)

Heparin is known to potentiate the inhibitory effect of PN-2 on FXIa by decreasing the K_i 10-fold from 400 pM to 40 pM (Scandura, Zhang et al. 1997; Zhang, Scandura et al. 1997; Badellino and Walsh 2000; Salvesen and Abrams 2004).

A recent report demonstrated the direct interaction between FXI and RNA and suggested that RNA may be the long sought natural "foreign surface" necessary for activation of contact factor pathway *in vivo* (Kannemeier, Shibamiya et al. 2007).

Patient mutations causing FXI deficiency

Mutations in FXI have been divided into three main categories: type I, II and III. Type I mutations disrupt normal mRNA splicing, type II cause premature polypeptide termination, and type III result in an amino acid substitution (Asakai, Chung et al. 1989). To date, at least 127 mutations have been associated with abnormalities caused by either a low level of FXI protein or a defective FXI gene product (Saunders, O'Connell et al. 2005). The population of Ashkenazi Jews have particularly high incidence of FXI deficiency. About 8% of this population carries mutations in the FXI gene. The type II mutation Glu117Stop and type III mutation Phe283Leu are common in this population. The Phe283Leu mutation affects the A4 domain of FXI (Asakai, Chung et al. 1989). The type III mutation leads to a significantly reduced secretion of FXI from cells grown in culture as a result of impaired protein dimerization (Meijers, Davie et al. 1992).

About 30% of mutations in FXI are found in the serine protease domain. For example, the substitution Gly555Glu results in a normal level of FXI antigen but

dramatically compromises the catalytic activity of FXIa (Zivelin, Ogawa et al. 2004). Residue Gly555 provides a backbone carbonyl oxygen necessary for the formation of the oxyanion hole in the enzyme FXIa. Replacement of Gly555 by any other amino acid leads to an increased size of side chain not compatible with the function of this residue (Schmidt, Ogawa et al. 2004; Schmidt, Sun et al. 2008).

Throughout this work, the numbering systems for full-length FXI and chymotrypsin were used in order to correlate findings with the previously published data on FXIa and related serine proteases of the coagulation cascade (Hartley 1964; Fujikawa, Chung et al. 1986). In mature FXI protein, the catalytic triad residues are His413, Asp462 and Ser557, which correspond to His 57, Asp 102 and Ser 195 in chymotrypsin (CT) (Hartley 1964; Matthews, Sigler et al. 1967; Blow, Birktoft et al. 1969). The residues in the heavy chain (HC) of FXI located outside of the catalytic domain were numbered as in mature FXI protein (Fujikawa, Chung et al. 1986).

Research goals

The aim of this project was to determine the relative contributions of the positively charged residues in FXI involved in interaction with heparin. Using site-directed mutagenesis, the aim was to identify heparin-binding residues within and in close proximity to a cysteine-constrained α -helix-containing region (⁵²⁷CQKRYRGHKITHKMIC⁵⁴²) and within a heparin-binding consensus sequence (⁵⁰³YRKLRLDKI⁵¹⁰) of the autolysis loop region in FXI.

My experimental design takes into account newly available structural information and will provide a better understanding of the role of the individual residues involved in

binding to heparin. The crystal structures of FXI and FXIa catalytic domain provide an excellent rationale for design of experiments to address the functional role of the specific residues involved in heparin binding. Mutational analysis of full-length FXI expressed in HEK293 cells will be based on the following criteria: conservation of the positively charged residues in FXI among various species; surface exposure of the residues based on the X-ray crystal structure of FXI and comparison with human plasma prekallikrein (PK), which does not bind heparin.

In order to determine whether the dimeric structure of FXI is required for high affinity binding to heparin, a monomeric FXI mutant in which Cys321 is mutated to Ser along with Lys331 mutated to Ala was used. This mutant exists predominantly in the monomeric form as determined by the size exclusion chromatography (SEC) and analytical ultracentrifugation (AUC) (Wu, Sinha et al. 2008). The monomeric form of FXI is normal with respect to FIX activation, whereas it displays defective activation by FXIIa, IIa and FXIa. The interaction of FXI_{C321S/K331A} with heparin has been assessed using ELISA and surface plasmon resonance (SPR).

Hypotheses to be tested

1. Factor XIa binds to heparin with higher affinity than FXI utilizing different residues reflecting a postulated conformational difference between the zymogen and the enzyme.
2. In addition to the residues within the A3 domain that mediate FXI binding to heparin, there are positively charged residues within and in close proximity to heparin binding consensus sequences that mediate FXI/FXIa binding to

heparin. These residues, located in FXI catalytic domain, are highly conserved in mammalian species but are dissimilar to those in human PK.

3. Unique homodimeric structure of FXI/FXIa is required for high affinity binding to heparin.

CHAPTER 2

EXPERIMENTAL DESIGN, MATERIALS, AND METHODS

Experimental design

Selection of candidate residues for mutational analysis

Potential heparin binding sequences can be identified by the presence of a high density of positively charged residues, particularly within identified heparin-binding consensus sequences in the protein. In particular, the amino acid sequence XBBXB (where B is a basic amino acid and X is any amino acid) is often indicative of the heparin-binding site in a protein (Cardin and Weintraub 1989). However, the basic residues can be located far apart in the linear sequence of a protein, but may still comprise a heparin binding site in a protein (Sali, Matsumoto et al. 1993; Matsumoto, Sali et al. 1995; Friedrich, Blom et al. 2001). Obviously, such a pattern is more difficult to identify, especially when no structural information is available.

Although residues K252, K253 and K255 in the apple 3 (A3) domain of the FXI heavy chain have been shown to be involved in binding to heparin by mutational analysis, no analysis of the residues in the light chain of FXI has been performed to date. The light chain of FXI contains two heparin binding consensus sequences (⁵⁰⁴RKLR⁵⁰⁷ and ⁵²⁹KRYR⁵³²). For mutational analysis, the degree of sequence conservation in FXI genes in various species was taken into account. Initial comparison of the genes coding for FXI was based on human, bovine, rabbit and mouse sequences. During the course of this work, the genomes of several organisms became available. The newly available genomes

include those of orangutan, chimpanzee, gray opossum, chicken, and horse. Therefore, the sequences from these species were also used in the analysis.

The amino acid alignment of the FXI catalytic domain is shown in **Figure 7**. In the first potential heparin-binding sequence in FXICD, the residue Arg507(147_{CT}) in FXI is strictly conserved among all species. The residue Lys505 (145_{CT}) is conserved in nine species out of ten. The residue Arg504(144_{CT}) is conserved in six species out of ten.

In the second potential heparin binding region located in the CD (⁵²⁹KRYR⁵³²) of FXI, lysine 529 (169_{CT}) shows the lowest sequence conservation. It is present only in three species in addition to human FXI. The Arg530 (170_{CT}) is conserved in nine species, while in the bovine protein it is replaced by glycine. Residue Arg532 (171_{CT}) is highly conserved, with the exception of the rabbit FXI sequence where it is replaced by a neutral glutamine.

The relative surface exposure of the residues chosen for mutational analysis was taken into consideration by analyzing the X-ray crystal structure of the full length FXI (Papagrigoriou, McEwan et al. 2006). The evaluation was performed using the GETAREA program (Fraczkiewicz and Braun 1998). This program provides the solvent accessible surface area (SASA) for each residue in the protein of known structure. The value of the surface exposure of a residue X is compared to that of the random coil value of the residue in the tripeptide Gly-X-Gly. According to the GETAREA program, the residues are considered to be solvent exposed if the ratio exceeds 50%. The residue is considered to be buried if the ratio is less than 20%.

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HS  IVGGTASVRGEWPWQVTLHHTSPTQRHLCGGSTIGNOWILTAAHCFYG.....VESPKILRVY
PT  IVGGTASVRGEWPWQVTLHHTSPTQRHLCGGSTIGNOWILTAAHCFYG.....VESPKILRVY
PA  IVGGTASVRGEWPWQVTLHHTSPTQRHLCGGSTIGNOWILTAAHCFYG.....VESPKILRVY
OC  IVGGASLPGGEWPWQVTLHHTSPTQRHLCGGSTIGNOWILTAAHCFYG.....IESPKILRVY
EC  VVGGRASVPGGEWPWQVTLHHTSPTQRHLCGGSTIGNOWILTAAHCFDE.....VESPKILRVY
BT  IVGGTQSVHGEWPWQVTLHHTSPTQRHLCGGSTIGNOWILTAAHCFNE.....VKSPNVLRVY
RN  VFGGAASVHGEWPWQVTLHHT...QGHLCGGSTIGNOWILTAAHCFSGVQHMNPTLTLLFSIGQRTETPKTLRVY
MM  VVGGAASVHGEWPWQVTLHHT...QGHLCGGSTIGNOWILTAAHCFSG.....IETPKKLRVY
MD  IVGGTQSVLAEPWQVSLHHTSPTQRHLCGGSTIGNOWILTAAHCFLEE.....LEAATDLHVY
GG  IIGGTDSSPGGEWPWQVSLHVKLSRQRHLCGGSTIGNOWILTAAHCFMS.....VQNPNIWRVY

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HS  SGILNQSEIKEDTSFFGVQEI I IHDQYKMAESGYDIALKLETTVNYTDSORPICLPKSGDRNVIYTD CWVTGWG
PT  SGILNQSEIKEDTSFFGVQEI I IHDQYKMAESGYDIALKLETTVNYTDSORPICLPKSGDRNVIYTD CWVTGWG
PA  SGILNQSEIKEDTSFFGVQEI I IHDQYKMAESGYDIALKLETTVNYTDSORPICLPKSGDRNVIYTD CWVTGWG
OC  SGILNQSEIKEDTSFFGVQEI I IHDQYKMAESGYDIALKLETTVNYTDSORPICLPKSGDRNVIYTD CWVTGWG
EC  SGILNQSEIKEDTSFFGVQEI I IHDQYKMAESGYDIALKLETTVNYTDSORPICLPKSGDRNVIYTD CWVTGWG
BT  SGILNQSEIKEDTSFFGVQEI I IHDQYKMAESGYDIALKLETTVNYTDSORPICLPKSGDRNVIYTD CWVTGWG
RN  GGI VNQSEINEDTSFFGVQEI I IHDQYKMAESGYDIALKLETTVNYTDSORPICLPKSGDRNVIYTD CWVTGWG
MM  GGI VNQSEINEDTSFFGVQEI I IHDQYKMAESGYDIALKLETTVNYTDSORPICLPKSGDRNVIYTD CWVTGWG
MD  AGI VNQSEINEDTSFFGVQEI I IHDQYKMAESGYDIALKLETTVNYTDSORPICLPKSGDRNVIYTD CWVTGWG
GG  AGI VNQSEINEDTSFFGVQEI I IHDQYKMAESGYDIALKLETTVNYTDSORPICLPKSGDRNVIYTD CWVTGWG

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HS  YRKLRDKIQNTLQKAKIPLVTNEECQKRYRSHKITHKMI CAGYREGGKDA CKGDSGGPLSCKHNEVWHLVGITSW
PT  YRKLRDKIQNTLQKAKIPLVTNEECQKRYRSHKITHKMI CAGYREGGKDA CKGDSGGPLSCKHNEVWHLVGITSW
PA  YRKLRDKIQNTLQKAKIPLVTNEECQKRYRSHKITHKMI CAGYREGGKDA CKGDSGGPLSCKHNEVWHLVGITSW
OC  YRKLRDKIQNTLQKAKIPLVTNEECQKRYRSHKITHKMI CAGYREGGKDA CKGDSGGPLSCKHNEVWHLVGITSW
EC  YRKLRDKIQNTLQKAKIPLVTNEECQKRYRSHKITHKMI CAGYREGGKDA CKGDSGGPLSCKHNEVWHLVGITSW
BT  YRKLRDKIQNTLQKAKIPLVTNEECQKRYRSHKITHKMI CAGYREGGKDA CKGDSGGPLSCKHNEVWHLVGITSW
RN  YTKSRDEVQSTLQKAKIPLVTNEECQKRYRSHKITHKMI CAGYREGGKDA CKGDSGGPLSCKHNEVWHLVGITSW
MM  YTKSRDEVQSTLQKAKIPLVTNEECQKRYRSHKITHKMI CAGYREGGKDA CKGDSGGPLSCKHNEVWHLVGITSW
MD  YTKSRDEVQSTLQKAKIPLVTNEECQKRYRSHKITHKMI CAGYREGGKDA CKGDSGGPLSCKHNEVWHLVGITSW
GG  YTKSRDEVQSTLQKAKIPLVTNEECQKRYRSHKITHKMI CAGYREGGKDA CKGDSGGPLSCKHNEVWHLVGITSW

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HS  GEGCAQRERPGVYTNVVEYVDWILEKKTQAV
PT  GEGCAQRERPGVYTNVVEYVDWILEKKTQAV
PA  GEGCAQRERPGVYTNVVEYVDWILEKKTQAV
OC  GEGCAQRERPGVYTNVVEYVDWILEKKTQAV
EC  GEGCAQRERPGVYTNVVEYVDWILEKKTQAV
BT  GEGCGQRERPGVYTNVVEYVDWILEKKTQAV
RN  GEGCGQRERPGVYTNVVEYVDWILEKKTQAV
MM  GEGCGQRERPGVYTNVVEYVDWILEKKTQAV
MD  GEGCARPGRAGVYTKVAEYVDWILEKKTQAV
GG  GEGCARPGRAGVYTKVAEYVDWILEKKTQAV

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Figure 7. Amino acid sequence alignment of the FXI catalytic domain from various species.

Strictly conserved residues are highlighted in black. Potential heparin binding sequences are in boxes. Alignment performed using CLUSTALW algorithm (Thompson, Higgins et al. 1994) . FXI sequences are from human human (HS, *Homo sapiens*), chimpanzee (PT, *Pan troglodytes*), orangutan (PA, *Pongo abelii*), rat (RT, *Rattus norvegicus*) rabbit (OC, *Oryctolagus cuniculus*), horse (EC, *Equus caballus*), cow (BS, *Bos taurus*), mouse (MM, *Mus musculus*), opossum (MD, *Monodelphis domestica*), and chicken (GG, *Gallus gallus*). Figure was generated using Esprint program.

Based on the relative surface exposure ratios (**Table 1**), residues Arg507 and Arg532 are the most exposed in both FXI and FXI_{acd}. Residues Arg504, Lys505 and Lys529 are also solvent exposed. The side chains of the residues Arg530 and Arg586 have the lowest extent of surface accessibility, with ratios of 8.3 % and 31.8 % respectively. Since the solvent exposure ratio of the Arg530 is below 20% this residue is considered buried in both FXI and FXI_{acd} structures. Residues Lys529 and Arg530 are part of an alpha-helical portion of the cysteine constrained region in FXI (Cys527-Cys542).

During the completion of this project, the crystal structure of the FXI catalytic domain became available (Jin, Pandey et al. 2005; Navaneetham, Jin et al. 2005). Residue Arg586 was implicated as a part of the positively charged surface patch, potentially involved in the interaction with the negatively charged molecules (Jin, Pandey et al. 2005). Residue Arg586 (224_{CT}) is conserved in nine out of ten species. Although Arg586 is not a part of the heparin binding consensus sequence, in three dimensional structure of it is located in close proximity to Arg532 and the mutation R586A has therefore been characterized.

Plasma prekallikrein was also chosen to serve as a model for the mutational analysis because it has high sequence homology to FXI. However, in contrast to FXI, human plasma PK is a monomeric protein, and it does not bind to heparin. Residues Lys505, Lys529 and Arg530 are conserved in human PK. Residues Arg532 and Arg586 in FXI are replaced by neutral glutamine in PK.

Table 1. Solvent accessibility of the candidate residues selected for mutational analysis

Residue # FXI	Chymotrypsin numbering	Secondary structure region	Relative solvent accessibility
Arg504	144	loop	47.0
Lys505	145	loop	83.6
Arg507	147	loop	88.2
Lys529	169	α -helix	65.3
Arg530	170	α -helix	8.3
Arg532	171	loop	80.8
Arg586	224	loop	31.8

Secondary structure elements are as in X-ray crystal structure of FXI (Papagrigoriou, McEwan et al. 2006) (PDB code 2F83). Accessibility ratios were obtained using GETAREA program (Fraczkiewicz and Braun 1998). The residues with accessibility ratios above 50 are considered exposed, while residues whose ratios are below 20 considered buried.

Surface plasmon resonance

Surface plasmon resonance (SPR) is a well established biophysical method for characterization of molecular interactions (Rich and Myszka 2000). This method allows direct real-time characterization of the interaction between the immobilized ligand and an analyte in solution without the use of labels (Cooper 2002). The light is incident upon a gold film through a prism and the reflected beam is collected and analyzed (**Figure 8**). During a binding event, a change in mass takes place which alters the angle of light at which SPR occurs. Thus, the binding progress curve or a sensorgram is measured in real time and is expressed in arbitrary resonance units (RU). One RU corresponds to a 0.0001° shift in the SPR angle (Johnsson, Lofas et al. 1991). The amount of ligand bound to the chip surface is directly proportional to the SPR signal within 100 nm above the chip surface.

There are several advantages to the use of SPR. First, SPR biosensors offer high sensitivity, accuracy, precision, and repeatability (Homola 2003). Second, no labeling is necessary to study interactions (Gesellchen, Zimmermann et al. 2005). Third, only a very small amount of the ligand (micrograms) is required for immobilization. Depending on the nature of the studied interaction, it is possible to obtain affinity, association and dissociation rate constants from a series of titrations (Fisher and Fivash 1994).

Figure 8. Scheme of optics and signal generation in SPR biosensor.

The SPR method takes advantage of the total internal reflection of light shone at the surface of the chip through a prism. The SPR angle changes from position I to II if there is an accumulation of the analyte molecules in the close proximity of the chip surface. The binding curve or sensorgram is measured in real-time as a function of resonance signal versus time. Figure taken from (Cooper 2002)

The Biacore2000 instrument, used in this study, consists of an integrated microfluidic cartridge (IFC) and a sensor chip. The sensor chip is composed of a thin gold film (47 nm) attached to glass. The most commonly used modification of a sensor chip contains an additional 100 nm layer of carboxymethyldextran (CM) , which enhances hydrophilicity and at the same time presents carboxyl groups for covalent attachment of various ligands (Johnsson 1990). The negative charge of a carboxymethyldextran hydrogel may cause undesirable electrostatic interaction with the positively charged proteins resulting in high non-specific binding. Since FXI is a positively charged protein, a sensor chip C1 without a carboxymethyldextran layer was used in this study (Karlsson and Falt 1997). To study heparin-protein interactions, streptavidin-coated coated sensor chip (SA) was used.

The microfluidic cartridge of the Biacore 2000 machine contains four flow cells (dimensions 2.1 mm × 0.55 mm × 0.05 mm) (Schuck 1997). Each flow cell can be derivitized individually. The specific refractive index change is obtained by subtracting the “bulk” response from the control flow cell which serves as a blank surface. The surface of the flow cell 1 was used a reference throughout the experiments presented herein.

The most commonly used chemistry for immobilization of biomolecules is via primary amines such as those of Lys residues and N-termini of proteins. The derivatization utilizes NHS (*N*-Hydroxysuccinimide) and EDC (ethyl-*N*-(3-diethylaminopropyl) carbodiimide) as coupling reagents. The details of the reactions involved are shown in **Figure 9** (Johnsson, Lofas et al. 1991).

Figure 9. Chemical strategy used for the immobilization of 1G5 antibody on C1 chip.

1, 2: EDC (ethyl-*N*-(3-diethylaminopropyl) carbodiimide) and NHS (*N*-Hydroxysuccinimide) activated carboxyl groups. 3: Ligand is covalently linked. 4: 1mM ethanolamine, pH 8.0 is added to block remaining NHS-activated carboxyl groups.

The covalent ligand attachment to the chip is facilitated by using low ionic strength (<50 mM) and a pH below the isoelectric point of the molecule (Canziani, Zhang et al. 1999). Following ligand coupling, unreacted NHS-esters are hydrolyzed by applying 1mM ethanolamine hydrochloride solution (pH 8.0). The non-covalent immobilization of ligands is often achieved via attachment of biotinylated molecules on streptavidin-coated chip surface. This strategy was used in this work for heparin immobilization.

Depending of the strength of the interaction between the ligand and analyte, the chip surface has to be regenerated for reuse in successive binding experiments (Nguyen, Tanious et al. 2007). For this purpose, strong denaturing conditions such as extreme pH or high salt concentrations are normally used. Harsh regeneration conditions, however, may damage or completely deactivate the ligand on the chip. Therefore, the regeneration conditions for each experimental system have to be carefully optimized. It is also important to minimize the signal contributions due to the changes in the matrix refractive index resulting from regeneration (Karlsson and Falt 1997). Once the conditions are optimized, the chip surface can be reused multiple times (Brigham-Burke, Edwards et al. 1992).

Materials

Proteins and antibodies

Plasma derived FXI, FXIa, ATIII and FXIIa, and FXI-deficient plasma were purchased from Haematologic Technologies, Inc., Essex Junction, VT. Monoclonal FXI

antibodies 1G5 and 5F7 (Sinha, Koshy et al. 1985) were purchased from Green Mountain Antibodies (Burlington, VT) under designation GMA-021 and GMA-070. Affinity purified HRP-conjugated Fc-specific anti-mouse antibody was purchased from SIGMA.

Cell culture reagents

DMEM (Dulbeco's modified Eagle medium) was purchased from Mediatech, Herndon, VA. Glutamine, penicillin/streptomycin and fetal bovine serum (Fetalplex) were obtained from Gemini Bio-Products (West Sacramento, CA).

Miscellaneous reagents

Neutravidin-coated black 96 or 384 well plates (Cat #15117 and #15402), bicinchoninic protein assay reagents, and QuantaBlu substrate were purchased from Pierce (Rockland, IL). Heparin-biotin was purchased from Neoparin (San Leonardo, CA). Non-treated 384-well and 96-well microtiter plates for BCA protein and kinetic assays were from Thermo Fisher Scientific (Waltham, MA). Plastic roller bottles were purchased from Corning (Lowell, MA). The site-directed mutagenesis kit (Quikchange II) was purchased from Stratagene (La Jolla, CA). O-Phenylenediamine (OPD), cyanogen bromide-activated-sepharose 4B, potassium thiocyanate, HEPES, Tween 20 were from SIGMA-ALDRICH (St. Louis, MO). Sodium chloride and APTT reagent were from Fisher Scientific. The chromogenic substrate S-2366 (L-pyroglutamyl-L-prolyl-L-arginyl-*p*-nitroaniline hydrochloride) was purchased from Chromogenix (Mölnådal, Sweden). NHS (N-hydroxysuccinimide) and EDC (ethyl-N-(3-

diethylaminopropyl)carbodiimide) were obtained from Biacore/GE Healthcare (Piscataway, NJ). All other reagents were of analytical grade and were the best quality commercially available.

Buffers

Acetate buffer, used for immobilization in SPR experiments, contained 10 mM sodium acetate (pH 4.5). Phosphate buffered saline (PBS) contained 20 mM sodium phosphate, 150 mM NaCl, 5 mM KCl. Hepes buffered saline (HBS) consisted of 10 mM Hepes, pH 7.4, 150 mM NaCl. HBST buffer was the same as HBS, containing 0.01% Tween 20. Tris buffered saline (TBS) contained 25 mM Tris (Tris-(hydroxymethyl)-aminomethane), 150 mM NaCl, pH 7.4. TBST was the same as TBS, containing Tween20, 0.05%. TBS-BSA was the same as TBS, containing 0.02% bovine serum albumin (BSA). TBST-BSA was the same as TBS-BSA, containing 0.02 % Tween20. Carbonate buffer (pH 9.5), containing 14 mM Na₂CO₃ and 36 mM NaHCO₃ was used for coating the ELISA plates with anti-FXI antibody.

Methods

Mutagenesis

Mutations were introduced in pJVCMV-FXI vector using the QuickChange II site-directed mutagenesis kit (Stratagene). The sequences of the mutagenic primers are presented in **Table 2**. The mutagenic primers were designed using a web-based program PrimerX (<http://bioinformatics.org/primerx/>) and were synthesized and purified by Invitrogen (Carlsbad, CA). The reaction mixture contained 10 mM dNTPs, 50 ng of wt

FXI cDNA, 200 nM mutagenic primers and 2.5 units of *Pfu* Ultra HF (Stratagene) polymerase. Each PCR step consisted of melting the DNA duplexes at 95°C for 5 min, followed by reannealing at 60°C for 1 min, and then by an extension reaction at 72°C for 14 min. The PCR reactions were performed using Perkin Elmer DNA Thermal Cycler (Foster City, CA). A total of 16 amplification cycles were performed. Upon completion of the PCR cycle, the template plasmid was digested with the *DpnI* restriction enzyme. The template DNA obtained from *E. coli* is naturally methylated in bacteria, while the DNA synthesized by PCR is not. The *DpnI* enzyme specifically recognizes and degrades methylated sequences, thereby leaving only the mutant DNA intact. The obtained constructs were transformed into competent XL-Blue *E. coli* competent cells (Stratagene) for DNA isolation and sequencing. The positive colonies were manually picked up with a sterile loop and grown overnight in 4 mL of LB medium containing 100 µg/mL ampicillin.

Plasmid DNA preparation

Midi-prep cultures of competent XL-Blue *E. coli* cells were grown in two steps. First, a starter culture was grown in 2 mL of Luria Broth (LB) containing 100 µg/ml ampicillin for 5-6 hours. Then, 50 µl of a starter culture was added to 50 ml of LB ampicillin media and grown overnight in 250 mL flask at 37°C. After 16-18 hours the OD₆₀₀ usually reached a value of 1.6-1.8. The media was transferred into 50 mL plastic tubes and centrifuged at 5,000 rpm for 10 min at 4°C.

Table 2. Oligonucleotide mutagenic primers used in the study

Mutation	Primer sequence 5' to 3'
R504AF	GGTGACTGGATGGGGGTAC <u>GCC</u> AAACTAAGAGACAAAATAC
R504AR	GTATTTTGTCTCTTAGTTT <u>GGC</u> GTACCCCATCCAGTCACC
K505AF	GGATGGGGGTACAGAG <u>CC</u> CTAAGAGACAAAATAC
K505AR	GTATTTTGTCTCTTAG <u>GG</u> CTCTGTACCCCATCC
R507AF	GATGGGGGTACAGAAA <u>ACTAGCC</u> GACAAAATACAAAATACTC
R507AR	GAGTATTTTGTATTTTGT <u>CGG</u> CTAGTTTCTGTACCCCATC
K529AF	CAACGAAGAGTGCCAG <u>GCC</u> AGATACAGAGGACATAAAATAAC
K529AR	GTTATTTTATGTCCTCTGTATCT <u>GGC</u> CTGGCACTCTTCGTTG
R530AF	CGAAGAGTGCCAGAAG <u>G</u> CATACAGAGGACATAAAATAACC
R530AR	GGTTATTTTATGTCCTCTGTAT <u>GC</u> CTTCTGGCACTCTTCG
R532AF	AGAGTGCCAGAAGAGATAC <u>G</u> CAGGACATAAAATAACCCATAAG
R532AR	CTTATGGGTTATTTTATGTCCT <u>GCG</u> TATCTCTTCTGGCACTCT
R586AF	GTGCTCAAAGGGAG <u>GCCC</u> CAGGTGTTTACACC
R586AR	GGTGTAACACCTGG <u>GGC</u> CTCCCTTTGAGCAC

Nucleotide changes are underlined. A-alanine, F-forward, R-reverse primer.

PureYield (Promega) midiprep kit was used to extract the plasmid DNA from the bacterial pellet. DNA was eluted in sterile dH₂O and stored at -20°C.

DNA sequencing

For the sequencing of the regions containing mutations in the FXI catalytic domain, reverse (REV) and light chain forward (LCF) primers were used. The plasmids were sequenced by ACGT Inc. (Wheeling, IL) or Genewiz Inc. (New Brunswick, NJ).

Mutant FXI mRNA sequencing

Total mRNA was extracted from transfected HEK 293 cells using Trizol reagent (Invitrogen). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using one-step SuperScript RT-PCR kit (Invitrogen). Upon completion of the reaction, agarose gel electrophoresis was performed and the DNA copies of the FXI mRNA from the cells were excised from the gel and sent for sequencing.

S-2366 hydrolysis

Serially diluted stock solutions of the chromogenic substrate S-2366 (12 mM to 0.06 mM) were prepared in dH₂O. Plasma-derived, wild-type and mutant FXIa proteins were used at a fixed final enzyme concentration of 0.67 nM. No Ca²⁺ was added to the buffer as it has no effect on amidolytic activity of FXIa (van der Graaf, Greengard et al. 1983). The reaction of hydrolysis was initiated by adding 80 µL of FXIa solution in TBS-BSA (25 mM Tris, 150 mM NaCl, pH 7.4) to the wells containing 40 µL of chromogenic substrate. Each measurement was performed in duplicate. The absorbance at 405 nm

resulting from the appearance of p-nitroaniline was measured for 15 min using Thermomax plate reader (Molecular Devices Corp., Sunnyvale CA) at 37°C.

Recombinant FXI expression

Human embryonic kidney 293 (HEK Flp-InTM-293, Invitrogen) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine, 5% (vol/vol) fetal bovine serum (FBS), 9%, penicillin (500 µg/ml) and streptomycin (500 µg/ml) in a humidified 5% (vol) CO₂ atmosphere at 37° C. Upon reaching 80%-90% confluence, cells were transfected with target DNA along with the vector conferring neomycin resistance using Lipofectamine 2000 reagent. The OPTI-Mem medium containing DNA-Lipofectamine 2000 complexes was added and incubated for 24 hours. The total amount of DNA was 4 µg per well in a 6 well plate or 2 µg per well in a 4 well plate. The cells were maintained in non-selective media for 48 hours, followed by dividing and changing media, containing the geneticin analogue G418 at concentration 500 µg/ml as a selection strategy.

Preparation of FXI immunoaffinity column

5F7 antibody solution was dialyzed overnight against 100 mM carbonate buffer, pH 8.3. Cyanogen bromide-activated cross-linked 4% beaded agarose (Sigma C9210) was first equilibrated in 50 mL of 1mM ice-cold hydrochloric acid to remove stabilizing agents (Springer 2001). The acid was removed by gentle suction on a Buchner funnel. The agarose was rapidly washed with 15 mL dH₂O and then with 5 mL of 100 mM sodium bicarbonate (pH 8.3) containing 0.4 mM NaCl. Agarose was then promptly

transferred directly to the antibody solution using a plastic spatula. After four hour incubation at room temperature, agarose was placed into a plastic column (BioRad) and the liquid was drained by gravity. Then 20 mL of 1mM Tris was added to the agarose to inactivate any remaining cyanogen groups. The column was stored in TBS buffer. The immunoaffinity column was used repeatedly for at least 50 purification cycles.

ELISA for detection of FXI expression

An ELISA was used to select the clones with the highest expression level. The polyclonal capture antibody was diluted 1:100 into 50 mM sodium carbonate buffer (pH 9.5) and 100 μ l were added to each well in a 96-well plate. After one hour incubation at RT, the coating antibody was removed and the wells were washed with PBS three times. The wells were blocked with 1% BSA solution in PBS. Cell culture supernatant samples were added and incubated for one hour. The wells were emptied, washed three times with TBS-BSA and then HRP-labeled anti-FXI antibody was added to the wells. After three washes with PBS, the OPD substrate was added to the wells and the absorbance at 450 nM was measured using a Thermomax microplate reader (Molecular Devices). All steps were performed at room temperature.

Recombinant FXI purification

Colonies of cells with the highest expression levels of FXI were gradually expanded into 6, 10 and then 15 cm culture dishes. Finally, cells were transferred into a 2L roller bottle where they were allowed to reach 75-90% confluency. When desired level of confluency was reached, serum free medium was added to cells. To minimize

proteolysis, the soybean and lima bean trypsin inhibitors (10 µg/mL) were added to the medium. Conditioned medium was collected every 48 h and supplemented with 5 mM benzamidine and 5 mM EDTA to minimize proteolysis. In order to remove cell debris, conditioned medium was subjected to filtration through a Supor filter membrane filter unit (Nalgene). The medium was stored at -30°C pending purification.

Purification of the mutants was performed using affinity chromatography with monoclonal anti-FXI 5F7 antibody coupled to sepharose beads. The conditioned medium (~0.5 liter) was applied to the column overnight at a flow rate 12-15 drops per minute. The bound material was washed with 100 mL (~50 column volumes) of TBS (25 mM Tris-HCl, pH 7.4, 150 mM NaCl), containing 5 mM benzamidine. The protein was eluted with 8-10 mL 2.0 M potassium thiocyanate in Tris/NaCl, pH 7.4 and concentrated in a Pierce I-Con concentrator with a 20,000 molecular weight cutoff (MWCO) filter membrane or Amicon Ultra 15 concentrator with 30,000 (MWCO). After each purification cycle, the column was washed with 100 mL of TBS buffer. In order to remove KSCN and benzamidine, concentrated proteins were dialyzed against TBS buffer using the dialysis cassettes with 20,000 MWCO (Pierce).

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using precast gradient gels 4-15% (BioRad). Samples were mixed with either non-reducing sample buffer or reducing sample buffer containing 5% β-mercaptoethanol, followed by boiling for five min. After cooling to RT, samples were loaded onto the gel. Gels were typically run at 150V, 35 mAmp for 1.2 hour at RT. The gels were rinsed with

dH₂O for 10 min and then placed into Imperial Protein Stain (Pierce) for 15-30 min, depending on the amount of sample per well.

Protein concentration determination

The bicinchoninic acid (BCA) assay was used to determine the protein concentration. This method is based on a two step reaction. During the first step, cuprous (Cu^{1+}) ion is formed upon reaction between the peptide backbone and a Cu^{2+} ion. In the second step, BCA reacts with the cuprous cation (Cu^{1+}) forming the purple-colored product which is measured at 562 nm. Serial dilutions of bovine serum albumin (BSA) at concentrations of 125, 250, 500, 750, 1000, 1500 $\mu\text{g/ml}$ were used as standards to determine protein concentration. Eight microliters of samples or standards (in quadruplicate) were added to a 384-well microplate (Nunc) followed by the 30 min incubation with 70 μl bicinchoninic acid (BCA) reagent at 37°C. The optical density was measured using Spectramax M2 plate reader (Molecular Devices Corp., Sunnyvale CA).

Activated partial thromboplastin time assay (APTT)

The standard curve for APTT assay was generated using serial dilutions of normal pooled plasma and FXI-deficient plasma. To 50 μl of normal pooled plasma was added 50 μl of FXI-deficient plasma, followed by addition of 25 μl of APTT reagent. After 2 min incubation at 37°C, the clotting reaction was initiated by addition of 25 μl of 50 mM CaCl_2 (final concentration 10 mM). The clotting times were measured using an Amelung MC4 coagulometer (Sigma Diagnostics). Each measurement was performed in triplicate.

ELISA of FXI binding to heparin

ELISA for studying protein-carbohydrate and protein-heparin interactions has been used in numerous studies (Pankhurst, Bennett et al. 1998; Barth, Schafer et al. 2003; Srinivasan, Viswanathan et al. 2008). ELISA was used in order to estimate the apparent dissociation constants for the wt and mutant FXI interaction with heparin. ELISA was also chosen as a highly sensitive method which avoids the use of radioactivity.

Black neutravidin-coated 96 or 384 well plate was washed three times with TBS buffer. Fifty microliters of 1 µg/ml of heparin-biotin solution in TBS was added to the wells and incubated at 4°C overnight. Heparin-biotin solution was aspirated using multichannel pipettor. This was done in order to minimize carryover of heparin solution into non-heparinized wells, which served as controls. After washing the plate, the non-specific binding sites were blocked with 1% BSA in TBS (25 mM Tris pH 7.3, NaCl 150 mM) overnight at 4°C. Mutant and wt FXI in TBST-BSA (25 mM Tris pH 7.3, NaCl 150 mM, 0.05% Tween 20, 0.02% BSA) concentrations FXI (0.1 to 90 nM) were added along with the wtFXI as a control, followed by 1 hr incubation at RT. The same concentrations of the proteins were added to the wells containing no heparin and served as blanks. Each of the following incubation steps was performed after three consecutive washes with TBS (25 mM Tris pH 7.3, 150 mM NaCl, 0.05% Tween 20). The monoclonal 5F7 antibody directed against FXI A1 domain was added (1 µg/ml) to the wells and incubated for 1 hour. Fresh solution of anti-mouse Fc-specific IgG horseradish peroxidase (HRP) at concentration 1 µg/ml was then added to the wells and incubated for 30 min. Finally, freshly prepared QuantaBlu fluorogenic substrate (Pierce) was added to the wells and developed for 10 min. The reaction was stopped by addition of 50 µl of the QuantaBlu

stop solution and fluorescence was measured (ex. 340 nm/ em. 430nm / cutoff 420nm) using Spectramax M2 microplate reader. The data were analyzed after subtracting the values for the non-specific binding obtained for each FXI concentration. The K_D apparent values were obtained by the non-linear regression data fitting procedure in Kaleidagraph program (Synergy Software) using the following equation:

$$[F] = B_{\max} \times [L] / (K_D + [L]),$$

where L denotes FXI concentration (nM), K_D is an apparent dissociation constant (nM), and $B_{\max}$ is the maximum number of binding sites, in RFU.

Basic SPR procedures

The 10X HBS (Hepes buffered saline, pH 7.4, 1.5 M NaCl) was diluted to 1X with ddH₂O and degassed for 10 min. After degassing, Tween 20 was added to a final concentration 0.01% to minimize non-specific binding of proteins to the tubing in SPR apparatus. After docking into a Biacore2000 system, the chip was equilibrated with running buffer three consecutive times using PRIME command. The C1 sensor chip was preconditioned two times by consecutive 6 sec injections of 0.1% SDS, 25 mM NaOH, 25 mM HCl at a flow rate 100 μ l/min. Streptavidin-coated chip (SA) was preconditioned by injecting 15 μ l of 0.05% SDS in dH₂O five consecutive times at a flow rate 50 μ l/min.

Protein dilutions were prepared in 1.5 mL plastic vials and subjected to 30 sec centrifugation prior to the binding experiments. The effects due to the changes in the matrix refractive index were minimized by allowing the baseline to reach equilibrium after regeneration for various amounts of time (Karlsson and Falt 1997). The blank buffer

injections were used in order to correct for any systematic signal drift during the kinetic binding experiment (Myszka 1999).

1G5 antibody binding experiments studied by SPR

The monoclonal antibody 1G5 was immobilized onto a carboxymethyl-dextran-free chip C1 using amine coupling chemistry to a surface density of approximately 200 RU. FXI was injected at a flow rate 20 μ l/min. A six minute association phase was followed by the 12 min dissociation phase. For each mutant, the binding sensorgrams for at least five protein concentrations were obtained. The concentrations were run in random order to ensure reproducibility between the cycles. Cycles were run in automated mode. The injections were performed in KINJECT mode including a two min wait time prior the injection start. The bound protein was removed from the chip by injecting 25 mM NaOH for 24 sec at the flow rate 20 μ l/min. The reference sensorgram obtained for the buffer blank injection was subtracted from the experimental data set to correct for any systematic drift.

Binding of proteins to heparin studied by SPR

Streptavidin coated sensor chip (SA) was used to immobilize heparin-biotin conjugate. Usually, 1 μ g/ml of heparin-biotin was injected into a flow cell 2, 3 or 4 at a flow rate of 2 μ l/min for 10 seconds which was sufficient to obtain response value of approximately 50 RU. In order to account for the non-specific binding, flow cell 1 was used as a control. Ten microliters of 1mM biotin hydrazide in HBS-T buffer was injected at a flow rate 10 μ l/min to block streptavidin sites on the flow cell 1. Each analyte

injection was preceded by a three minute hold period to allow the baseline to stabilize. Each injection was performed using KINJECT command with the time of association and dissociation as indicated in the particular experiment (see Chapter 3 for details). FXI diluted in HBS-T buffer (10 mM HEPES, 150 mM NaCl, pH 7.4, 0.01% Tween 20) at varied concentrations was injected at a flow rate 20 μ l/min with a five min association phase followed by a five min dissociation phase. Removal of residual FXI after each binding experiment was achieved by a 40 second regeneration cycle of 0.02% of SDS at a flow rate 20 μ l/min.

Affinity SPR analysis

In the case when the interaction reached an equilibrium plateau during the association phase of the injection ($dR/dt=0$), the affinity K_D can be obtained by plotting R_{eq} vs. analyte concentration C (Karlsson and Larsson 2004). The apparent dissociation constant K_{Dapp} was determined by either non-linear regression analysis of the binding data or from the Scatchard analysis (Dahlquist 1978). By plotting $R_{eq}/Conc$ (RU/nM) versus R_{eq} , (RU), K_{Dapp} was also calculated as the negative reciprocal of the slope obtained from the straight line (Karlsson, Michaelsson et al. 1991; Weitz, Zoche et al. 1998).

Kinetic SPR analysis

For kinetic data analysis, several models are provided by the BIAeval4.1 software (Biacore). Throughout this work, two models were used: the first assuming a ratio of one

to one between the analyte and a ligand (Langmuir) and second, a bivalent analyte model, assuming two independent ligand binding sites present in the analyte molecule.

The simplest 1:1 interaction follows the equation: $A+B \leftrightarrow AB$. The mathematical model referred to as Langmuir isotherm (Langmuir 1918) is described by the equations below (Karlsson and Falt 1997). It is assumed that the interaction between the ligand and an analyte follows a pseudo-first order reaction, there is no depletion of analyte in solution and that the analyte concentration remains constant during the injection (Karlsson, Michaelsson et al. 1991). The following differential equation describes the simple 1:1 interaction:

$$dAB/dt = k_a \times [A] \times [B] - k_d \times [AB],$$

where k_a ($1/M \times s$) is the association rate constant, k_d (s^{-1}) is the dissociation rate constant, $[A]$ is the concentration of the injected analyte, $[B]$ is the concentration of the immobilized ligand, and $[AB]$ is the concentration of the complex AB. (Karlsson, Michaelsson et al. 1991). The maximum response R_{max} is proportional to the total ligand concentration and $(R_{max} - R_A)$ is proportional to the free ligand concentration $[B]$. The proportionality factor is cancelled out as it would appear in all terms of the equation. The equation above can be rewritten into:

$$dR/dt = k_a [A] \times (R_{max} - R) - k_d \times R$$

The global data analysis yields k_a , ($1/M \times s$), R_{max} (RU), and k_d (s^{-1}). From the kinetics experiments, the dissociation constant K_D can be obtained, since $K_D = k_d/k_a$.

The bivalent analyte model assumes the presence of two identical binding sites on the solute molecule which can bind to one or two ligand molecules with a single binding site, following the reaction equation: $A+2B \leftrightarrow AB+B \leftrightarrow AB_2$.

The following differential equations are used for this model:

$$dB/dt = - ((2 \times k_{a1}) \times [A] \times [B] - k_{d1} \times [AB]) - (k_{a2} \times [AB] \times [B] - (2 \times k_{d2}) \times [AB2]),$$

$$dAB/dt = ((2 \times k_{a1}) \times [A] \times [B] - k_{d1} \times [AB]) - (k_{a2} \times [AB] \times [B] - (2 \times k_{d2}) \times [AB2]),$$

$$dAB2/dt = (k_{a2} \times [AB] \times [B] - (2 \times k_{d2}) \times [AB2]);$$

where [A] and [B] denote the concentrations of a bivalent analyte and a ligand respectively; [AB] and [AB2] are the concentrations of the 1:1 and 1:2 complexes respectively, k_{a1} and k_{a2} are the association rate constants for the first and second binding events respectively; k_{d1} and k_{d2} are dissociation rate constants for the first and second binding event respectively.

Data fitting procedure for both kinetic models was performed using a global algorithm as the most accurate method (Rich and Myszkka 2005). The association and dissociation phases of the interaction for each concentration of protein were analyzed simultaneously. The usefulness of kinetic data depends on the quality of the fit between a theoretical model and the experimental data (Myszkka 1999). The quality of the fitting models was assessed by the visual estimation of the residual plot and by the statistical parameter χ^2 . The χ^2 value is the mean square of the differences between the theoretical curve and the experimental data points and is calculated according to the equation:

$$\chi^2 = \sum (r_f - r_x)^2 / (n - p),$$

where r_f indicates the fitted value at a given point, r_x indicates the experimental value at that point, n is the number of data points, and p indicates the number of fitted parameters. Ideally, χ^2 value should be equal or below one. However, χ^2 values below 10 may be acceptable.

Electron Microscopy

Negative stain electron microscopy was carried out in the laboratory of Dr. Thomas Walz (Harvard Medical School). Samples were prepared by conventional negative staining with 0.75% (w/v) uranyl formate as described before (Ohi, Li et al. 2004). Images were collected with a Tecnai T12 electron microscope (FEI, Hillsboro, OR) equipped with an LaB6 filament and operated at an acceleration voltage of 120 kV. Images were recorded on imaging plates at a nominal magnification of 67,000x and a defocus value of -1.5 μm using low dose procedures. Imaging plates were read out with a Ditabis micron imaging plate scanner (DITABIS Digital Biomedical Imaging System AG, Pforzheim, Germany) using a step size of 15 μm , a gain setting of 20,000 and a laser power setting of 30%, 2 x 2 pixels were averaged to yield a pixel size of 4.5Å on the specimen level. The BOXER, the display program associated with the EMAN software package was used to process the images (Ludtke, Baldwin et al. 1999). The wtFXI particles were windowed into 64 x 64 pixel images and the FXI_{C321S,K331A} and PK particles into 50 x 50 pixel images. Using the SPIDER software (Frank, Radermacher et al. 1996), the particles were rotationally and translationally aligned and subjected to 10 cycles of multi-reference alignment.

CHAPTER 3

RESULTS

Preparation of the recombinant FXI mutants

Expression and purification

In order to determine the relative contributions of the positively charged residues in the catalytic domain (CD) of FXI in interaction with heparin, I have produced a number of mutants. The candidate residues were mutated to alanine using site-directed mutagenesis. Mutations were introduced into FXI-PJVCMV vector using the Quickchange method (Stratagene). The correctness of the mutations was confirmed by DNA sequencing of the plasmids prior to transfection. A pair of the downstream and upstream primers was used for sequencing to verify each mutation.

The wild-type (wt) FXI and the mutants were stably transfected and expressed in HEK293 cells. Proteins were purified from the conditioned serum-free medium using immunoaffinity chromatography. The average protein yield after purification was ~ 0.5 mg of protein per one liter of medium. The results of SDS-PAGE of the mutants are shown in **Figure 10** and provide the evidence that all mutants were >95% homogenous. All mutants appear identical to the wtFXI and plasma FXI and migrated as single bands at MW ~160,000 Da on non-reducing SDS-PAGE (**Figure 10, A**). The mutant proteins were stored at -80 °C for a period of two to three years. As demonstrated by the reducing SDS-PAGE (**Figure 10, B**) under the storage conditions, no autoactivation of FXI was observed.

A.

B.

Figure 10. SDS-PAGE of the recombinant FXI proteins.

Panel A: Non-reducing conditions. Lanes: M: Molecular weight markers, 1: wtFXI, 2: plasma FXI, 3: R504A, 4: K505A, 5: R507A, 6: K529A, 7: R530A, 8: R532A, 9: R586A; Panel B: Reducing conditions. Legends are the same as in Panel A.

Protein concentration determination

The protein concentration was measured using bicinchoninic (BCA) assay adapted for the 384-well microtiter plate format. Serial dilutions of bovine serum albumin (BSA) from 0.125 to 1.5 mg/ml were used to generate a standard curve. A new standard curve was prepared every time the protein concentration was measured. A representative standard curve for the BCA assay is shown in **Figure 11**.

Characterization of the recombinant FXI mutants

Clotting assays

Activated partial thromboplastin time (APTT) assays were performed to test the physiological function of the alanine-substituted FXI mutants. The standard curve was generated using serial dilutions of wtFXI in FXI-deficient plasma. As indicated in **Table 3**, all mutant FXI proteins showed the clotting activity similar to that of wtFXI.

FXI binding to 1G5 antibody studied by SPR

In order to assess the structural integrity of the mutant proteins, the binding interaction kinetics between the monoclonal antibody 1G5 and the FXI mutants was determined by SPR. **Figure 12** illustrates a representative immobilization sensorgram of 1G5 monoclonal antibody in 10 mM acetate buffer (pH 4.5) onto the surface of a C1 sensor chip. The immobilization level (~200RU) was determined by the difference in response units (RU) obtained before the injection of the antibody onto NHS/EDC activated chip surface and the endpoint of the ethanolamine injection.

Figure 11. Standard curve for protein concentration determination.

A representative standard curve was generated for the bicinchoninic acid assay (BCA) adapted for the 384-well microplate format. Pre-diluted BSA standards were incubated with bicinchoninic acid working solution for 30 min at 37°C. The absorbance at 562 nm was measured using Spectramax M2 microplate reader (Molecular Devices).

Table 3. Clotting activity of FXI mutants in APTT assays

Mutant	% activity
WT	100±3
FXI _{R504A}	89±3
FXI _{K505A}	102±4
FXI _{R507A}	105±5
FXI _{K529A}	95±4
FXI _{R530A}	92±3
FXI _{R532A}	100±4
FXI _{R586A}	88±3

Figure 12. Immobilization of 1G5 antibody on C1 sensor chip.

Shown is a continuous sensorgram of the injections of NHS/EDC mixture, 1G5 antibody, and ethanolamine. The amount of the antibody covalently bound to the chip was determined by the difference in RU prior antibody injection and after ethanolamine injection at the positions indicated by the arrows. Usually, approximately 200 RU of antibody was immobilized onto the chip.

The average antibody immobilization level for the experiments varied slightly, but was kept below or equal to 200 RU (in order to minimize potential mass transport limitation in kinetic analysis (Myszka 1999). The ethanolamine-blocked flow cell served as a reference for detection of non-specific binding. Prior to each binding experiment, C1 chip was preconditioned using 45 s injection of 25 mM NaOH at a flow rate 20 μ l/min in order to remove any non-specifically bound material and stabilize the baseline.

The real-time SPR sensorgrams obtained for the wtFXI binding to 1G5 monoclonal antibody are shown in **Figure 13**. A four minute wait period preceded the injections of the analyte in order to achieve a stable baseline. The response was highly reproducible over the wide concentrations of FXI that ranged from 0.6 nM to 150 nM. In order to remove bound FXI, low pH, high salt (2M NaCl), 0.01% SDS or 25 mM NaOH were tested as regeneration reagents. Sodium hydroxide was the only reagent effective for a complete removal of the bound FXI from the antibody on the chip. Injection of sodium hydroxide, however, introduced significant upward drift in the baseline response following regeneration. Therefore, an additional six min wait period was necessary after each regeneration injection was complete. Sensorgrams of the control channel were subtracted from the sensorgrams obtained on the experimental channel. The entire data set was corrected for systematic drift by subtracting the sensorgram of the buffer blank injection. The corrected sensorgrams were analyzed using Biaeval 4.1 software (Biacore).

Figure 13. SPR analysis of wtFXI binding to 1G5 antibody.

Injectons at the various protein concentrations were performed at a flow rate of 20 $\mu\text{l}/\text{min}$ in HBST buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.01% Tween 20). Panel A: The experimental binding sensorgrams are shown in black. The data fitting curves were derived from the two models: blue- Langmuir 1:1; red- bivalent analyte. The corresponding residuals are shown for Langmuir model (Panel B), and bivalent analyte (Panel C)

A.

B.

C.

Figure 13 illustrates a comparison between the two different fitting models overlaid on the same set of the experimental data: 1:1 interaction and a bivalent analyte model. Based on the visual assessment, the Langmuir model is inadequate at the higher concentrations of FXI (**Figure 13, A**). The residual plot for the Langmuir model (**Figure 13, B**) shows systematic deviations for both association and dissociation phases. In contrast, the residual plot for the bivalent analyte model (**Figure 13, C**) shows less systematic deviation than the Langmuir 1:1 model. Thus, the bivalent analyte model provides significantly better curve fitting as assessed by more random distribution of residuals as well as lower χ^2 value (Karlsson and Falt 1997). This observation is consistent with FXI being a dimeric molecule with two potentially independent antibody binding sites. The bivalent analyte model was used for analysis of the experimental data for all the mutant proteins. The kinetic parameters and χ^2 values for the data fitting are summarized in **Table 4**. The rate constants for all mutants were in the same order of magnitude. Small variations between the mutants are expected because of the differences in the electrostatic interactions of the proteins resulting from substitutions of the positively charged groups by a neutral alanine. Mutant FXI_{R530A} is the only protein which demonstrated about 2-fold decrease in the first rate constant (k_{a1}) compared to the wtFXI. This may be due to the fact that FXI_{R530A} is the buried residue in the FXI structure (see Table 1, chapter 2).

Table 4. Kinetic parameters of mutant FXI binding to 1G5 monoclonal antibody determined by SPR

Mutant	ka1, 1/Ms	kd1, 1/s	ka2, 1/RU	kd2, 1/s	χ^2
WT	1.24×10^5	1.85×10^{-3}	7.49×10^{-4}	3.47×10^{-3}	0.71
FXI _{R504A}	1.51×10^5	1.72×10^{-3}	6.10×10^{-4}	4.9×10^{-3}	0.61
FXI _{K505A}	1.97×10^5	1.05×10^{-3}	4.63×10^{-4}	3.57×10^{-3}	0.43
FXI _{R507A}	1.17×10^5	1.95×10^{-3}	8.3×10^{-4}	3.4×10^{-3}	0.44
FXI _{K529A}	1.29×10^5	1.75×10^{-3}	5.11×10^{-4}	4.35×10^{-3}	0.52
FXI _{R530A}	6.30×10^4	1.04×10^{-3}	5.17×10^{-4}	2.18×10^{-3}	0.96
FXI _{R532A}	1.66×10^5	1.02×10^{-3}	3.75×10^{-4}	5.17×10^{-3}	0.55
FXI _{R586A}	2.0×10^5	1.51×10^{-3}	4.12×10^{-4}	3.72×10^{-3}	0.67

This change, albeit small, may result from a minor structural perturbation caused by the R530A mutation. The distribution of residuals for all the mutants was similar to that for the wtFXI. The χ^2 values were below unity for wtFXI and all mutants, which provides additional evidence that the bivalent model correctly described the experimental data. Ideally, χ^2 value should fall within the noise value of the Biacore equipment, which is ~ 0.3 RU (Morton and Myszka 1998).

Heparin binding ELISA

Sandwich-type ELISA was used in order to estimate the apparent dissociation constants for FXI interaction with heparin and estimate the effects of the introduced mutations (Engvall 1980). For the ELISA-based binding experiments, a monoclonal 5F7 antibody which recognizes the A1 domain of FXI was used for detection of the heparin-bound FXI. The bound 5F7 antibody was detected by a horseradish peroxidase (HRP) conjugated IgG molecule. The use of a sensitive fluorogenic substrate could detect FXI binding at concentrations as low as 100 pM. In the ELISA setup, used in the present work, the background fluorescence level due to antibody binding to heparin was about four times lower compared to the fluorescence signal obtained at the lowest FXI concentration in the heparinized wells. The non-specific FXI binding to the plate was estimated by adding the same concentrations of FXI to the wells containing no heparin. The signal arising from the non-specific binding was subtracted from the total binding signal.

As demonstrated in **Figure 14**, the wtFXI demonstrated saturable binding with an apparent dissociation constant of ~ 2.1 nM, while FXIa showed $K_D \sim 1.9$ nM.

Figure 14. wtFXI and FXIa binding to heparin studied by ELISA.

Open squares: wtFXI, filled squares: wtFXIa. RFU: Relative fluorescence units.

Data represent the means of two independent experiments each performed in duplicate.

Error bars are $\pm$ S.D.

These data indicate that the heparin binding affinity of the zymogen XI is very close or identical to that of the enzyme XIa. This observation is in conflict with the previously published data (Ho, Badellino et al. 1998). The discrepancy is likely to be due to insufficient heparin retention by the amine-coated plates used in the previous studies. **Figure 15** demonstrates the effect of mutations R504A, K505A, R507A in FXI on heparin binding studied by ELISA. The mutant FXI_{Arg504} (Panel A) demonstrated moderate loss in total number of binding sites (28%), with the K_D value of 7.2 nM. Mutant FXI_{K505A} (panel B) demonstrated an apparent K_D value of 3.9 nM, while retaining almost 90% of total number of binding sites. Mutant FXI_{Arg507} (panel C) demonstrated a significant decrease (65%) in total number of binding sites with a K_D value ~ 11 nM (5-fold decrease in affinity). **Figure 16** shows the effect of mutations K259A and R530A introduced into cysteine constrained region formed between Cys527 and Cys542. As shown in **Table 5**, FXI_{K529A} and FXI_{R530A} mutants demonstrated K_D values very similar to that of wtFXI. **Figure 17** shows the ELISA data for mutations of the two residues, Arg532 and Arg586 located in close proximity (approx 6Å) to each other in FXI structure, but more than 50 residues apart in amino acid sequence of FXI. Dissociation constants and B_{max} values compared to those of the wtFXI, demonstrate that R532A resulted in ~40% decrease in total number of binding sites as well as an increased K_D value of ~21 nM. The Arg586Ala mutation resulted in an increased K_D of 13.3 nM, without a significant change in B_{max} . These data are in agreement with the fact that both K529 and R530 are conserved in human PK, whereas R532 and R586 are not.

Figure 15. Effect of mutations R504A, K505A, and R507A in FXI on heparin

binding studied by ELISA.

Panels: A, wtFXI vs. FXI_{R504A}; B: wtFXI vs. FXI_{K505A}; and C wtFXI vs. FXI_{R507A}.

Symbols: wtFXI, open circles; mutants, closed circles. RFU: relative fluorescence units.

Data represent the means of two independent experiments each performed in duplicate.

Error bars are $\pm$ S.D.

Figure 16. Effect of mutations K529A and R530A on FXI heparin binding studied by ELISA. Panels: A, wtFXI vs. FXI_{K529A}; B: wtFXI vs. FXI_{R530A}; Symbols: wtFXI, open circles; mutants, closed circles. Data represent three independent experiments performed in duplicate. Error bars are $\pm$ S.D.

Figure 17. Effect of mutations R532A and R586A in FXI on heparin binding studied by ELISA.

Panels: A, wtFXI vs. FXI_{R532A}; B: wtFXI vs. FXI_{R586A}. Symbols: wtFXI, open circles; mutants, closed circles. Data represent the means of three independent experiments each performed in duplicate. Error bars are $\pm$ S.D.

Table 5. Heparin-binding properties of FXI mutants based on the ELISA

Protein	K _{Dapp} , nM	B _{max}
FXI _{WT}	2.1±0.1	1±0.02
FXI _{R504A}	7.2±0.3	0.72±0.05
FXI _{K505A}	3.9±0.3	0.89±0.02
FXI _{R507A}	11.1±0.4	0.35±0.01
FXI _{K529A}	3.2±0.1	0.95±0.01
FXI _{R530A}	2.7±0.2	0.96±0.03
FXI _{R532A}	21.1±3	0.62±0.08
FXI _{R586A}	13.3±2	0.94±0.05

In order to ensure that the ELISA results are not affected by the type of the antibody used in the assay, two different antibodies in the same ELISA set were compared. A monoclonal antibody 1A6, against the A4 domain of FXI, (a gift from Dr. David Gailani) was used in the ELISA assay along with the 5F7 antibody against the A1 domain of FXI. Similar results were obtained with both antibodies for wtFXI binding to heparin (**Figure 18**). The apparent dissociation constant for heparin binding using the 5F7 antibody was 2.1 nM for wtFXI and 27 nM for FXI_{R532A} mutant, whereas when the 1A6 antibody was used, the comparable values for wtFXI and FXI_{R532A} mutant were 2.4 and 43 nM respectively. Thus, two completely different antibodies corroborated the ELISA data.

Previously, it has been reported that heparin could activate FXI during the equilibrium binding experiments performed in the microtiter wells (Ho, Badellino et al. 1998). After incubation with heparin, the supernatants of wtFXI as well as the mutant FXI proteins were examined using S-2366 as an indicator of proteolytic activity of FXIa. Supernatants were diluted into TBS buffer containing 0.2% BSA to a final concentration of FXI of 18 nM. Under the conditions used for ELISA at the highest concentration of FXI (90nM) tested, the maximum activation was ~ 0.1% relative to total amount of the protein present in the well. This value is at least two orders of magnitude lower than that previously reported i.e. 75% activation in similar assays (Ho, Badellino et al. 1998). The discrepancy may be due to differences in the strategy used for heparin immobilization in microtiter plates.

Figure 18. Comparison of two different antibodies in ELISA-based heparin binding assay.

Open symbols: wtFXI; filled symbols: FXI_{R532A}; squares: 1A6 antibody, circles: 5F7 antibody. Data are average of a single experiment performed in duplicate.

Hydrolysis of S-2366

In order to ascertain the correctness of the protein folding in the mutants, defective in binding to heparin, the hydrolysis of the small synthetic substrate S-2366 has been studied. The catalytic activity of the mutant proteins, which showed defective heparin binding, was tested by measuring their ability to hydrolyze the small peptidyl substrate S-2366. As summarized in **Table 6**, the mutants demonstrated normal K_m and k_{cat} values. The time course of each reaction was monitored by measuring the release of p-NA at 405 nm. The K_m values of the mutants were comparable to those of wtFXIa. Also, the k_{cat} values demonstrated only minor variations compared to the wtFXIa. The data for the mutants FXI_{R504A} and FXI_{R507A} agree with the recently published data describing the same mutations in autolysis loop of FXIa (Rezaie, Sun et al. 2006). From the structural information available, loop 170 plays a minor role in substrate recognition of FXIa (Jin, Pandey et al. 2005).

Table 6. Amidolytic activity of FXIa mutants towards S-2366.

Enzyme	K_m , mM	k_{cat} , (sec ⁻¹)	k_{cat}/K_m , mM ⁻¹ sec ⁻¹
WT	0.35±0.02	111±3.4	317
FXI _{R504A}	0.30±0.01	119±6.3	396
FXI _{R507A}	0.42±0.02	125±6.5	298
FXI _{R532A}	0.37±0.01	110±7.4	297

The data are averages of three separate experiments, each performed in duplicate.

SPR analysis of FXI binding to heparin

In order to characterize kinetics of the heparin binding of the mutant FXI proteins, surface plasmon resonance (SPR) was employed as an additional biophysical method. The biotin-LC-hydrazide-blocked flow cell served as a control to account for the non-specific binding of the proteins to the sensor chip. The binding sensorgrams obtained for wtFXI and plasma prekallikrein (PK) are shown in **Figure 19**. All sensorgrams were double referenced by subtracting the signal for the control surface as well as blank buffer injection. Plasma prekallikrein displayed no binding at a concentration of 30 nM and a very low binding response at a concentration of 300 nM. The wtFXI, injected at concentration of 30 nM demonstrated very rapid binding during the early association phase. A short burst (about 15 sec) in the beginning of the association is followed by a slow increase which does not reach saturation during 5 min injection. In an attempt to increase the association phase, the association interval was extended to 10 min which still did not result in saturation. Similar kinetic behavior was also observed at higher (90nM) FXI concentration (**Figure 20**). At the end of the injection, the signal rapidly dropped followed by a slower dissociation phase which did not reach baseline level. A residual amount of FXI bound to heparin could not be removed by applying concentrated salt solutions such as 1M NaCl or 2M Mg₂SO₄. Among many regeneration conditions tested, 0.02% SDS was the most efficient in removing all bound FXI from heparin.

As evident from the global data fitting analysis in **Figure 20**, the interaction does not conform to a 1:1 model provided by the Biaeval 4.1 software (Biacore). The residuals show highly non-random distribution and the χ^2 value is unacceptably high (>200).

Figure 19. Interaction of wtFXI and PK with heparin studied by SPR.

Injections were performed in HBS buffer (10 mM HEPES, 150mM NaCl, pH 7.4). Association phase and dissociation phases were 5 min each. The specific response was double referenced against the biotin-blocked streptavidin flow cell and buffer blank injection.

Figure 20. SPR analysis of the wtFXI interaction with heparin.

Injections were performed at a flow rate of 20 $\mu\text{l}/\text{min}$ in HBST buffer (10 mM HEPES, pH 7.4, 150mM NaCl, 0.01% Tween 20). The association phase and dissociation phases were 5 min. Experimental sensorgrams are represented by the black lines. Theoretical fit to 1:1 model is shown in red.

Langmuir model $\chi^2 = 277$.

An attempt was made to fit the data using several models available in Biaeval4.1 software (Biacore), none of which provided significant improvement. This is in marked contrast with the 1G5 antibody binding experiments, which show significantly more random distribution of residuals and very low χ^2 values.

In order to compare the heparin binding data for FXI by SPR with other known proteins, ATIII was used as a control protein which interacts with heparin with a high degree of specificity. **Figure 21** shows the sensorgrams obtained for the various concentrations of ATIII using the same heparin-coated biosensor chip. A five-minute association phase was followed by monitoring the dissociation for five minutes. Even at lower concentrations, the equilibrium response was reached very rapidly. The attainability of the steady state level permitted the analysis of the binding data using Scatchard plot. As shown in **Figure 21**, panel B, based on the slope analysis, the K_D of ATIII-heparin interaction was found to be ~ 60 nM.

Part of the reason for complexity of the sensorgrams of FXI binding to heparin could arise from its dimeric structure which results in multivalent binding interaction or cooperativity in binding. SPR analysis however is not a suitable tool to study cooperative binding.

A.

B.

Figure 21. SPR analysis of ATIII interaction with heparin.

Panel A: Real-time sensorgrams of ATIII binding to heparin-coated streptavidin (SA) chip. The measurements were performed at a flow rate of 20 $\mu\text{l}/\text{min}$. The equilibration delay of three min preceded each injection. Panel B: Scatchard analysis of the equilibrium binding response. Linear regression analysis yielded the equation: $y = 2.56 - 0.0166 \cdot x$, from which K_D was obtained.

Heparin binding of monomeric FXI

Human FXI is a covalent homodimer composed of two identical subunits. The covalent bond is formed by Cys321 located in the Apple 4 (A4) domain. The disruption of the covalent linkage does not lead to complete dissociation of the subunits. Several monomeric species of FXI have been prepared and characterized by Dr. Wenman Wu in our laboratory (Wu, Sinha et al. 2008). The monomeric FXI_{C321S/K331A} mutant was prepared and characterized by gel filtration chromatography and analytical ultracentrifugation and migrates as a single band on a non-reducing SDS-PAGE (**Figure 22**). This mutant was utilized in order to assess its heparin binding properties using both ELISA and SPR. Utilizing the ELISA method, the monomeric FXI demonstrated a significant defect in heparin binding (**Figure 23**). While the apparent dissociation constant is increased to 30nM (~15-fold), the total number of binding sites is ~ 40% times of that of wtFXI. This observation has been confirmed by the SPR data presented in **Figure 24**. Although the quantitative analysis was precluded by the complexity of the sensorgram, qualitative analysis indicates that the monomeric protein displays slower association rate, almost reaching equilibrium at the end of the injection. The mutant FXI_{C321S/K331A} which has no additional mutations in any of the heparin binding regions demonstrated a major defect in binding to heparin. These unexpected observations suggest that the dimeric structure of FXI is necessary for the high affinity binding to heparin. An attempt has been made to produce a dimeric FXI_{K331A} protein with the intact Cys321 residue. However, a single mutation K331A resulted in a protein expressed predominantly in a monomeric form (data not shown).

Figure 22. SDS-PAGE of monomeric FXI.

Lanes: 1: wtFXI, 2, FXI_{C321S/K331A}, non-reducing; 3,4: wtFXI, FXI_{C321S/K331A}, reducing.

Figure 23. ELISA analysis of monomeric FXI binding to heparin.

Symbols: open circles, wtFXI; closed circles: monomeric FXI C321S/K331A;
RFU: relative fluorescence units.

Figure 24. SPR analysis of monomeric FXI binding to heparin.

wtFXI (30nM, black line) and C321S/K331A (60nM, gray line) FXI were injected at a flow rate of 20 μ l/min in HBST buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.01% Tween 20). The association phase and dissociation phases were 5 min each.

Electron microscopy

In order to assess the structural integrity of the monomeric form of FXI, negative staining electron microscopy (EM) was used to analyze the overall shape of the molecules and variation in their population. The negatively stained EM images of proteins were collected by Dr. Calvin Yip in the laboratory of Dr. Thomas Walz at Harvard Medical School. The total of 4,269 particles of wtFXI were selected and classified into 50 classes. As demonstrated in **Figure 25 (panel A)**, all of the six representative wtFXI class averages show very similar homogenous X-shaped particles, ~ 8.5 x 14 nm in size, suggesting that the wtFXI adsorbed to the grid in a single preferred orientation. All wtFXI class averages are consistent in size and shape with the FXI dimer seen in the X-ray structure (Papagrigoriou, McEwan et al. 2006). Images of negatively stained monomeric FXI_{C321S/K331A} (**Figure 25. B**) show a homogeneous particle population, about 7 x 11.5 nm in size, but the particles appear smaller than those of wtFXI. The class averages of FXI_{C321S/K331A} demonstrate more structural variability compared to those of wtFXI. This is most likely due to the fact that FXI_{C321S/K331A} adsorbs to the grid in different orientations. This interpretation is supported by images of negatively stained PK (**Figure 25, C**), a molecule with high sequence homology to FXI but existing as a monomer. The PK particles are highly similar in size and shape to those observed with FXI_{C321S/K331A}. The EM analysis suggests that no major structural rearrangements occur in the monomeric form of FXI. However, smaller structural changes which could not be visualized by the negative stain EM can no be ruled out.

Figure 25. Representative class averages of the negatively stained electron microscopy images of wtFX, monomeric FXI, and PK.

Panels: A, wtFXI; B, monomeric FXI _{C321S/K331A}; C, PK. Fifty class averages were obtained for each protein. The total number of 4,269, 4,150, and 4,601 particles was analyzed for wtFXI, FXI _{C321S/K331A}, and PK respectively.

CHAPTER 4

DISCUSSION

During this thesis project, I have successfully expressed and characterized a number of FXI mutants with alanine substitutions in place of positively charged residues in order to identify residues important for heparin binding. The candidate mutations were chosen based on the presence of clusters of positively charged residues in the amino acid sequence of FXI, their conservation among various species as well as the structural information which became available during the project. For the first time it has been demonstrated that the unique homodimeric structure of FXI is essential for high-affinity binding to heparin. This is an unexpected observation, considering that the dimer interface in FXI is the apple 4 domain, which is remote from the heparin binding site.

Studies presented in this thesis, demonstrate that FXI and FXIa bind to heparin with virtually identical affinity. For the first time it has been demonstrated that the residues within and in close proximity to heparin binding consensus sequences in the FXI catalytic domain make significant contributions to heparin binding. This study demonstrated that a single mutation of Arg532 to alanine leads to ~40% decrease in the total number of the heparin binding sites and ~ 5 fold increase in the apparent K_D . Also, residue Arg507 in the autolysis loop region and Arg586 are important for heparin binding. **Figure 26** illustrates the localization of the newly identified heparin binding regions along with previously identified residues in the A3 domain. It appears that the heparin binding region in the apple 3 domain (Lys252, 253 and 255) is located within 50Å from the residues Arg532 and Arg586 in the FXI_{CD} on the same side of a FXI subunit. Such

arrangement would allow accommodating a single heparin chain on each side of the FXI dimer.

Figure 26. Heparin binding regions in FXI.

Abbreviations: HC, heavy chain, CD: catalytic domain. Residues involved in heparin binding are coloured in magenta. Heparin binding sites on the adjacent subunit are located on the opposite side of the dimer.

It is somewhat surprising that some of the mutations lead to loss both in affinity and total number of binding sites. Since the stoichiometry of FXI-heparin interaction could not be determined from ELISA or SPR, it is difficult to rationalize the specific effects of these mutations. It appears clear, however, that FXI-heparin interaction is a complex process which is likely to proceed in more than a single step.

Currently, there are very few published studies that address the significance of dimeric protein structure in heparin-binding. One example is the dimeric PWWP module from hepatoma-derived growth factor, which was shown to bind to heparin with affinity

of ~13 nM, which is two orders of magnitude higher than that of the monomeric PWWP domain (Sue, Lee et al. 2007).

Based on the data presented herein and the structural information available for FXI, there are three possible models which can describe heparin-FXI interactions (**Figure 27**). A single heparin chain can bind across two individual subunits of FXI. Two heparin chains can be bound to each of FXI subunits. Two heparin chains can bind to the catalytic domain and heavy chains in each FXI subunit, resulting in 4:1 stoichiometry.

The similarity between DNA and heparin suggest that direct comparisons can be made. Both heparin and DNA consist of repeating subunits of negatively charged moieties. DNA molecule consists of nucleotides connected by a pyrophosphate backbone, while heparin contains sulfated glucuronic and iduronic acids. Both DNA and heparin form extended helical structures in solution (Watson and Crick 1953; Wilkins, Stokes et al. 1953; Rabenstein 2002). Long-range electrostatic forces play an important role in initial contacts between DNA and protein regardless of a particular nucleotide sequence (Oda and Nakamura 2000). This predominantly non-specific binding is characterized by fast association rates and is sensitive to changes in salt concentration. The concept of facilitated target location or docking onto DNA was initially proposed by Von Hippel and Berg (von Hippel and Berg 1989). Upon encountering nucleic acid, the protein can undergo one-dimensional diffusion along the DNA strand as supported by single molecule experiments (Kabata, Kurosawa et al. 1993; Shimamoto 1999). The second step involves formation of specific hydrogen and van der Waals bonds with the specific nucleotide sequence. This type of interaction is less affected by changes in ionic strength and is characterized by slow dissociation rates (Oda and Nakamura 2000).

Figure 27. Proposed models describing FXI-heparin interaction.

Panel A: Single heparin chain bound across two subunits. Panel B: Two heparin chains bound to two FXI subunits. Panel C: two heparin chains bind to the light and heavy chains in each FXI subunit, leading to 4:1 stoichiometry.

Similar mechanisms are likely to be applicable for protein-heparin interactions and, in particular for FXI. The question of specificity of FXI interaction with heparin remains to be addressed.

An interesting fact is that many proteins that bind to DNA are homo- or heterodimers. The affinity for DNA is often dependent on their dimeric structure. Many transcription factors, including p53, bind to DNA in a cooperative manner. Disruption of the dimerization interface in p53 leads to a significant loss in affinity for DNA (Dehner, Klein et al. 2005). Another example is a HetR protein from cyanobacteria which exists as a covalent dimer linked via a single disulfide bond. Disruption of covalent dimerization in HetR completely abrogates binding to DNA (Huang, Dong et al. 2004).

Another possible explanation for defective heparin binding of the monomeric FXI is the existence of additional binding sites in other domains which together with the residues in CD and heavy chain of FXI form a composite binding site. It is tempting to speculate that a single heparin chain can bind across two subunits in FXI molecule. Similar proposal has been made for the Slit-Robo complex interaction with heparin (Fukuhara, Howitt et al. 2008). Although Slit-Robo is a heterodimeric protein complex, each of the two molecules in this complex possesses positively charged surface patches which form a composite heparin binding site in this complex.

Although more thorough investigation is required, it is possible that a cooperative interaction takes place between the two subunits in FXI dimer upon its interaction with heparin. Cooperative interactions are quite common phenomena in biological systems (Whitty 2008). The most well studied example is the binding of oxygen to hemoglobin

(Ciaccio, Coletta et al. 2008). Although, only one oxygen atom binds per single hemoglobin subunit, the heparin molecule is likely to have more than one protein binding site. These differences invite more detailed investigation addressing the minimal heparin chain length required for FXI binding. In order to obtain quantitative information about a possible cooperative interaction, more rigorous experimental approaches are necessary to be undertaken. For example, filter-binding experiments using radiolabeled nucleic acids have been extensively used to study cooperative interactions between proteins and DNA. One of the more appropriate models that may be tested is the McGhee–von Hippel model of non-specific binding of large ligands to one-dimensional homogeneous lattices (McGhee and von Hippel 1974). This model was originally formulated for protein-DNA interactions. This model suggests an overlap of the protein binding sites on the one-dimensional lattice provided by the DNA. This overlap in binding sites leads to an apparently negatively cooperative effect observed in titration experiments (Kowalczykowski, Paul et al. 1986). This phenomenon does not exclude positive cooperativity which often antagonizes the overlap effect. Because of this complexity, the analysis of the data can also result in a misleading conclusion that there is no cooperativity in the system (Kowalczykowski, Paul et al. 1986; Ma and Lieber 2001).

In this work, I have been cautious about analyzing SPR data and over-interpreting results arising from these studies (Rich and Myszka 2005). The complexity of SPR sensorgrams generated for heparin-binding interactions may arise for several reasons. First, many heparin-protein interactions show low specificity. Second, there is likely more than one FXI binding site on a heparin molecule. Third, there may be more

than a single heparin binding site in a single FXI subunit. Finally, FXI may undergo aggregation upon interaction with heparin.

A major obstacle in the SPR data analysis was the lack of the steady state response in binding of FXI to heparin. In addition, no suitable kinetic model could be used for data fitting analysis. In the case of heparin binding experiments with wtFXI, a short initial burst was followed by a slower increase in RU without reaching saturation. Similar observations have been made for interactions of human cytomegalovirus (HCMV) protease with small peptidyl substrates studied by SPR (Geitmann and Danielson 2004). In contrast to FXI, the interaction of antithrombin III with heparin reached equilibrium very rapidly during the analysis. Although no high quality kinetic data was obtained, it was possible to obtain the apparent affinity constant for ATIII-heparin interaction based on the analysis of the Scatchard plot. Unlike the case with ATIII, FXI injections did not provide steady state responses over several concentrations. There are many examples in the literature of the use of SPR methodology to study protein-DNA and protein-heparin interactions (Yu, Munoz et al. 2005; Zhang, McLellan et al. 2007). Only a small fraction of researchers demonstrate, however, how well the experimental data fit theoretical models that are chosen for analysis (Giannetti, Halbrooks et al. 2005). While some authors provide reference to the magnitude of the residuals or χ^2 values for the data fitting, many do not show or mention them in their work. The visual inspection of SPR sensorgrams in the literature often indicates complex binding patterns, which are often oversimplified or over-interpreted as pointed out by Rich and Myszka (Rich and Myszka 2005).

According to the SPR data, neither low nanomolar nor higher FXI (90nM) concentrations yielded saturable binding. This, however, was not the case when ATIII-heparin interaction was studied using SPR. Also, the study of FXI interaction with 1G5 antibody produced high quality data. Concentrations up to 150 nM gave reproducible responses which were successfully fit to a bivalent analyte model, even though no equilibrium was reached at lower concentrations tested. This is indicative of the high degree of protein purity and lack of spontaneous aggregation. Currently there is no algorithm for analyzing cooperative interactions by SPR (Ma and Lieber 2001). Thus, my attempts to obtain quantitative information from the SPR data on heparin-FXI interactions did not fail because of the low quality data. Rather, the analysis failed because of the complex nature of these interactions.

The complexity in protein-heparin interactions is exemplified by some of the structures of protein-heparin complexes and molecular docking calculations. Even relatively short heparin fragments are capable of engaging in more than a single binding mode. For example, co-crystal structures available for thrombin-heparin and thrombospondin-1-heparin complexes have demonstrated that the molecules in the same asymmetric unit have more than one heparin binding mode (Carter, Cama et al. 2005; Tan, Duquette et al. 2008). Thus the complex SPR data presented herein are likely indicative of multiple heparin-binding modes present in FXI.

Many protein-carbohydrate interactions show characteristics of multivalent interactions. The characteristics of such interactions are relatively weak individual binding constants which become orders of magnitude stronger upon presenting multiple epitopes (Kiessling and Pohl 1996). The reduction in entropy plays the key role in

polyvalent interactions (Mammen, Choi et al. 1998). It is possible that FXI interaction with heparin and, perhaps, other negatively charged molecules is a complex polyvalent process.

Future directions

The future studies should address the individual contributions of the residues in the catalytic domain of the enzyme FXIa in binding to heparin. The same sets of experiments described in this dissertation can also be performed with heparan sulfate as a more physiologically relevant polysaccharide involved in control of hemostasis. Mutations identified in this work can provide useful information about the relative contributions of the residues involved in enhancement of the inhibition of FXIa by PN2 in the presence of heparin. The residues in the heavy chain of FXIa have been previously demonstrated to be important in FXI inhibition by ATIII in the presence of heparin. The roles of the newly identified residues in the catalytic domain of FXIa may provide additional information of this important interaction.

A potentially interesting project would be to introduce the positively charged residues identified in this work into plasma prekallikrein. Although challenging, this strategy may provide substantial evidence about the specific roles that these residues play in two highly structurally homologous serine proteases with different functions.

Although proven useful in this study, the ELISA approach also has several potential drawbacks. First, the ELISA is an indirect method to study interactions of biomolecules. Second, weak interactions may be lost during the washing procedures between steps. Thus, one of the future goals is to study the interaction of FXI and FXIa

with heparin in solution phase. This would avoid any potential surface-related artifacts. A powerful technique utilized to study these interactions is fluorescence polarization or fluorescence anisotropy (Heyduk, Ma et al. 1996). These methods would permit acquisition of quantitative information about the interaction in solution. Such an approach has been successful in studying many protein-nucleic acid interaction systems. This approach has also proved feasible in studying heparin-protein interactions (Ingham, Brew et al. 1993; Andrievskaia, Potetinova et al. 2007).

The defects in heparin binding resulting from mutations of specific residues could not be categorized as direct or indirect at this point. One of the most intriguing questions which remain to be addressed is whether residues of FXI identified in this work are in direct contact with heparin molecule. Structural studies utilizing X-ray crystallography, for example, would help to distinguish between the direct and indirect interactions (Carter, Cama et al. 2005).

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APPENDIX

LIST OF ABBREVIATIONS

A1, A2, A3, A4	Apple domains 1, 2, 3, and 4 in factor XI
aa	Amino acids
APC	Activated protein C
APTT	Activated partial thromboplastin time
ATIII	Antithrombin III
Bmax	Maximum specific binding
BPTI	Bovine pancreatic trypsin inhibitor
BSA	Bovine serum albumin
CD	Catalytic domain
CT	Chymotrypsin
DMEM	Dulbeco's modified Eagle medium
DVT	Deep vein thrombosis
ELISA	Enzyme-Linked Immunosorbent assay
FV	Factor V
FVa	Activated factor V
FVII	Factor VII
FVIIa	Activated factor VII
FVIII	Factor VIII
FVIIIa	Activated factor VIII
FIX	Coagulation factor IX
FIXa	Activated factor IX
FX	Factor X
FXa	Activated factor X
FXI	Factor XI
FXIa	Activated factor XI
FXII	Factor XII
FXIIa	Activated factor XII
GLA	Gamma-carboxyglutamic acid

GlcN	D-Glucosamine
GlcNS	N-sulfo-D-glucosamine
HC	Heavy chain
HK	High molecular weight kininogen
HRP	Horseradish peroxidase
IdoA	L-Iduronic acid
K_{Dapp}	Apparent dissociation constant
K_m	Michaelis constant
LC	Light chain
PCR	Polymerase chain reaction
PBS	Phosphate buffer saline
PDB	Protein data bank
PE	Pulmonary embolism
PK	Plasma prekallikrein
pNA	Para-nitroaniline
PN2	Protease nexin 2
S-2366	Pyro-Glu-Pro-Arg-p-nitroaniline
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPR	Surface plasmon resonance
TBS	Tris buffered saline
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
UFH	Unfractionated heparin
V_{max}	Maximum reaction velocity at saturating substrate concentration
von WF	von Willebrand factor