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STRUCTURAL STUDIES OF THE FACTOR X ACTIVATING COMPLEX OF THE INTRINSIC PATHWAY OF BLOOD COAGULATION

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Thesis submitted to
the School of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Biochemistry

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ABSTRACT

STRUCTURAL STUDIES OF THE FACTOR X ACTIVATING COMPLEX OF THE INTRINSIC PATHWAY OF BLOOD COAGULATION

Judith Sharron Atkins
Department of Biochemistry
University of Ottawa, 1992

In the blood coagulation pathway the zymogen factor X (FX) is converted to its enzymatic form (FXa) by the FX activating complex which is a membrane bound multi-protein complex comprised of the cofactor factor VIII (FVIII), the enzyme factor IXa (FIXa), the substrate FX, as well as calcium and phospholipid. Activation of FX to FXa is essential for production of fibrin, which is the final product of the coagulation pathway. Defects or deficiencies in FVIII or FIXa hinder production of FXa and results in the bleeding disorder, haemophilia. Although much is known about the individual components of the FX activating complex, the mechanisms by which these proteins interact remain to be ascertained. In addition, the protein and phospholipid binding sites within each protein and the molecular forces involved in these associations have not been fully elucidated. Structural aspects of the FX activating complex have mostly been probed using purified coagulation factors and synthetic phospholipid bilayers. Detailed studies using biological surfaces, such as platelet membranes are still forthcoming.

To examine structural aspects of the FX activating complex, affinity chromatography was utilized as an analytical rather than as a preparative tool. This novel application was used to characterize the molecular forces that are involved in maintaining the integrity of the membrane-bound FX activating complex. In conjunction with radioligand binding studies, this procedure was also employed to identify binding domains within FVIII for FIXa, FX and phospholipid. The data demonstrated that both chains of FVIII; FVIII light chain (FVIII-LC) and FVIII heavy chain (FVIII-HC), contained binding domains for FIXa. Binding of FIXa to FVIII-LC was observed to be mediated by hydrophobic forces, whereas divalent cation-dependent forces mediated binding to FVIII-HC. FVIII-LC was also found to contain a binding domain for FX. This interaction occurred independently of FVIII-HC and was maintained by hydrophobic forces. FVIII-LC contained hydrophobic binding domain(s) for phospholipid. Hydrophobic forces were also found to play a heretofore unrecognized role in the association of FIXa or FX with phospholipid. These data indicate that multiple forces are involved in forming and maintaining the integrity of the FX activating complex. These results also emphasize that the mechanism by which FVIII serves as a cofactor is the culmination of multiple structural interactions.

Data from radioligand binding studies suggested that FVIII acted as a high affinity binding protein for FX ($K_d = 10^{-8}$ M) on the surface of synthetic phospholipid membranes. To ascertain whether this also applied to biological surfaces, the association of FX with platelet plasma membranes was investigated using equilibrium binding studies. The effect of platelet storage on binding of FX was also examined. Unstimulated platelets did not bind FX. In contrast, specific binding of FX to stimulated platelets ($K_d = 10^{-8}$ M) occurred when platelets had been stored for less than 4.5 hours from phlebotomy. The affinity of binding of FX to fresh platelets was not significantly affected by the presence of FVIII ($K_d = 10^{-8}$ M), nevertheless, the storage-dependent decline in platelet binding of FX was not observed in the presence of FVIII. The high affinity and lability of the FVIII-independent binding site suggests that FX binds to a specific, and as yet unidentified, platelet membrane component. The information obtained in the aforementioned studies extends our current understanding of the FX activating complex.

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LIST OF ABBREVIATIONS

CPM	Counts per minute
DPG	Diphosphatidylglycerol (also called cardiolipin)
EDTA	Ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(ß-aminoethyl ether)n,n'-tetraacetic acid
FIX	Factor IX
FIXa	Factor IXa
FX	Factor X
FXa	Factor Xa
FV	Factor V
FVa	Factor Va
FVIII	Factor VIII
FVIIIa	Factor VIIIa
FVIII-HC	The heavy chain of FVIII
FVIII-LC	The light chain of FVIII
GLA-domain	Gamma-carboxyglutamic acid containing domain
Gla residues	Gamma-carboxyglutamic acid residue
K_d	Dissociation constant
Lyso-PC	Lysophosphatidylcholine
Lyso-PE	Lysophosphatidylethanolamine
Lyso-PS	Lysophosphatidylserine
M_r	Relative molecular mass
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
Pi	Inorganic phosphate ion
PI	Phosphatidylinositol
PL	Phospholipid
PLV	Phospholipid Vesicles
PPi	Inorganic pyrophosphate ion
PS	Phosphatidylserine
Tris	Tris(hydroxymethyl)aminomethane
vWF	von Willebrand factor

CHAPTER 1

BACKGROUND

CHAPTER 1: Background

1.1.0 Introduction

A number of essential biochemical events and pathways, such as blood coagulation, require formation of multi-protein complexes that interact with membranes. The mechanisms and forces involved in forming and maintaining the integrity of such membrane-bound multiprotein complexes have not been clearly defined. Shortage of highly purified constituent proteins, inadequate model systems or limitations of techniques that are capable of examining associations at the molecular level have hampered these investigations. For those interested in studying protein-lipid and protein-protein interactions within such structures, the enzymatic complexes of the blood coagulation pathway may represent a paradigm for membrane-bound macromolecular complexes. Purified proteins of the blood coagulation pathway are readily available. Their amino acid sequences have been delineated as well as the sequences of their cDNAs. Synthetic PLV have proved excellent as a simplified model system to study the association of various coagulation proteins with membranes. In addition, the recent availability of monoclonal antibodies directed towards specific coagulation proteins makes possible the use of immuno-affinity chromatography to examine protein-protein and protein-lipid associations. A number of bleeding disorders result from deficiencies or dysfunctions of specific coagulation factors. Information obtained through investigations of coagulation complexes and their constituent proteins may lead to a better understanding of membrane bound multi-protein complexes in general and, more importantly, may lead to improvements in treatment for bleeding diatheses in clinical settings.

1.2.0 Thrombosis, Haemostasis and Coagulation

The fluidity of plasma is maintained by dynamic interactions between components of the vessel walls and blood (plasma proteins and cellular elements). However, if a vessel wall is punctured or perforated, bleeding occurs and the net balance of these interactions is shifted. The process by which blood loss is stopped, termed haemostasis, involves two major overlapping events: vessel wall contraction and formation of a thrombus or clot. Thrombogenesis results from the adhesion and aggregation of platelets to form a plug and activation of the coagulation pathway which leads to formation of a cross-linked fibrin network. The fibrin matrix stabilizes and helps maintain the platelet plug until the damaged vessel wall is repaired. As vessel repair occurs, enzymes of the fibrinolytic pathway degrade the fibrin network, allowing dissolution of the clot.

Fibrin is the end-product of the coagulation pathway. In humans, the coagulation pathway is comprised of thirteen proteins which circulate in the blood in the inactive (zymogen) form (Table 1.1). Damage to the vascular wall causes activation of the initial zymogens of the contact phase of the coagulation pathway (Figure 1.1) and, thus, induces the chain of reactions which lead to fibrin production. Proteins of the mid-phase of the coagulation pathway are organized into two "arms", the intrinsic and extrinsic branches which function independently of each other until they converge at production of FXa. The final common pathway of coagulation involves FXa catalyzed activation of prothrombin to thrombin. Thrombin converts fibrinogen to fibrin which, as already stated, is required for thrombogenesis. Lastly, factor XIIIa catalyses intermolecular γ-glutamyl-ϵ-lysine bridge formation between fibrin side chains [1], thus strengthening the fibrin skeleton and stabilizing the thrombus. Activated proteins of the coagulation pathway are localized to the damaged area presumably by binding to platelets and other surfaces in the immediate vicinity, such as endothelial cells. This

Table 1.1. Proteins of the Coagulation Pathway

Coagulation Factor (Zymogen State)	Alternate Names	year of Isolation	Activated Form	Main Function(s)
high molecular weight kininogen	Fitzgerald, Flaujeac or Williams Factor or Contact Activation Cofactor	1975[2-4]	HMWKa	cofactor/carrier protein for FXI and prekallikrein cofactor for FXIIa[5]
prekallikrein	Fletcher Factor	1972[6]	kallikrein	activates FXI, FXII, HMWK (releasing bradykinin) cleaves plasminogen, prorenin and C1 in fluid phase
XIII	Fibrinoligase or Fibrin Stabilizing Factor	1961[7]	FXIIIa	cross-links fibrin[1], actin, α_2-macroglobulin, fibronectin, collagen[5] and α-plasmin inhibitor
XII	Hageman Factor	1955[8]	FXIIa FXIIf	activates FXI and prekallikrein activates FVII by FXIIf[9]
XI	Plasma Thromboplastin Antecedent, PTA	1953[10]	FXIa	activates FIX to FIXa
X	Stuart-Prower Factor	1956[11]	FXa	activates prothrombin and FVIII
IX	Christmas Factor	1952[12-14]	FIXa	activates FX to FXa
VIII	Antihaemophilic Factor	1972[15]	FVIIIa	cofactor in activation of FX to FXa
VII	Serum Prothrombin Conversion Accelerator (SPCA) or Stable Factor	1951[16]	FVIIa	activates FX and FIX
V	Proaccelerin or Labile Factor	1947[17]	FVa	cofactor in the activation of FX cofactor in the activation of protein C[18]
III	Tissue Factor or Tissue Thromboplastin	1944[19]	-----	cofactor for FVII in activation of FX and FIX
II	Prothrombin	1962[20]	Thrombin	cleaves FXIII, FV, FVIII, prothrombin, protein C activates platelets and endothelial cells
I	Fibrinogen	1830[21]	Fibrin	stabilizes blood clot

Table 1.1. Continued...

Coagulation Factor	molecular weight	plasma conc. (μg/ml)	In Vivo half-life (hours)	site of synthesis/ release	gene location (Chromosome)	incidence of deficiency
high molecular weight kininogen	120,000	70-90	150[5]	????	3[22]	rare[5]
prekallikrein	85,000	35-50	35[5]	liver[23]	7 (mouse)[24]	rare[5]
XIII	320,000	10-20	150[5]	subunit A:blood cells & precursors subunit B:liver[26]	subunit A:6[25] subunit B:1[27]	100(tot)[5]
XII	80,000	15-47	40-70	liver[28]	5[5]	1:500,000[5]
XI	160,000	2-7	40-80[5]	liver[29]	4[30]	1:100,000
X	59,000[5]	5-10[31]	25-60[5]	liver	13[32]	1:500,000
IX	56,722[33]	3[5]-5[34]	24[5]	liver	X	1:25,000
VIII	260,000[35]	0.05[36] -0.2[37]	12[5]	tissues from liver to endothelium	X	1:10,000
VII	48,000	0.5	6[5]	liver	13[5]	1:500,000
V	330,000	10	24[5]	tissues from liver to endothelium	1[38]	1:1,000,000[39]
III	44,000	0[5]		tissues from brain to monocytes[5]	1 [5]	rare
II	70,000	100	50-80[5]	hepatic parenchymal cells	11[40]	35(tot)[5]
I	340,000	2000-4000	100-150[5]	hepatic parenchymal cells	4[41]	1:2,000,000[5]

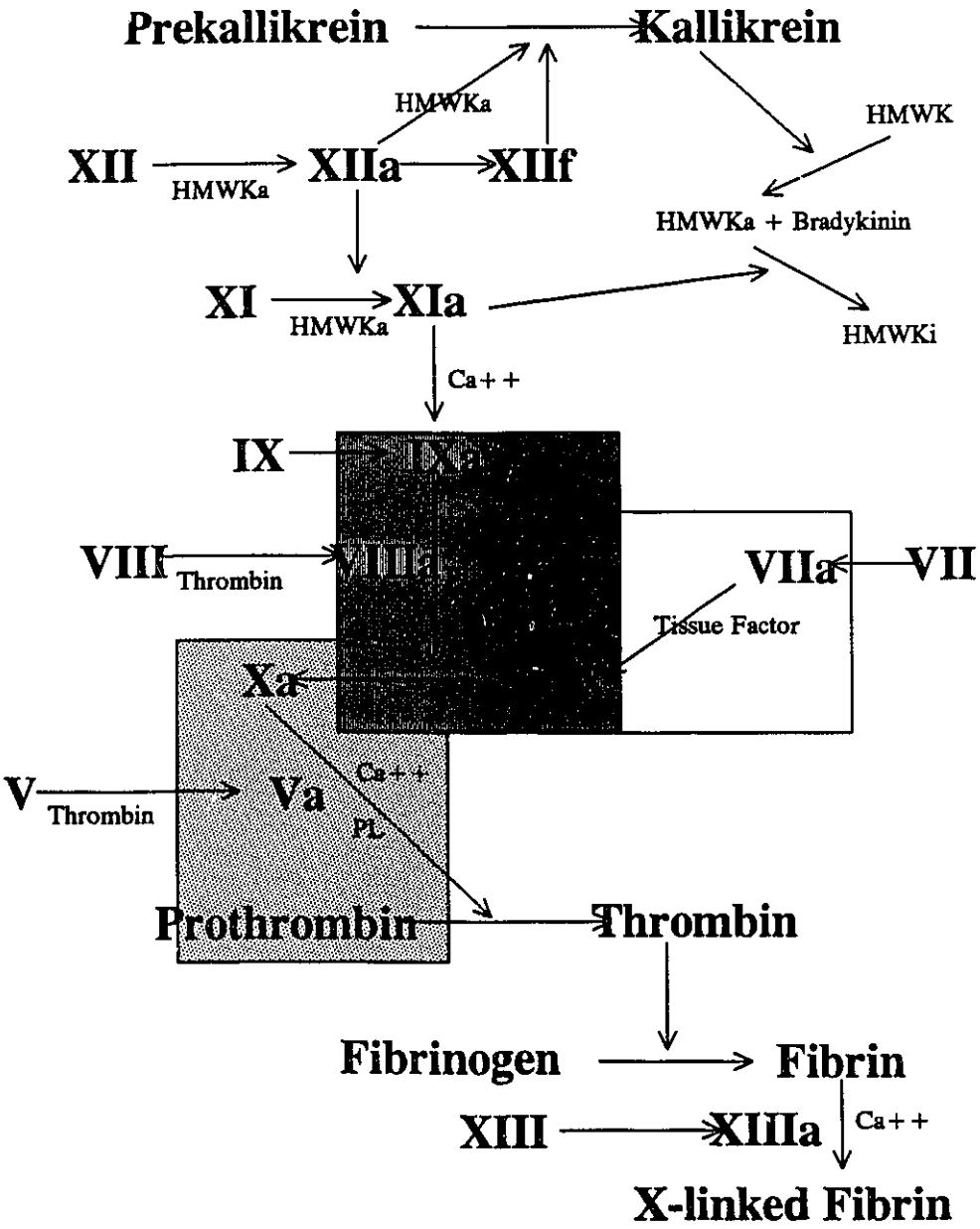

Figure 1.1. A simplified schematic diagram of the coagulation pathway. This pathway can be arbitrarily divided into three components. The contact phase of coagulation involves the zymogens prekallikrein, high molecular weight kininogen, FXII and FXI. The mid-phase of coagulation involves the following zymogens and cofactors: FVIII, FIX, FX, FVII and tissue factor and the final, common pathway, of coagulation involves the zymogens FV, prothrombin, fibrinogen and FXIII.

helps localize thrombogenesis to the site of the wound and prevents formation of thrombi at sites where no vascular injury is present. Formation and dissolution of the clot is also controlled by a network of multiple feed-back reactions and controls which operate at many levels. For example there are at least ten circulating plasma proteins that function to inhibit blood coagulation (Table 1.2).

During the course of coagulation, three membrane-bound multi-protein complexes are formed: (i) the intrinsic FX activating complex, (ii) the extrinsic FX activating complex and (iii) the prothrombinase complex (Figure 1.1). Each structure is comprised of three proteins that possess specific functions. For example, the intrinsic FX activating complex is comprised of FVIII (the cofactor), FIXa (the enzyme) and FX (the substrate). Formation of these macromolecular enzymatic complexes has been demonstrated to occur on the surface of PL membranes. It is believed that, in vivo, platelets provide the PL surface [42-44]. However, there is recent evidence that endothelial cells may also support coagulation complex formation [45-47]. The role of platelets in thrombogenesis will be discussed further in chapter 4. Formation of the intrinsic FX activating complex and the prothrombinase complex requires calcium. Calcium anchors FIXa, FX, FXa and prothrombin to the PL surface [32, 48-51] and is required to maintain the structural integrity of FVIII and FV [52, 53]. Activation of FX to FXa is central to the blood coagulation process [54, 55]. Inhibition of FXa formation may lead to clinical complications, such as prolonged bleeding, which if untreated, may lead to permanent tissue damage and in severe cases, death. In vivo, the intrinsic FX activating complex appears to be the major activator of FX, as the most common bleeding disorders are caused by defects in this complex, specifically by the deficiency or dysfunction of FVIII or FIXa (Table 1.1).

Table 1.2. Plasma Borne Blood Coagulation Inhibitors

Inhibitor	Molecular Weight	Plasma Conc. (μg/ml)	Function
Activated protein C	56,000	4	inactivates FVa, FVIIIa and plasminogen activator inhibitor[58]
Protein S	69,000	25	cofactor of activated protein C[58]
Activated Protein C inhibitor	57,000	5	inhibits activated protein C[59]
Heparin			cofactor for antithrombin III, heparin cofactor II, APC inhibitor[60]
Heparin cofactor II[s?]	65,600	9	inhibits thrombin[61]
Antithrombin III[s]	65,000	180-300	deficiency may cause venous thromboembolic disease[20, 62] inhibits thrombin, FXIIa, FXIa, FXa, FIXa, kallikrein, plasmin
α_2-macroglobulin	725,000	1500-3500	inhibits kallikrein, thrombin and plasmin[63]
C1 inactivator	104,000	150-300	deficiency associated with hereditary angioneurotic edema[64] inhibits C1, C1s, C1r, kallikrein, FXIIa, FXIIf, FXIa and plasmin
α_1-antitrypsin[s]	50,000	2000-4000	deficiency correlated with development of pulmonary emphysema[65] inhibits FXIa, FXa, thrombin and plasmin[5]
α_2-plasmin inhibitor[s?]	68,000	50-70	inhibits plasmin[66], deficiency causes visceral bleeding after trauma and oozing of wounds
Plasminogen activator inhibitor	52,000	0.05 (variable)	inhibits activation of plasminogen by t-PA and urokinase
Extrinsic pathway inhibitor (or LACI)	38,000	0.1	inhibits FVII-tissue factor-FXa complex

s. Member of the Serpin family which includes: α_1-Antichymotrypsin, Ovalbumin and angiotensin.
s?. May be a member of the Serpin family.

1.3.0 A Brief History of Haemophilia

Over 1,700 years ago it was written in the Talmud, that a distinguished Rabbi exempted a newborn boy from circumcision because his brothers had died from this operation [56]. This is the earliest documented description of a bleeding disorder with symptoms matching those of haemophilia [57]. The first officially recorded case of haemophilia was in 1793 by an unknown author [67]. The criteria for a diagnosis of haemophilia were not commonly agreed upon until 1911. At this time a report was published which described the symptoms in detail [68]. The next major milestone in the identification of the defect afflicting individuals with haemophilia, occurred in 1937 with identification of the presence of a blood borne "anti-haemophiliac factor", termed FVIII [69]. FVIII was found to co-purify and circulate with vWF in plasma as a non-covalent complex [70]. The association of FVIII with vWF occurs with high affinity ($K_d = 10^{-12}$ M) [71]. In 1972 the procoagulant activity of FVIII was structurally separated from the ristocetin cofactor (FVIIIR:RCF) activity of vWF [15]. However, the molecular form of FVIII was not fully characterized until 1984 when the complete amino acid sequence was deduced [72] from the mRNA-derived cDNA sequence of FVIII [73].

The isolation and characterization of FIX was not complicated by the presence of a carrier protein. In 1947, while studying two patients with bleeding disorders, presumed to be classical haemophilia, Pavlovsky found that infusion of blood from one patient could correct the bleeding time in the recipient patient [74]. Although he did not conclude that the patients had different types of haemophilia, Pavlovsky's study provided clues for others and in 1952 Aggeler et al. [13] and Biggs et al. [14] independently discovered that there were two sex-linked recessive types of haemophilia that are clinically indistinguishable from each other [75]. Classical haemophilia, also called haemophilia A, and haemophilia B are due to the deficiency or dysfunction of blood coagulation FVIII or FIX, respectively. Haemophilia B is also called Christmas disease

[14]. In 1956 a deficiency or dysfunction of another blood borne agent, the Stuart-Prower factor, later termed FX, was reported to cause a bleeding diathesis that was corrected by the infusion of plasma [76-79].

As early as 1969 [80], it was recognized that haemophilia-like symptoms were also caused by FVIII inhibitors, which are defined as pathologic circulating human autoantibodies or alloantibodies that specifically inhibit the coagulant activity of FVIII [81, 82]. Unlike genetically transmitted haemophilia, FVIII inhibitors arise in both males and females, and similar to other autoimmune diseases, a 3:1 female to male ratio has been observed [83]. These antibodies arise in approximately 5-20% of multitransfused individuals with severe haemophilia A in response to FVIII infusion [81, 82, 84, 85] and may occur spontaneously in nonhaemophiliacs [85], such as postpartum women, patients with autoimmune diseases, and in older adults without underlying disease [82].

1.4.0 Advances in Clinical Treatment of Haemophilia

Between 1950 and 1970 great advances in research led to the development of treatments for both haemophilia A and B. Prior to this time, the mean age of death in haemophilia A-afflicted individuals was less than 20 years [86]. Initially, treatment consisted of plasma infusions. This procedure was inefficient and expensive considering that plasma contains only 100 [86, 87] to 200 [37, 86] ng of FVIII per millilitre. Some researchers estimate the concentration of FVIII in plasma to be as low as 300 pM [88]. A great advance in haemophilia therapy occurred in 1964 with the discovery of cryoprecipitate by Judith Pool and her colleagues [89]. Cryoprecipitate is relatively easy to prepare. It is comprised of serum proteins that precipitate from plasma which has been frozen and then thawed. These proteins constitute the fraction of plasma that is insoluble at 4°C. Cryoprecipitates contain 40% to 80% of the original plasma FVIII

activity [90]. In addition, FVIII is present at much higher concentrations than in plasma, thus, drastically reducing the required infusion volume. To further purify FVIII and to further reduce the infusion volume, processes were developed to concentrate the FVIII obtained in cryoprecipitates. Unfortunately, up to 60% of the original FVIII activity is lost during the additional purification procedures [90]. Currently, commercial FVIII concentrates are prepared from pools of plasma obtained from thousands of donors [86]. In the United states, alone, 500 million units of FVIII concentrate are produced, which represents approximately 300 g of purified FVIII [91]. FVIII concentrates contain less biological contaminants than plasma, however concentrates are still highly impure, containing less than 1% of the total protein as FVIII [86]. Another drawback, from a transfusion perspective, is that FVIII concentrates carry a risk of transmission of infectious agents such as hepatitis or HIV.

FVIII concentrates are used for prophylaxis and home treatment of individuals with haemophilia A. However, some hemophiliacs are treated only when they have an uncontrolled "bleed" [57]. This results in chronic bleeding into the joints which can lead to tissue damage and joint problems in later life [57]. Current treatment for haemophilia B involves infusion of commercial FIX concentrates prepared by relatively crude plasma fractionation or immunoaffinity chromatography [92-94]. Many problems exist with the current treatment regiment for haemophilia A and B. Some risks posed by concentrates, such as HIV, may be worse than the disease itself, thus, new treatment strategies must be found.

Treatments which are not based on plasma purification of FVIII or FIX are also being explored, such as infusion of PLV plus FXa [95] and oral administration of a peptide fraction (M_r of 1,000 to 20,000) of bovine FVIII devoid of procoagulant and platelet aggregating activities but possessing anti-haemorrhagic activity [96]. These treatments are especially relevant to patients who produce inhibitor antibodies.

However, the most promising development in the clinical treatment of haemophilia is the potential to produce recombinant FVIII [86] or FIX [97]. The complete cDNA for FVIII has been isolated and expressed in mammalian cells [86, 98] and is currently undergoing clinical trials. With the advent of recombinant techniques it may be possible in the near future to use retrovirally-mediated transfer of FIX or B-domainless FVIII for gene therapy. Unfortunately, at the present time, it is difficult to express FVIII and FIX in quantities great enough for widespread clinical use. Further basic research is required to determine which regions of FVIII are critical for cofactor activity. Information on the functions of specific structural domains within FVIII and FIX, essential for formation of the FX activating complex, will be useful in the construction of smaller recombinant FVIII- and FIX-like molecules that could be expressed in larger quantities.

1.5.0 Structure and Function of the FX Activating Complex and its Protein Components

Simplified model systems that support coagulation have been used to examine macromolecular complexes of the coagulation pathway. Currently, information about the FX activating complex is based upon results obtained from such studies in which the FX activating complex was reconstituted in vitro from purified components. From these data, it was proposed that membrane-bound FIXa and FX interact with each other and at the same time each interact with membrane-bound FVIII (Figure 1.2). However, the mechanism by which the FX activating complex assembles has not been determined, nor have the molecular forces required to maintain the integrity of this complex on a membrane surface been defined. The mechanism by which FVIII exerts its cofactor function has also not been characterized. The domains of each protein that associate with other constituents of this complex have not been identified. Furthermore, it has not been resolved whether FVIII serves as a binding protein for FIXa and FX on the surface

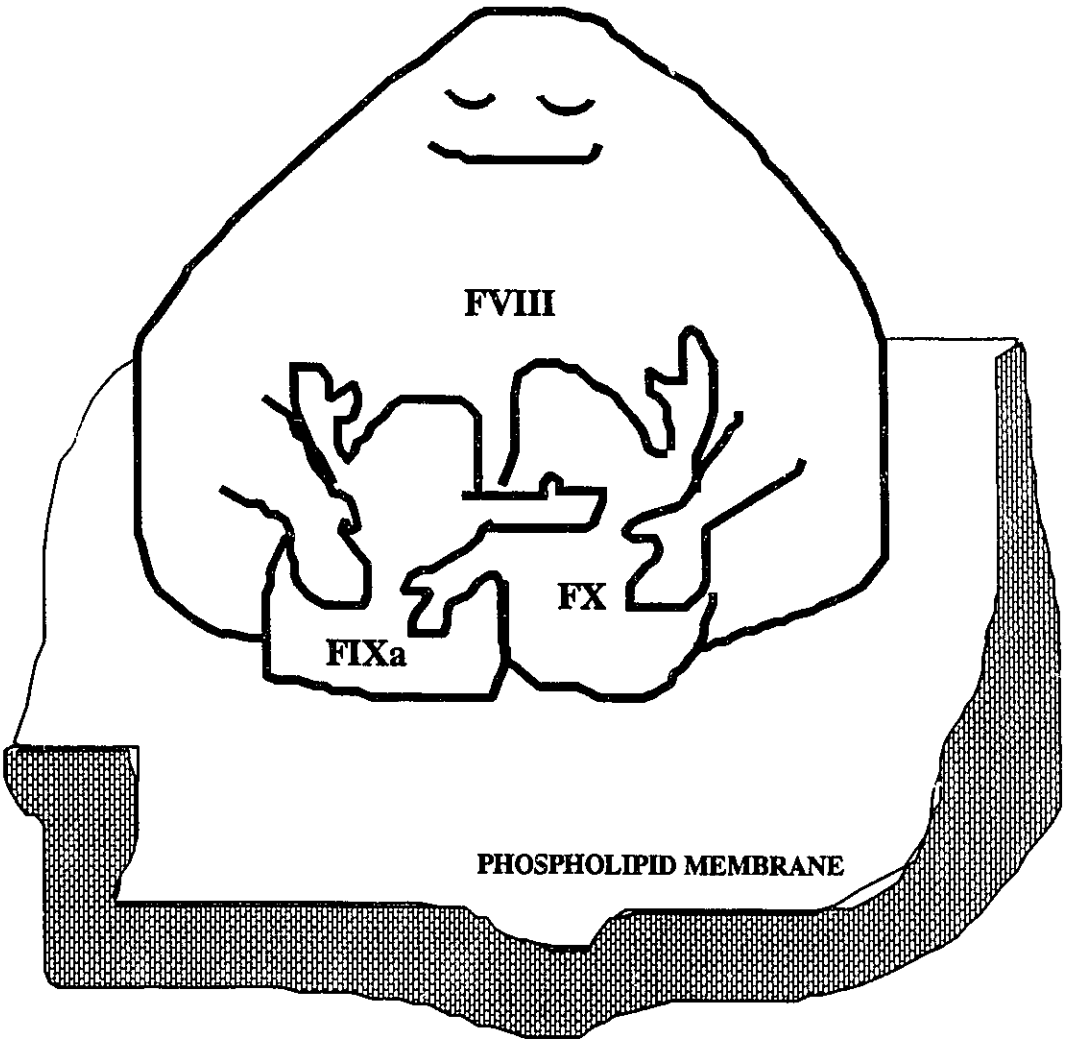

Figure 1.2. Schematic model of the currently accepted structure of the FX activating complex. In this model, each of FVIII, FIXa and FX interact with the remaining two proteins on the surface of a PL membrane.

of PL membranes. In vivo, the FX activating complex is believed to form on cellular surfaces, such as the plasma membrane of activated platelets. It is not known, in vivo, whether other factors, such as proteinaceous receptors or accessory binding proteins present on cellular surfaces may be involved in formation or function of the FX activating complex. In this regard, further work is warranted to better define the role platelets play in the formation and function of the FX activating complex.

Within the FX activating complex, FX is the substrate, and FVIII serves as a non-proteolytic cofactor to the enzymatic component, FIXa [99, 100]. Calcium and PL are also required for efficient activation of FX by this complex. In vitro, FIXa, alone, is able to activate FX [101, 102] and increases the V_{max} of the reaction [102]. However, activation of FX by FIXa is increased 2,000 to 200,000 fold in the presence of the remaining components of the FX activating complex [103]. FVIII, in conjunction with FIXa, acts to increase the V_{max} of activation of FX from 4 orders of magnitude [104] to 200,000 fold [102]. Calcium increases the rate of FXa formation over ten fold [54]. Localization of coagulation factors on acidic PL containing membranes increases the activation of FX by 550- to 200,000-fold [105] by decreasing the Km of the reaction [102, 103, 106-108]. In vitro, PLV containing acidic PLs, such as PS, have been shown to support clotting activity with efficiencies very close to that of activated platelets [109]. Further investigations concluded that PL membranes provided a surface upon which this multi-protein enzyme complex forms [42, 110-112]. Once bound to the membrane, coagulation factors may move by bulk phase diffusion or by "sliding" in the plane of the membrane, and thus come together to form enzymatic complexes such as the FX activating complex [113].

1.5.1 FVIII

FVIII is synthesized and secreted by a number of tissues within the body

(Table 1.1). In plasma, FVIII is found as a bi-molecular complex with vWF. During coagulation the FVIII-vWF complex dissociates [114] and FVIII associates with PL [115]. Two forms of FVIII have been isolated from circulating blood, a single chain and a two chain form. The single chain form of FVIII has a molecular weight ranging from 280,000 to 340,000 [35]. The two chain form of FVIII is derived from the single chain polypeptide and consists of an amino-terminal derived heavy chain (FVIII-HC) of variable length (90,000-210,000) and a carboxy-terminal derived light chain (FVIII-LC) of about 80,000 [81, 116, 117]. It has not been determined whether processing to the two chain form is an intra-cellular or an extra-cellular event [37, 99]. Post-translational modifications of the FVIII molecule, such as removal of a 19 amino acid signal peptide, glycosylation and sulfation, occur before release into the circulation [99].

FVIII-LC forms a divalent cation-dependent complex with FVIII-HC. Exposure to concentrations of EDTA greater than 100 μM [118] dissociates FVIII-LC and FVIII-HC and causes loss of procoagulant activity [52, 53, 116, 119-121]. Neither FVIII-HC or FVIII-LC have coagulant activity when separated [119, 121]. However, FVIII structure [52, 53] and activity [53] can be reconstituted after chelation by reintroducing Ca^{2+}, Sr^{2+}, Mn^{2+}, or Mg^{2+} [52, 53] into the incubation mixture. Highly purified citrated FVIII contains calcium at a ratio of approximately 1.0 mole/mole FVIII with M_r of 220,000, indicating the presence of at least one high affinity calcium binding site [52]. It has been suggested that calcium is the divalent cation responsible for the stabilization of FVIII in vivo, however, this has not been confirmed or disproved.

FVIII is comprised of 2332 amino acids. The primary sequence exhibits 3 distinct structural domains, including a triplicated region (the A domains), a unique region of 909 amino acids (the B domain), and a carboxy-terminal duplicated region (the C domain), that are arranged in the order A1-A2-B-A3-C1-C2 [119]. The A domains of FVIII exhibit 35% homology with the A domains of ceruloplasmin, and FV [122].

The functions of the A domains of these proteins have not been elucidated. The C domains of FVIII exhibit 18% homology with domains of the discoidin lectins isolated from *Disctyostelium* [72] and 42% sequence homology with the C domains of FV, a blood coagulation protein similar in structure and function to FVIII [123]. The functions of the C domains of FVIII and FV have not been delineated, but based on the sequence similarity with *Disctyostelium* lectin, it has been postulated that they may play a role in anchoring FVIII and FV to PL membranes [123]. The region encompassed by residues 2303 to 2332 of FVIII shares 58% and 70% sequence homology with *Disctyostelium* lectin and FV, respectively [123]. Such high conservation of sequence suggests that this region may play an important functional role [123]. FVIII also possesses two highly acidic regions, one in the heavy chain between domains A1 and A2 (residues 331-372) and one in the light chain (residues 1649-1689). The second acidic region may form a binding site for vWF [99, 124]. Furthermore, the second acidic region contains a sequence (residues 1663-1669) to which an antibody binds. This antibody inhibits the procoagulant activity of FVIII [125], suggesting that the second acidic region also possesses critical structural significance for FVIII function [125]. The two acidic regions are not contained in FV [126, 127] and may contribute to the unique functions of FVIII [124].

The role of the B domain of FVIII is uncertain. The B domain appears to be cleaved away during activation and may not itself possess procoagulant activity [81]. Recombinant DNA techniques have been used to construct rFVIII in which 767 [121] to 880 [104] residues of the B domain were removed. This B-domainless FVIII binds to PLs in a manner similar to native FVIII [119], suggesting that the B domain is not involved in PL binding [119]. Furthermore, B-domainless FVIII could still be thrombin activated indicating that highly glycosylated B domain is not needed for activity [104,

121]. In vitro experiments suggest that B-domainless FVIII may be more susceptible to proteolytic attack, suggesting that this domain of FVIII may function to protect FVIII from proteolysis by low amounts of circulating active proteases, such as thrombin [121]. Deletion of the B domain in recombinant FVIII does not affect binding of vWF to FVIII [99] and, therefore, is likely not involved in this interaction [128].

It is currently believed that FVIII is activated to FVIIIa by thrombin cleavage at both Arg372 [87, 116, 122, 129-131] and Arg1689 [81, 116, 122, 130]. Thrombin proteolysis, thus, results in the separation of the domains A1 and A2 [132], producing fragments with M_r of 54,000 and 44,000 from the amino-terminal FVIII-HC (M_r of 92,000), while a fragment with a M_r of 72,000 was derived from the carboxy-terminal FVIII-LC (M_r of 80,000) [81, 87, 116, 122, 130, 131, 133]. Thus, proteolytic activation of FVIII appears to result from the removal of the acidic region contained in FVIII-LC. In contrast, removal of the FVIII-HC acidic region, by activated protein C mediated cleavage at Arg336, results in inactivation of FVIII [122, 132]. This implies that the resultant 36 amino acid fragment possesses a functional significance [122]. It is possible that the heavy chain acidic region may be involved in association of the two chains of FVIII, possibly by coordination of a calcium ion [134] or may be important for interaction with FIXa or FX [122]. FXa cleaves FVIII at residue 1721 and thrombin also cleaves FVIII at residue 740, however mutation of these residues had no observed effect on in vitro procoagulant activity [130].

1.5.2 FIX

FIX is synthesized in the liver as a single chain zymogen of 461 amino acids [33, 135]. A number of post-translational modifications occur before secretion [135], including glycosylation [136, 137], cleavage of the signal peptide and propeptide [135, 137], vitamin K-dependent carboxylation of the 12 N-terminal Glu residues to form Gla

residues [136, 137], and β-hydroxylation of Asp64 to form β-hydroxyaspartic acid [136, 137]. Thus, the final form of human FIX consists of a single polypeptide containing 416 amino acids [33]. It has been reported that circulating FIX has a M_r of 54,000 [75] to 65,000 [93]. From the cDNA it has been determined that FIX has a M_r of 56,722, including 17% carbohydrate [33] (Table 1.1).

FIX is a member of a family called vitamin K-dependent proteins. Like other vitamin K-dependent coagulation proteins, FIX is comprised of a number of structural domains [75]. The first 46 amino-terminal residues comprise the GLA-domain, residues 47 to 127 comprise two epidermal growth factor-like domains, residues 128 to 195 comprise the activation peptide [75], and residues 196 to 415 comprise the trypsin-like serine protease domain [75, 138]. It has been demonstrated that the PL binding site(s) of FIXa are contained within the GLA-domain [32, 50, 51, 75, 102, 109, 139]. These studies employed decarboxylation, chemical modification or vitamin K deficiency (which prevents gamma carboxylation of Gla residues) to show that the absence of Gla residues result in decreased FIXa catalyzed FX activation [50, 51, 75, 138, 140, 141]. Moreover, competition experiments demonstrated that isolated peptides of the GLA-domains of FIX and FX prolonged the clotting time of normal plasma and prevented thrombus formation in vivo [51, 142].

The 12 glutamic acid residues located within the GLA-domain are post-translationally modified to negatively charged Gla residues [33, 48, 75, 143] by a vitamin K-dependent carboxylase system associated with the microsomes of hepatocytes [75]. These residues are located at positions 7, 8, 15, 17, 20, 21, 26, 27, 30, 33, 36 and 40 [33]. The Gla residues of FIX bind calcium [144, 145]. Moreover, binding of calcium produces a conformational change in the FIX molecule [48]. The Fab fragments of antibodies that are specific for the calcium-bound conformation of FIX, can inhibit PL binding in the presence of calcium [49], suggesting that calcium induced

conformational changes are essential for normal FIX activity. Furthermore, chemical modification of Gla residues blocks these conformational changes resulting in loss of procoagulant activity [140, 146]. FIX binds to PLV in the presence of $CaCl_2$, but not $MgCl_2$ indicating that binding is specifically dependent upon calcium [49]. It is, thus, hypothesized that calcium forms divalent-cation bridges between Gla residues of vitamin K-dependent proteins and acidic PLs, thereby anchoring these proteins to PL membranes [32, 48, 50, 51, 75, 102, 109, 139]. It is not known whether the GLA-domain of FIXa contains binding sites for FVIII or FX, however, mutations in the GLA-domain of FIX have been found to inhibit FVIII dependent activation of FX [147].

The functions of the epidermal growth factor domains have not been characterized, but it has been postulated that they are responsible for protein-protein interactions. The first epidermal growth factor-like region, contained in the light chain of FIXa has been postulated to bind FVIII [148]. However, it has also been suggested that FVIII binds to the heavy chain of FIXa [149]. Nonetheless, the FIX of some haemophilia B patients contain mutations in the first epidermal growth factor region, emphasizing the functional importance of this domain [147]. The serine protease domain contains the catalytic triad Ser365, Asp269, His221 [75] and possesses 71% sequence homology with trypsin [34].

The zymogen FIX must be converted to its enzymatic form to express proteolytic activity. Activation of FIX is mediated by FXIa and requires two steps (Figure 1.1). The first step involves cleavage between Arg145 and Ala146 which results in the two chain zymogen form (FIXα) [48, 136, 144] which has a molecular weight similar to the above [150]. The second cleavage, between Arg180 and Val181, results in formation of activated FIX (FIXaβ) by the release of an activation peptide, 35 amino acids in length, with molecular weight 11,000 [48, 136, 144]. FIXa has a M_r of 45,000 [42, 151] to 49,000 [152]. With a light chain of 18,000 (145 amino acids) [33] and a

heavy chain of 28,000 (236 amino acids) [33, 151]. The two chains of FIX(a) are held together by a disulphide bond [33] from Cys132 to Cys322 [33]. It has been reported that the regions of FIX necessary for interaction with both FVIII and PL are more accessible in FIXaβ, than in FIXα [139, 153]. Although the binding affinities of FIXα and FIXaβ for PLV have been shown to be similar, once bound, structural differences between the two proteins still exist [139]. Thrombin is able to cleave FIX between Arg327-Val328 and between Arg338-Ser339 [155], but only in the absence of calcium [154, 155]. These cleavages result in the loss of FIXa procoagulant activity [155]. One study found that FIXa interacts with antithrombin III in a 1:1 stoichiometry which resulted in inhibition of FIXa activity [136], however, another study observed antithrombin III inhibition only in the presence of heparin [156]. Phenylmethyl sulfonyl fluoride and diisopropylfluorophosphate (DFP) inhibit the activity of FIXa by blocking active serine side chains [156].

1.5.3 FX

FX is synthesized in the liver as a single chain peptide [106, 157] of 486 amino acids [32]. Similar to FIX, post-translational modifications occur, where FX is glycosylated, the signal peptide and propeptide are cleaved, the first 11 N-terminal glutamic acid residues undergo vitamin K-dependent carboxylation to form Gla residues (residues 6, 7, 14, 16, 19, 20, 25, 26, 29, 32, 39), and Asp63 is β-hydroxylated to form β-hydroxyaspartic acid [157]. Thus, the circulating form of FX contains 446 amino acid residues [32]. FX is generally isolated as two covalently coupled polypeptides. This form of FX (FXα), is believed to be produced by an extracelluar event that cleaves FX between Arg139 and Ser140 [32]. The light chain of FX is comprised of the GLA-domain and epidermal growth factor-like domains (residues 1 to 139) and the activation peptide and serine protease domain constitute the heavy chain

(residues 140 to 446) [32]. The two chains of FX are covalently coupled by a disulphide bond between Cys132 to Cys299 [158]. The light chain of FX has a M_r of 16,211 determined from its amino acid sequence [106], whereas experimentally, the light chain has been determined to have a M_r ranging from 16,900 [32] to 18,000 [153]. The heavy chain of human FX has been reported to have a M_r range of 38,000 [153] to 42,100 [32]. Analytical velocity ultracentrifugation has been used to study the hydrodynamic properties of bovine FX and indicate that FXα behaves like an elongated rod with an axial ratio of 7:9 [106]. Human FX, as a whole, has been reported to have a M_r ranging from 56,000 [153] to 59,000 [32, 106] to as high as 66,000 [159].

FX contains significant sequence homology with other vitamin K dependent coagulation proteins, such as FIX and protein C [106, 160]. Like these other vitamin K dependent coagulation proteins, FX can be divided into specific structural domains: one GLA-domain, two epidermal growth factor-like domains, a connecting region also called the activation peptide domain, and one serine protease which is commonly called the catalytic domain [32]. These domains possess 71%, 49%, 9%, and 48% sequence homology, respectively, with the corresponding domains of FIXa [143]. Similar to other vitamin K-dependent proteins, the PL binding site(s) of FX are contained within the GLA-domain. In addition, binding of calcium to the GLA-domain of FX [106, 161, 162] induces a conformational change in the molecule [141, 153, 163]. Calcium is also believed to form divalent cation bridges between Gla residues of FX and the acidic polar head groups of membrane PLs, such as PS [32, 50, 51, 102, 109]. It has not been determined if the GLA-domain of FX is involved in the association with FVIII or FIXa. The functions of the epidermal growth factor-like domains have also not been delineated. The activation peptide is cleaved away during activation. This fragment contains an RGD sequence which has been implicated in binding of proteins to integrins. However, the significance of this sequence in the activation peptide of FX has still to be

investigated. Similar to FIX, the serine protease domain of FX contains the catalytic triad Ser324, Asp227, His181 [32] and is involved in the proteolytic activation of the next enzyme in the coagulation pathway.

Activation of FX requires two cleavages. First, FX must be converted to the two chain zymogen form, FXα [32]. The activated, FXβ, form is created by another cleavage at Arg190 [32]. FXa, like thrombin and most other coagulant serine proteases [136, 156], is inactivated by antithrombin III [106]. However, α_1-antitrypsin or α_2-macroglobulin may also be significant inhibitors of FXa in vivo [106].

1.6.0 Techniques Used to Characterize the FX Activating Complex

To date, most studies examining the FX-activating complex have not directly examined associations between constituents of the FX activating complex. They have relied mostly on activation of one of the proteins [54, 117, 164, 165], or they have used techniques such as fluorescence [166], NMR [161] and relative light scatter [103, 167], to infer associations based on changes in spectroscopically determined parameters (Table 1.3). Many of the available techniques, such as calorimetry, ultracentrifugation and NMR, require relatively large quantities of proteins (Table 1.3). Commonly used techniques such as gel filtration, equilibrium dialysis and ultracentrifugation are limited by the similarity in size of the interacting molecules (Table 1.3). Studies utilizing recombinant and molecular biology techniques in which selected regions of the protein have been deleted or modified, provide important information concerning structurally and functionally essential domains. However, it is difficult to produce sufficient quantities of coagulation proteins in mammalian cells. Table 1.3 provides a survey of techniques that have been used to study the FX. Various advantages and disadvantages of these techniques are also summarized.

To overcome limitations encountered in previous studies, alternative

Table 1.3. A survey of techniques used to examine the FX activating complex

Technique/Description	Example	Advantages and Disadvantages versus Analytical Ligand Chromatography
ANALYTICAL LIGAND AFFINITY CHROMATOGRAPHY involves adsorption and subsequent elution of a particular protein or molecular complex from a ligand which has been bound to a solid support matrix	-present study -interaction of factors II, VIII and X with immobilized PLV[172]	A: -not limited by molecular size similarities -allows for direct measure of an interaction -not dependent on functional activity (such as enzyme kinetics or immunoassay) -uses μg (or less) quantities of both affinity column and mobile interactors D: -cannot differentiate between interactions involving the same molecular force(s) with similar affinities
DSC DATA (CALORIMETRY) measurement of thermal denaturation (change in enthalpy) of protein or change in lipid packing	-change in FIX and FX conformation upon binding of calcium[153]	A: can examine secondary structure D: requires larger quantities of protein to perform accurate assays e.g. Calorimetry=1-5 mg/ml[153], most machines require min. 0.5 ml
FLUORESCENCE SPECTROSCOPY changes in conformation determined by shifts in fluorescence intensities and wavelengths by native and attached fluorophores	-measurement of FVIII-HC and FVIII-LC reassociation and orientation after separation by chelation[166]	A: can estimate structural distances D: Required 1 to 15 μg of each chain or 239-294 μg of FVIII[166]
^{113}CD-NMR examination of the behaviour of nuclei of atoms (esp stable isotopes) placed in a magnetic field gives information on the structural properties of a molecule	-examination of the binding of ^{113}Cd to FX and prothrombin GLA-domains[151]	A: can examine secondary structure D: requires relatively large quantities and high high concentrations of protein
GEL-FILTRATION separation of molecules or complexes based on molecular weight	-separation of soluble versus PL[173] or platelet[174] bound coagulation factors	A: is relatively rapid and simple D: limited by similarities in size of interacting molecules
ULTRACENTRIFUGATION separation of molecules or complexes based on sedimentation co-efficients which are dependent on molecular weight and conformation	-binding of FVIII to PLV[55, 68] -structure of FV[175]	A: can determine approximate size (dimensions) of molecules D: -limited by similarities in size of interacting molecules -requires relatively large quantities of proteins e.g. 86 to 143 μg[55]
EQUILIBRIUM DIALYSIS examination of the size, complex formation of molecules and metal binding capabilities	-Ca^{2+} binding to FIXa lacking Gla residues[48, 145] -Ca^{2+} binding to GLA-domainless FX[160]	A: is relatively simple to perform D: limited by similarities in size of interacting molecules

EQUILIBRIUM BINDING determination of the affinity of binding between molecules, PL vesicles or cells	-binding of FVIII[176] or FIX[177] to platelets -binding of FIXa[107], or FX[107] to PLV	A: estimation of affinity of interactions D: can only infer interactions in a system such as platelets
RELATIVE LIGHT SCATTER examination of structure of molecules or formation of complexes by scattering of coherent light as it is reflected (scattered) by molecules.	-binding of FIX and FX to PLV[105, 167]	A: D: changes in size of cells or PLV due to changes in conditions could give artifactual results
ELISA examination of binding and affinity between molecules and immobilized ligand or PL	-binding of FVIII[119], FIX[178] or FX[178] to PL -binding of FVIII to vWf[71]	A: fast, uses relatively small quantities of protein D: may get steric inhibition if assay using antibodies
IRMA the presence of specific molecules is determined using radio-labelled antibodies	-influence of FVIII on binding of FIX and FX to platelets[179] -FVIII binding to PL[114]	A: fast, uses relatively small quantities of protein D: Fab fragment or whole antibody may block binding
RECOMBINANT/MOLECULAR BIOLOGY manipulation of the amino acid sequence of a protein to examine structure, function and interactions with other molecules or to determine an unknown amino acid sequence	-examination of activation sites in FVIII[122] -examination of the function of B-domainless FVIII[104] -determination of the amino acid sequence and structure of FVIII[72]	A: get precise structural information D: difficult to generate relatively large quantities of desired protein in mammalian cells
ACTIVATION ASSAYS determining the interaction of molecules based on the "activation" of a final substrate	-interaction of constituents of the FX activating complex[164] -influence of Ca^{2+} and PL on FIXa activation of FX[34] -FX activation by FIXa and Platelets[165] -regeneration of FVIII from its isolated subunits[117]	A: -confirms functional viability D: -can only infer interaction in systems such as platelets -can get artifactual results due to alternative activation of molecule being assayed

approaches were explored to examine associations between the constituents of the FX activating complex. As described in Chapters 2 and 3, I have used a novel adaption of affinity chromatography, termed analytical ligand affinity chromatography. Affinity chromatography techniques have been commonly used to isolate and purify many biologically important proteins, such as human blood coagulation FVIII [35], DNA-polymerase [168], as well as specific cell types such as adipose cells [169]. In addition, affinity chromatographic techniques have been used to study enzyme mechanisms, isolate specific receptors in cells, and study a vast array of protein interactions such as formation of macro-molecular enzymatic complexes [170].

The binding of proteins to other proteins or biological surfaces, such as a PL bilayer, is the result of combinations of simultaneous molecular forces, namely electrostatic forces (e.g. hydrogen bonding, van der Waals forces and charge-charge interactions) and hydrophobic forces. Analytical ligand affinity chromatography involves specific adsorption and subsequent elution of a particular protein or molecular complex from a ligand bound to a solid support matrix [170]. Elution is based on the selective dissociation of the above molecular interactions [170, 171]. By exploring the effect of agents known to disrupt specific molecular interactions, it is possible to identify the basic forces involved in the association between molecular complexes, such as proteins and PL membranes. In the present study, analytical ligand affinity chromatography was used to examine protein-lipid and protein-protein associations within the FX activating complex of the intrinsic pathway of blood coagulation. This technique uses a series of buffers containing salts, chelating agents and/or organic solvents to selectively disrupt interactions, thereby obtaining information on the molecular forces involved in maintaining the FX activating complex. None of the previously used techniques are able to deliver this type of information as quickly or as efficiently as analytical ligand affinity chromatography.

1.7.0 Rationale

Although FVIII [55, 88, 103], FIXa [54, 107, 180] and FX [54, 107, 172, 180] have been shown to bind directly to synthetic PLV, the PL binding regions on each protein and the types of molecular interactions involved in their association with PL have not been fully defined. Characterization of the molecular forces involved in binding of FVIII, FIXa and FX to PL is crucial to understanding how these factors associate with membranes. Formation of the FX activating complex likely involves interactions between the constituent proteins, however, the protein binding domains and the types of molecular interactions involved in their association have not been fully delineated. Moreover, the association of FVIII, FIXa and FX with platelets has not been fully characterized. Although it is clear that the FX activating complex is comprised of FVIII, FIXa, FX, calcium and PL, very little information exists on the structure, the mechanism, or the forces that bring together and maintain the integrity of this membrane-bound multi-protein complex. Investigation of these details will not only advance our understanding of FX activation, but will also provide an insight into the forces that form and maintain other membrane-bound macromolecular complexes, such as those involved in other coagulation complexes.

1.8.0 Objectives

The general aim of these studies was to investigate the structure of the FX activating comple . Specifically I wished:

1. To determine the functions of structural domains of human FVIII as they pertain to formation of the FX activating complex. The goal was to determine which chain(s) of human FVIII are involved in (a) binding of PL membranes, (b) binding of human FIXa, (c) binding of human FX.
2. To characterize the types of molecular interactions involved in the formation of

the FX activating complex. Specifically, to characterize the type of interaction(s) involved in (a) protein-lipid associations between human FVIII, human FIXa, or human FX and membrane PL and (b) protein-protein associations between FVIII and FIXa or FX.

3. To examine formation of the FX activating complex on platelets, beginning with the study of FVIII-, FIXa-, and FX-platelet interactions.

1.9.0 References

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CHAPTER 2

THE ASSOCIATION OF HUMAN COAGULATION FACTORS VIII, IXa AND X WITH PHOSPHOLIPID VESICLES

CHAPTER 2: The Association of Human Coagulation Factors VIII, IXa and X with Phospholipid Vesicles

2.1.0 Introduction

Formation of macromolecular enzymatic complexes of the coagulation pathway, such as the FX activating complex, are believed to involve PLs of cellular membranes, such as that of stimulated platelets. Therefore, characterization of the molecular forces involved in binding of FVIII, FIXa and FX to PL is crucial to understanding how these factors associate with biological membranes. As previously stated, non-covalent molecular interactions, such as those which occur between proteins and PLs, can be divided into two basic categories: (i) electrostatic, such as hydrogen bonding, van der Waals forces and charge-charge interactions and (ii) hydrophobic. Although FVIII [1-5], FIXa [6-9] and FX [6-11] have been shown to bind directly to synthetic PLV, few studies have investigated the relative roles that these forces play in the association of the aforementioned factors with PL. One study has examined the types of molecular forces involved in the association of FVIII with PLV, concluding that binding of FVIII to PL was primarily dependent on hydrophobic interactions [12]. Conversely, the binding of FIXa and FX to PL was postulated to reflect mainly electrostatic interactions which are the result of divalent cation bridge formation between PLs of the membrane and Gla residues of the protein [13]. Whether electrostatic interactions play a role in binding FVIII to membranes and whether hydrophobic interactions play a role in binding FIXa and FX to membranes is currently unresolved.

2.1.1 The association of FVIII with synthetic phospholipid membranes

The primary sequence(s) responsible for binding of FVIII to PL have not been elucidated. It is also possible that more than one domain of the protein participates in

PL binding. Determination of the molecular forces involved in this association will provide information as to the types of amino acids that constitute the binding domain(s) and may facilitate identification of the primary sequences.

Previous work has shown that binding of FVIII to PL is rapid and of high affinity [14] with a K_d of approximately 10^{-9} M, [3, 14]. However, FVIII did not bind with the same intensity to all classes of PLs [14]. Data, based on PL reduction of antigenic sites on FVIII, suggested the following order of avidity [5]:

$$PS \geq PA > > DPG > PE > > Lyso\text{-}PE > PC > Lyso\text{-}PS > Lyso\text{-}PC$$

Optimal binding of FVIII to vesicles composed of mixed PLs was observed when 15 to 20% of the lipid was PS [15]. In addition, the binding affinity of FVIII for PLV decreased proportionally with PS levels when the PS content of PS:PC vesicles fell below 30% [3]. Vesicles composed totally of non-acidic PLs, such as 100% PC, were found not to bind FVIII [1, 4]. These results suggested that the negative charge on PS is essential in forming FVIII-PL complexes [5]. Although acidic PLs are required for optimal binding, FVIII-PL interactions do not require the addition of calcium [1]. Moreover, high concentrations of calcium dissociate the protein from PL [5]. This may be due to competition for acidic PL membrane binding sites or due to lateral phase separation of acidic PLs by calcium. The exact mechanism is not known. Binding of FVIII to PL was found to stabilize [16-19] and make FVIII more resistant to protease degradation [5, 20-22]. However, calcium is not required in the protective action of PL on FVIII [5, 14]. The requirement for calcium in membrane binding suggests that electrostatic interactions may be involved in the association of FVIII with PL.

2.1.2 The association of FIXa or FX with synthetic phospholipid membranes

As discussed in Chapter 1 (section 1.5.2 and 1.5.3) FIXa and FX are members of the vitamin K-dependent group of proteins. These proteins are believed to bind to

PL membranes via divalent cation bridges formed between Gla residues of the protein and acidic PL head groups of the membrane [13, 23-27], such as PS. Similar to FVIII, binding of FIXa and FX to PL was reported to be rapid and of high affinity, with K_d values in the nanomolar range and the affinity of binding was found to be greatly affected by the PS content of the membranes [26]. In addition, FIXa was found to have a slightly higher affinity for PS:PC vesicles than did FX [7, 8]. Differences in PL binding between the zymogen and activated forms of FIX and FX were also observed [8] and may represent a potential mechanism to control interactions with other proteins within the coagulation pathway.

The types and proportion of PL classes required for optimal binding of FIXa and FX to synthetic membranes has been extensively studied. Binding requires the presence of calcium [28] and acidic PLs [10]. In addition, FIXa and FX seem to compete for a single class of binding site on PS:PC vesicles [6, 7]. Both FIXa and FX bind to the different acidic PL classes with avidity as follows [10]:

$$PS > > PA > PE = PG$$

FIXa and FX have the greatest avidity for PS [10]. Additional studies have shown that the number of FX binding sites on a membrane increases linearly [7] as the PS content of the membrane is increased from 0 to 20% [10, 23]. However, at PS levels greater than 20%, the amount of FX bound was no longer proportional, suggesting that the protein packing density on the membrane surface may limit FX-membrane binding [10, 23]. Light scattering and fluorescence experiments indicate that FIXa and FX bind to clusters of acidic PLs at the membrane surface [10, 23]. It is not clear whether the acid PL clusters must exist for binding or whether binding induces cluster formation.

Monolayer techniques demonstrated that relatively small pressure changes occurred upon binding of FX to PS containing monolayers [23]. This suggests that FX-PL complex formation is mediated primarily through the head groups of the acidic PLs

and that little or no polypeptide insertion into the membrane bilayer interior occurs [23]. However, these studies also indicated that the surface pressure changes of PS:PC monolayers induced by FX, were biphasic. This indicates that two distinct surface adsorption processes occurred [23] which, in turn, suggests FX binding occurs in two stages and that more than calcium bridge formation may be involved.

In general, coagulant activity is promoted by membranes containing acidic PLs (PS, PE, PA, PI, PG). It is not known whether there is a specific requirement for neutral PLs, sphingolipids or cholesterol. However, the fluidity of the membrane appears to alter the rate of coagulation. Membranes in the more rigid gel state take longer to promote coagulation than more fluid membranes [29]. Coagulation proteins may take longer to bind and to diffuse in more rigid membranes, thereby increasing the time required to assemble each multi-protein complex. The studies presented in this chapter, concentrated on defining the molecular forces involved in the binding of FVIII, FIXa and FX to PL membranes. From this series of experiments, it was also determined which chain(s) of FVIII contained binding domains for PL.

2.2.0 Materials and Methods

2.2.1 Proteins: Human FVIII (specific activity 5200 Units/mg) was purified from whole blood using a modification of the method of Ganz et al. [30], described elsewhere [31]. Human FX (specific activity 75-125 Units/mg) and human FIXa (specific activity 200 Units/mg) were purchased (Enzyme Research Laboratories, South Bend, IN) as was myoglobin (Sigma, St. Louis, MO). Radiolabelling of myoglobin was carried out using $Na^{125}I$ (Dupont Canada Inc., NEN Products, Mississauga, Ontario) and IodoBeads (Pierce Chemicals, Rockford, IL) as outlined earlier [30]. Free ^{125}I was separated from radiolabelled protein by gel filtration using Sephadex G-25 (Bio-Rad, Richmond, CA).

Proteins were quantified using the method of Bradford [32].

2.2.2 Phospholipid Vesicles: To facilitate analysis of PLV elution patterns, PLV were radioisotopically labelled. L-dipalmitoyl-2(palmitoyl-9,10-^{3}H(N))-PC (Dupont Canada Inc., NEN Products, Mississauga, Ontario) was added directly to the lipid mixture prior to drying. Alternatively, radiolabelled PLV were produced by encapsulation of ^{125}I-myoglobin. Briefly, PLV were prepared by drying 2 mg of PL (PS:PC, 40:60, w/w, Sigma, St. Louis, MO) at room temperature under a nitrogen stream. The dried PL was then put on ice and, when appropriate, approximately 1 X 10^{6} CPM of ^{125}I-myoglobin (specific activity 2-100 CPM/ng) was added. The volume was brought to 500 μl using an ice cold buffer comprised of 1% NaCl and 50 mM Tris-HCl, (pH 7.4). PLV were generated using a micro-probe sonicator (Fisher Sonic Dismembrator, Model 30) at 20% relative power output. The ^{3}H-PL or PL-myoglobin mixture was sonicated, on ice, three times for 5 minute intervals with rest intervals of 2.5 minutes between sonications. The above procedure was adapted from the PLV preparation method of Broden et al. [2]. This procedure yields ^{3}H-PC tagged PLV with a specific activity of 200,000 CPM/mg PL or ^{125}I-myoglobin-tagged PLV with a specific activity of approximately 100,000 CPM/mg PL.

To assess the possibility of myoglobin leakage from PLV, myoglobin-tagged PLV were prepared as described above and gel-filtered, eluting with Buffer A, comprised of 1% NaCl, 4 mM $CaCl_2$, and 50 mM Tris-HCl, (pH 7.4), through Sepharose CL-2B (Pharmacia, Uppsala, Sweden). Myoglobin encapsulated in PLV eluted at the void volume, while unbound myoglobin eluted much later. Fractions containing the encapsulated myoglobin were pooled, divided and gel-filtered through Sepharose CL-2B; each column employed one of the elution buffers, described in section 2.2.5, for the interaction studies. All Sepharose CL-2B columns were pre-

equilibrated with the corresponding buffer used for elution. Myoglobin leakage was assessed by comparing the amount of myoglobin remaining associated with PLV to the amount of "free" myoglobin. Once corrected for myoglobin leakage, similar results were obtained using either ^{3}H-labelled or myoglobin-labelled PLV.

2.2.3 Affinity Columns: All affinity columns (bed volume of approximately 2.4 mls) were prepared using purified proteins covalently coupled to Affi-Gel 10 (Bio-Rad, Richmond, CA) as the solid support matrix. This solid support matrix employs a 10 carbon spacer arm which is linked to the agarose backbone by extremely stable neutral ether bonds and to the ligand by an amide bond, thus leakage of the ligand from the column is negligible [33, 34]. Covalent binding of the proteins to Affi-Gel 10 was carried out at pH 7.4 at 4°C according to the manufacturer's directions. The binding efficiency, which varied from approximately 83% to 95%, was monitored by comparing the amount of protein remaining in solution with the amount of protein initially added. After binding, all columns were washed with Buffer A until the absorbance at 280 nm reached zero. FX-affinity columns were constructed using fifty Units (400 μg) of FX. Fifty Units (250 μg) of FIXa were used to construct FIXa-affinity columns. A 100-fold molar excess (115 μg) of diisopropylfluorophosphate (Sigma, St. Louis, MO), a serine protease inhibitor, was added to FIXa prior to addition of Affi-Gel 10 to prevent autoproteolysis of FIXa. FVIII-immunoaffinity columns were constructed using a murine monoclonal antibody (5 mg, ESH-8, American Diagnostica Inc., Greenwich, CT) directed towards human FVIII-LC. This anti-FVIII-LC antibody was covalently bound to Affi-Gel 10. Purified FVIII (70-200 Units) was then circulated through the anti-FVIII column for two hours, to immobilize it by interaction with the FVIII-specific antibody. All affinity columns were "blocked" with 0.1 M ethanolamine, or by equilibrating with the primary amine Tris [35], contained in Buffer A.

2.2.4 Effects of the anti-FVIII antibody on the procoagulant and lipid binding properties of FVIII: The effect of the anti-FVIII antibody on FVIII procoagulant activity was examined using a one-stage clotting assay, a modification of the partial thromboplastin time [36]. Briefly, the procoagulant activity of FVIII, contained in a lyophilized plasma reference standard (Pacific Haemostasis, Bakersfield, CA), was assayed in the presence and absence of the anti-FVIII antibody, using a Coag-a-mate XC (General Diagnostics, Morris Plains, NJ). The anti-FVIII antibody was added in a 1:1 molar ratio with FVIII.

To assess whether this antibody inhibits binding of FVIII to PL, microtitre wells (Immulon 1 Removawell disposable microELISA plates, Fisher Scientific, Ottawa, Ontario) were coated with PS (10 μg/well). PS was taken to dryness in a flask under a stream of N_2. Ice cold methanol was added and the mixture was sonicated for 2 minutes, on ice, as described above. Once aliquoted into the microtitre wells, the plates were placed in a sealed incubator containing desiccant (Drierite, Fisher Scientific, Ottawa, Ontario) and the methanol was evaporated overnight under N_2. The microtitre wells were then blocked for 1 hour at 37°C using 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.5% gelatin. Approximately 7.25 ng of radio-iodinated FVIII (specific activity 15,690 CPM/ng) was added to each well in the presence or absence of a 10 fold molar excess (35 ng/well) of the anti-FVIII antibody and incubated for 2 hours at 37°C. The plates were then washed three times with Buffer A and the amount of ^{125}I-FVIII bound to each well was determined using a Beckman 5000 gamma counter (Beckman Instruments (Canada) Inc., Toronto, Ontario).

2.2.5 Interaction Studies: Freshly prepared excess labelled PLV were circulated through FVIII- and FX-affinity columns (3 cm X 1 cm, height X diameter) in the presence of 2-3 ml of Buffer A, at a flow rate of approximately 10 to 30 ml per

hour/ml gel for 1 hour at room temperature to allow binding. Experiments utilizing FIXa-affinity columns were treated in a similar manner, but PL binding was performed at 4°C to prevent degradation of the FIXa enzyme. Unbound PLV were washed from the columns by eluting with Buffer A. The remaining ^{125}I-myoglobin tagged PLV were eluted by subjecting the immobilized protein-PLV complexes to a series of washes by buffers comprised of the following salts, chelating agents and solvents: i) 1 M NaCl, 50 mM Tris-HCl (pH 7.4); ii) 25 μM EDTA, 1 M NaCl, 50 mM Tris-HCl (pH 7.4); iii) 10 mM EGTA, 1 M NaCl, 50 mM Tris-HCl (pH 7.4); and iv) 50% ethylene glycol, 1 M NaCl, 0.1 M lysine, 50 mM Tris-HCl (pH 7.4). The optimal concentrations of NaCl, EDTA and EGTA were deduced from preliminary experiments. The quantity of PLV tagged with radioactivity in each eluted fraction was monitored using a Beckman 5000 gamma counter (Beckman Instruments (Canada) Inc., Toronto, Ontario).

Binding studies were each performed a minimum of three times. To control for non-specific binding of ^{125}I-myoglobin and PLV to the FVIII-affinity column, columns were made of blocked Affi-Gel 10 and/or the FVIII-specific monoclonal antibody without FVIII. Radio-labelled myoglobin in the presence or absence of PLV was circulated through these control columns as described above. Elution profiles obtained from columns comprised only of blocked Affi-Gel contained no significant peaks and were taken as baseline values. Radio-labelled myoglobin eluted from columns constructed using only the FVIII-specific monoclonal antibody was quantified and these values were subtracted from the experimental data. To control for non-specific binding of ^{125}I-myoglobin and PLV to FIXa- and FX-affinity columns, experiments were performed using the above experimental columns with ^{125}I-myoglobin in the absence of added PL and/or using affinity columns comprised of blocked Affi-Gel. Elution profiles obtained from control columns, other than the FIXa- and FX-affinity columns, contained no peaks and the values were taken as baseline. The ^{125}I-myoglobin eluted from FIXa-

and FX-affinity columns was quantified and subtracted from experimental data. Although the proportions remained constant, the absolute quantity of PL eluted from each affinity column varied due to a decrease in the PL binding capacity with use. For example, the absolute quantity of PL eluted from FX-affinity columns was 5380 ± 3090 ng PL/nmol bound FX (Mean ± SD, n=5), whereas the percentage of total eluted PL, dissociated using the buffer containing NaCl, was 25 ± 5% (Mean ± SD, n=5). Therefore, results from representative binding experiments are presented and reported as absolute quantities (Mean ± SD) and as percent of total eluted PL (Mean ± SD).

2.3.0 Results

The relative contribution of electrostatic and hydrophobic forces in the binding of FVIII, FIXa or FX to PLV was investigated using ligand affinity chromatographic analyses and a series of specially formulated elution buffers. Each buffer was designed to disrupt a distinct type of molecular interaction. Specifically, a buffer containing 1 M NaCl was used to disrupt simple ionic interactions, buffers containing 25 μM EDTA or 10 mM EGTA were used to disrupt divalent cation-dependent interactions and, lastly, a buffer comprised of 50% ethylene glycol was used to disrupt hydrophobic interactions. The experiments were carried out in two stages: disruption of electrostatic forces, followed by disruption of hydrophobic forces.

2.3.1 Effects of the anti-FVIII antibody on the procoagulant and lipid binding properties of FVIII: To ensure that the anti-FVIII antibody (ESH-8) does not affect interactions between FVIII and the PLV, the effect of ESH-8 on the procoagulant activity and PL binding capacity of FVIII was investigated. A one-stage clotting assay was used to determine whether the FVIII-specific antibody inhibited FVIII-dependent

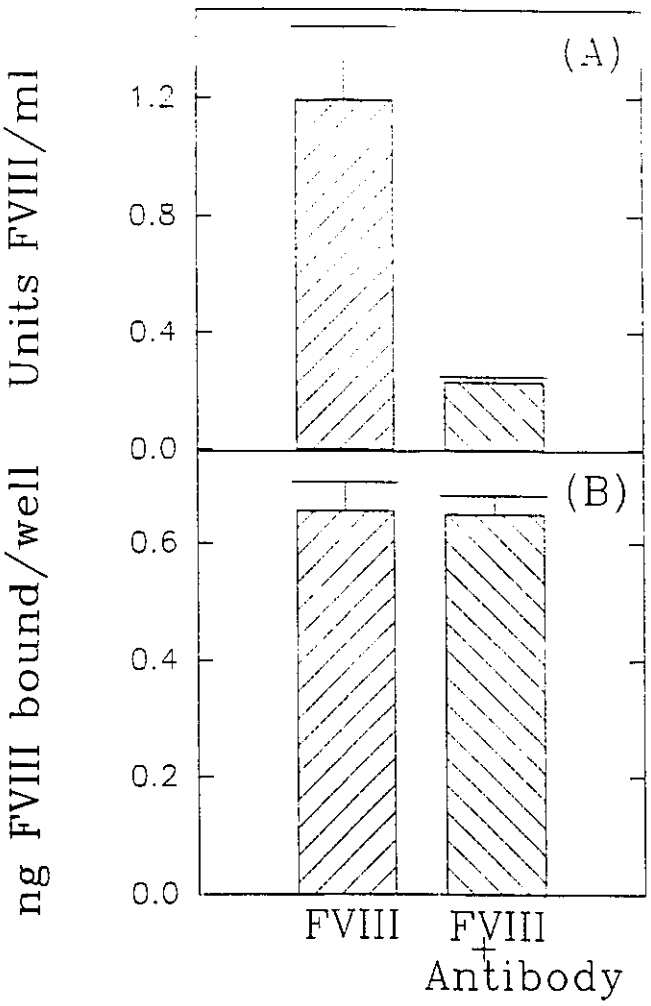

Figure 2.1. Characterization of the anti-FVIII antibody. (a) The procoagulant activity of FVIII was assessed in the absence (n=4) and presence (1:1 molar ratio, n=8) of the anti-FVIII antibody using a one-stage clotting assay. (b) The ability of FVIII to bind to PL in the absence (n=6) and in the presence of a 10-fold molar excess of the anti-FVIII antibody (n=5) was examined using PS coated microtitre wells (10 μg/well).

activation of FX, and thus clot formation. An 80% inhibition in FVIII procoagulant activity was observed (Figure 2.1). Thus, this antibody interferes in FVIII-dependent activation of FX, possibly by binding to FVIII thereby physically blocking formation of the multi-protein enzyme complex on the surface of the PL membrane. Data derived from the PL binding assays are also presented in Figure 2.1. A 10-fold molar excess of the anti-FVIII antibody did not inhibit binding of FVIII to the PS coated-microtitre wells. Therefore, the anti-FVIII antibody used in this series of experiments does not appear to inhibit FVIII procoagulant activity by blocking the interaction of FVIII with PL.

2.3.2 Characterization of the molecular interactions involved in the association of FVIII with phospholipid vesicles: The types of molecular interactions involved in the association of FVIII with PL were investigated. It was observed that washing FVIII-affinity columns with the buffer containing 1 M NaCl did not elute significant amounts of bound PLV (Figure 2.2a, Table 2.1). This suggests that simple ionic electrostatic interactions do not play a major role in binding FVIII to PLV. Association of the two chains of FVIII is divalent cation-dependent [21, 37, 38]. Thus, exposure of FVIII to the buffer containing 10 mM EGTA (a chelating agent) will cause dissociation of the two chains of FVIII [38]. Only FVIII-HC is eluted at this point, as FVIII-LC is immobilized on the affinity column by the FVIII-LC specific antibody. A small amount (15.2%) of PLV are also eluted from the FVIII-affinity column at this step (Figure 2.2a, Table 2.1). This result suggests that FVIII could possess a calcium dependent binding site for PLV or possibly that FVIII-HC possesses a hydrophobic binding site for PLV. These alternatives cannot be resolved from the existing data.

Contributions from electrostatic forces in the association of FVIII with PLV were eliminated by washing the FVIII-affinity column with buffers containing 1 M NaCl and/or 10 mM EGTA. Therefore, PLV remaining bound to the FVIII-affinity column must associate with FVIII by hydrophobic interactions. The ethylene glycol containing buffer disrupts hydrophobic interactions. As shown in Table 2.1, this buffer eluted the largest proportion of PLV (84.8%) from the FVIII-affinity column. These results indicate that hydrophobic interactions constitute the predominant molecular force involved in the association of FVIII with PLV. Elution of 84.8% of bound PLV from the FVIII-affinity column subsequent to removal of FVIII-HC by washing with the EGTA-containing buffer further localizes the major PLV binding domain to FVIII-LC. Reversal of the order of application of the buffers causes complete elution of PLV by ethylene glycol due to disruption of the FVIII-LC-antibody interaction.

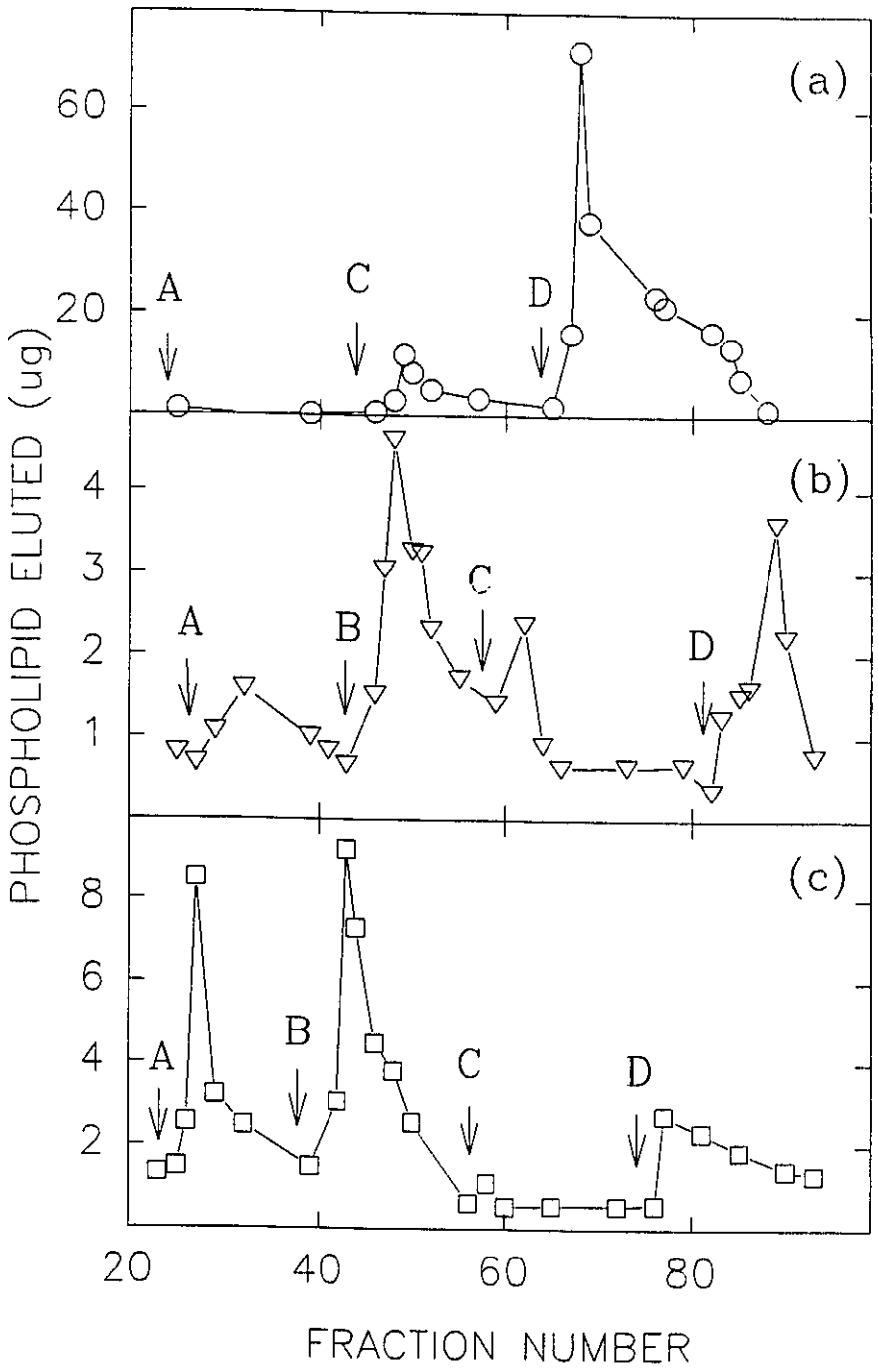

Figure 2.2. Elution profile of phospholipid vesicles from FVIII-, FIXa- and FX-affinity columns. PLV, labelled with ^{125}I-myoglobin, were applied to (a) FVIII-, (b) FIXa- and (c) FX-affinity columns. Elution buffers as indicated in the Figure were sequentially applied to each column: (A) 1 M NaCl, 50 mM Tris-HCl (pH 7.4); (B) 25 μM EDTA, 1 M NaCl, 50 mM Tris-HCl (pH 7.4); (C) 10 mM EGTA, 1 M NaCl, 50 mM Tris-HCl (pH 7.4); and (D) 50% ethylene glycol, 1 M NaCl, 0.1 M lysine, 50 mM Tris-HCl (pH 7.4). Fractions of 1 mL were collected and eluted radioactivity was measured.

Table 2.1. Dissociation of Phospholipid Vesicles from FVIII

Order of Application	Elution Buffer[a]	μg Phospholipid eluted/ nmol bound FVIII	% of Total eluted Phospholipid
1	1 M NaCl	0	0
2	10 mM EGTA	35	15.2
3	50% Ethylene Glycol	200	84.8

a. Buffers contained the listed components plus 50 mM Tris-HCl (pH 7.4) and 1 M NaCl.

2.3.3 Characterization of the molecular interactions involved in the association of FIXa with phospholipid vesicles: Experiments to characterize the molecular forces involved in the interaction between FIXa and PLV were carried out in a manner similar to the above experiments, except that elution with a buffer containing 25 μM EDTA was added to the procedure. In these experiments, the buffer containing 1 M NaCl eluted 3.6% of the total bound PLV from a representative FIXa-affinity column (Table 2.2). This suggests that simple ionic interactions may participate in the association of PLV with FIXa, but are not the major force involved in binding. Divalent cations, such as calcium, have been implicated to play a central role in the association of FIXa with PLV by anchoring FIXa to PL membranes through formation of divalent cation bridges between PS of the membrane and Gla residues of FIXa [13]. However, if FIXa associates with PLV purely by electrostatic interactions, then disruption of the divalent cation salt bridges by chelation of divalent cations should cause elution of the remaining PLV bound to the FIXa-affinity column. The present results demonstrate that this is not the case. Of the total bound PLV, 60.6% were eluted by a buffer containing 25 μM EDTA (Table 2.2). Subsequent elution with a buffer containing 10 mM EGTA, a

stronger chelating agent than 25 μM EDTA, dissociated an additional 7.1% of the bound PLV (Table 2.2). In total, elution with buffers containing chelating agents dissociated 67.7% of the bound PLV, not 95% as would be predicted. The remaining PLV (28.7% of the total bound) were eluted by washing with the buffer containing ethylene glycol (Table 2.2). Thus, results of these experiments demonstrate that forces other than simple ionic or salt bridge formation, i.e. hydrophobic forces, play a role in the interaction of FIXa with PLV. Reversal of the order of application of the elution buffers; ethylene glycol followed by the EGTA containing buffer, eluted all PLV from the FIXa affinity columns.

Table 2.2. Dissociation of Phospholipid Vesicles from FIXa

Order of Application	Elution Buffer[a]	ng Phospholipid eluted/ nmol bound FIXa	% of Total eluted Phospholipid
1	1 M NaCl	88	3.6
2	25 μM EDTA	1485	60.6
3	10 mM EGTA	173	7.1
4	50% Ethylene Glycol	704	28.7

a. Buffers contained the listed components plus 50 mM Tris-HCl (pH 7.4) and 1 M NaCl

2.3.4 Characterization of the molecular interactions involved in the association of FX with phospholipid vesicles: The molecular forces involved in the association of FX with PL were similarly investigated. The buffer containing 1 M NaCl eluted 23.1% of the total bound PLV from a FX-affinity column (Table 2.3), this is a much larger proportion than that eluted by the same buffer from FIXa-affinity columns (Table 2.2).

Similar to FIXa, simple ionic interactions are not the major force involved in binding of FX to PLV.

A subsequent wash using the buffer containing 25 μM EDTA eluted 53.5% of the total bound PLV from FX (Table 2.3). Washing the FX-affinity column with the EGTA containing buffer eluted an additional 2.3%, resulting in a total of 55.5% of PLV being eluted by chelating agents. Thus, a total of 78.8% of the PLV bound to the FX-affinity column were eluted by the disruption of electrostatic forces compared to a total of 71.3% from the FIXa-affinity column. The data of Table 2.3 demonstrate that 21.2% of the PLV were dissociated from FX by washing with the buffer containing ethylene glycol. This value is similar to the 28.7% of PLV eluted by this buffer from the FIXa-affinity column. Similar to FIXa, the data demonstrate that hydrophobic forces, in addition to simple ionic and salt bridge formation, are involved in the association of FX with PLV. Reversal of the order of application of the elution buffers; ethylene glycol followed by the EGTA containing buffer, eluted all PLV from the FX affinity columns.

Table 2.3. Dissociation of Phospholipid Vesicles from FX

Order of Application	Elution Buffer[a]	ng Phospholipid eluted/ nmol bound FX	% of Total eluted Phospholipid
1	1 M NaCl	1154	23.1
2	25 μM EDTA	2671	53.5
3	10 mM EGTA	116	2.3
4	50% Ethylene Glycol	1060	21.2

a. Buffers contained the listed components plus 50 mM Tris-HCl (pH 7.4) and 1 M NaCl.

2.4.0 Discussion

The studies presented, herein, examined the molecular forces involved in the association of FVIII, FIXa and FX with synthetic PL membranes. As previously mentioned, the association of FVIII with PLV is dependent upon PS [1, 3, 4, 14]. The requirement for PS in membrane binding suggests that electrostatic interactions (such as simple ionic or divalent cation-dependent) may play a role in binding PLV to FVIII. However, recent studies have demonstrated that divalent cations are not required for binding of FVIII to PL membranes [1, 12]. FVIII possesses no sequence homology with vitamin K-dependent coagulation proteins [39, 40], such as FIXa and FX, and does not contain Gla residues. Thus, although binding of FVIII to PL membranes is PS-dependent, it is unlikely that FVIII associates with PL membranes in a manner similar to FIXa or FX. The above suggests that divalent electrostatic interactions, such as salt bridge formation, may not be involved in the association of FVIII with PLV. It was, therefore, investigated whether electrostatic forces are involved in the association of FVIII with PL membranes.

In the present study, specific elution of PLV from FVIII by the buffer containing 1 M NaCl was not observed, suggesting that simple ionic interactions are not likely involved in the association of FVIII with PL. Elution of a small proportion of PLV (15.2%) was observed by washing with the EGTA containing buffer. The two chains of FVIII are held together in a divalent cation dependent manner [21, 22, 37, 38]. It is believed that, in vivo, this association is dependent upon calcium [21, 37]. Concentrations of EDTA ranging from 100 μM [22] to 60 mM EDTA [38] and 200 μM EGTA [22] have been demonstrated to dissociate the two chains of FVIII. In the present study, the light chain of FVIII is immobilized on the FVIII-affinity column by a light chain specific antibody. Thus, application of the buffer containing 10 mM EGTA will elute only the heavy chain of FVIII. Association of the light chain of FVIII with

the antibody is not affected by this concentration of EGTA. Elution of PL from FVIII by the buffer containing EGTA indicates either that FVIII (light chain or heavy chain) possesses a calcium-dependent binding site for PLV or that FVIII-HC possesses a hydrophobic binding site for PLV. Previous reports demonstrating that binding of FVIII to PL membranes is not calcium-dependent [12] suggest that the latter option is more likely.

The buffer containing ethylene glycol was applied last as elution of FVIII from antibody columns is generally accomplished using hydrophobic reagents; 50% ethylene glycol being the most common [30, 41, 42]. Ethylene glycol does not alter the activity of FVIII [30]. In addition, as electrostatic interactions were disrupted by the prior washes, PLV eluted by ethylene glycol must be associated with FVIII by hydrophobic forces. In the current study, the largest proportion of bound PLV (84.8%) was eluted from the FVIII-affinity column by this buffer, demonstrating that hydrophobic interactions are the predominant molecular force involved in the association of FVIII with PLV. Prior treatment of the FVIII-affinity column with the buffer containing EGTA caused elution of FVIII-HC, therefore subsequent elution of 84.8% of bound PLV by the ethylene glycol containing buffer further localizes the major PLV binding domain to FVIII-LC. This finding is supported by a report in the literature which suggested that the PL binding domain may be located on FVIII-LC [43]. Thus, the present results not only indicate that a PL binding domain is located on FVIII-LC, but also demonstrate that hydrophobic interactions play a major role in the association of FVIII and PLV.

It is currently accepted that binding of vitamin K-dependent coagulation factors to PL membranes is mediated through calcium bridges formed between the GLA-domain of these proteins and the acidic polar head groups of membrane PS [13]. Many groups have studied calcium-dependent binding of vitamin K clotting factors, such as FIX [6-

8], FIXa [7] and FX [6-8, 10] to PL surfaces. With the exception of Burri et al. [8], who examined the effect of NaCl on binding of FX to PLV, these studies did not explore, nor eliminate the possibility that other forms of molecular interactions participate in the association of FX with PL. Using relative light scattering to quantify the amount of FX dissociated from PLV, Burri et al. reported that only 2% of PL-bound FX remained attached to the PLV after treatment with 1 M NaCl [8]. However, they also report that the mass of the PLV decreased with increasing concentrations of NaCl [8] which could explain such a large apparent dissociation of FX from the PLV.

The types of molecular interactions involved in the association of FIXa and FX with PL were systematically examined. Data obtained in the present study indicate that a buffer containing 1 M NaCl eluted only 3.6% (Table 2.2) and 23.1% (Table 2.3) of the total bound PL adsorbed to the FIXa- and FX-affinity columns, respectively. This suggests that molecular interactions which are not disrupted under high salt conditions, such as divalent cation-dependent interactions, play a more significant role in binding of these proteins to PL membranes.

Previous publications have reported dissociation of FIXa and FX from PL using concentrations of EDTA starting from 2 mM [8], currently, dissociation was observed using EDTA concentrations as low as 6.25 μM to 25 μM (Tables 2.1 and 2.2). Thus, if the interaction between FIXa or FX and PL was totally divalent cation dependent, application of the 25 μM EDTA or the 10 mM EGTA containing buffer would be expected to completely dissociate FIXa/FX from PL. The buffer containing 10 mM EGTA was applied to the FIXa- and FX-affinity columns to demonstrate almost complete disruption of divalent cation-dependent interactions by 25 μM EDTA. Results presented in Tables 2.1 and 2.2 indicate that a sum of 67.7% and 55.5% of the total adsorbed PLV were eluted from FIXa- and FX-affinity columns, respectively, by the

buffers containing chelating agents. Elution of the largest proportion of PLV from FIXa- and FX-affinity columns by these buffers indicates that the predominant form of interaction between FIXa or FX and PLV is divalent cation-dependent.

The buffers used in these studies contain high concentrations of salt (1 M NaCl) and chelating agents (25 μM EDTA and 10 mM EGTA) and would be expected to disrupt all electrostatic interactions occurring between coagulation proteins and PLV. However, it was observed that after treatment with the above buffers, there remained bound to FIXa- and FX-affinity columns a proportion of PLV which could only be eluted by washing with 50% ethylene glycol. This indicates that hydrophobic interactions are involved in the association of PLV with FIXa or FX and that there may be hydrophobic regions exposed on molecules of FIXa and FX. This view is supported by reports that suggest that the aromatic cluster positioned at the C-terminal end of the GLA-domain is essential for PL binding [13]. It is speculated that hydrophobic sites on FIXa and FX may be exposed due to conformational changes induced by binding to PL. Although the purpose of such regions are unknown, based on information provided by Harlos et al. [44], they could be involved in binding of FIXa and FX to other proteins such as specific membrane receptors or, alternatively, to other coagulation proteins such as FVIII. The observed hydrophobic binding is not due to non-specific binding of the radio-labelled PLV to the neutral spacer arms of the Affi-Gel 10 solid support matrix as this type of binding was controlled for by using columns comprised of ethanolamine- or Tris-blocked gel.

FIXa and FX possess significant sequence homology [7, 40], especially in the GLA-domain region which contains the Gla residues reported to be critical for binding of these proteins to PL membranes. Previous studies have reported that FIXa and FX bind to PLV with similar K_d values [7] of 10^{-7} M [8] and that binding of both these factors to PL is divalent cation-dependent [13, 23, 45, 46]. The above suggests that

FIXa and FX not only bind PLV in a similar manner, but may possess identical PL binding sites. However, FIXa contains twelve Gla residues [47] while FX contains only eleven [48]. In addition, high calcium concentrations (100 mM) do not equally dissociate these factors from PLV [13]. Bloom has reported that both FIXa and FX bind to PS-lined microtitre wells, but with different affinities (8.4×10^8 versus 1.56×10^{10} M^{-1}, respectively) [9] and that FX binds to PC-lined microtitre wells while FIXa does not [9]. These data suggest that PL binding site(s) on FIXa and FX may not be identical. Under conditions of the present study, elution profiles of PLV from FIXa- and FX-affinity columns are not identical (Figures 2.2b and 2.2c, Tables 2.2 and 2.3).

Coagulation proteins, such as FVIII, FIXa and FX, contain many potential sites for interaction with other molecules [35, 49]. In addition, binding between biological molecules usually involves simultaneous ionic and hydrophobic interactions, labelled the "compound affinity" effect [34]. Generally, these two weak forces mutually reinforce each other so that the resultant strength of interaction is much greater than the sum of the two individually and the use of elevated concentrations of salt and organic solvents is frequently required for complete elution [34]. Heterogeneous elution from affinity columns under various conditions has been observed for a number of biological molecules [8, 12, 50] and is likely due to the "compound affinity" effect. In the current study, analytical ligand affinity chromatography was used to examine the types of molecular interactions which are involved in the association of FVIII, FIXa or FX to PLV. Binding of each of these coagulation proteins to PLV appears to involve more than one type of molecular interaction.

Results of these analyses indicate that the association of FVIII and PLV is mediated through the light chain of FVIII and further demonstrate that this interaction is predominantly due to hydrophobic interactions. These data establish that FIXa and FX associate with PLV predominantly in a divalent cation-dependent manner and

demonstrate that simple ionic electrostatic interactions are also involved. In addition, contrary to the prevailing hypothesis, it was demonstrated that hydrophobic forces play a role in the association of FIXa or FX with PLV. Furthermore, despite their sequence homology, FIXa and FX may not associate with PLV in an identical fashion and may possess distinct and different PL binding sites in addition to the GLA-domain.

After completion of these studies, two reports from competing laboratories were published that suggested the M_r=80,000 light chain of FVIII binds to PS [4] and to PS:PC (1:1, w/w) vesicles [43]. However, it was found that FVIII-LC bound less tightly than the intact FVIII molecule, suggesting the possibility of a conformational change or denaturation of the polypeptide during their purification procedure, [4]. In addition, it was found that the binding of FVIII to PS, resulting in inhibition of coagulant activity, can be blocked by antibodies that interact with the light chain (the C2 domain) of FVIII [5, 14, 51]. Further studies, based on inhibition of binding by antibodies, suggest that residues 2303 to 2332, contained within the C2 domain, may play a significant role in binding PL and/or constitute a PL binding domain [52, 53].

2.5.0 References

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CHAPTER 3

ASSOCIATION OF HUMAN BLOOD COAGULATION FACTORS IXa AND X WITH FACTOR VIII

CHAPTER 3: Association of Human Blood Coagulation Factors IXa and X with Factor VIII

3.1.0 Introduction

It is hypothesized that PL-bound FVIII exerts its cofactor function by physically interacting with PL-bound FIXa and FX [1, 2]. In addition, it has been proposed that FVIIIa serves as a receptor for FIXa, analogous to FVa acting as a receptor for FXa [3]. FVIII may also function as a high affinity binding site for FX on the surface of PL membranes, thus facilitating FX activation. In addition, binding of FVIII may structurally alter FX or FIXa to increase the catalytic efficiency of FIXa. Few studies have directly investigated and characterized the association of FVIII with FIXa or FX. Reports in the literature suggest that FVIII binds to both the light chain of FIXa, which contains the GLA and epidermal growth factor domains [4] and the heavy chain of FIXa, which contains the catalytic subunit [5]. However, it has not been resolved whether one or both chains of FVIII are involved in the association with FIXa. Likewise, it is not clear which domains of FVIII are essential for its cofactor function, nor have the molecular forces involved in these associations been characterized. In the previous chapter, analytical ligand affinity chromatography was used as a tool to examine binding of FVIII, FIXa and FX to PL membranes. In the present chapter, this technique was used to delineate the molecular forces involved in the association of FVIII with FIX and FX. In addition, analytical ligand affinity chromatography and radioligand binding studies were employed to determine which chain(s) of FVIII contain binding domains for FIXa and FX. Data obtained from radioligand binding studies were also used to determine binding parameters that characterize the association of FVIII with FX in the presence of PL.

3.2.0 Materials and Methods

3.2.1 Coagulation Factors: Unless stated otherwise, all proteins were prepared as listed in section 2.2.1-2.2.4. Additional human FVIII was supplied as commercial concentrate or as albumin-free concentrate (Monoclate, Armour Pharmaceuticals, Blue Bell, PA). Radiolabelling of FVIII (specific activity 15,000-40,000 CPM/ug), FIXa (specific activity 80-700 CPM/mg) and FX (specific activity 70-350 CPM/mg) and protein quantification was carried out as described in section 2.2.1.

3.2.2 Phospholipid Vesicles: The method for preparing PLVs was described previously (section 2.2.2). PLVs used in the current experiments were not radiolabelled and were, thus, prepared in the absence of L-dipalmitoyl-2(palmitoyl-9,10-^{3}H(N))-PC and ^{125}I-myoglobin.

3.2.3 Affinity Columns: Affinity columns were prepared as described under section 2.2.3. Immunoaffinity columns comprised of FVIII-LC were prepared by washing FVIII-affinity columns with a 10 mM EGTA-containing buffer, described below, which dissociates FVIII-HC from FVIII-LC, leaving FVIII-LC immobilized on the column [6, 7].

3.2.4 Ligand Affinity Chromatographic Analyses: Radiolabelled protein, as indicated, was incubated with freshly prepared PLVs (2- to 200-fold excess) for 20 minutes in Buffer A. The radiolabelled protein-PL mixture was then circulated through the specified affinity column at a flow rate of 10 to 30 ml/hour/ml gel for 1 hour. Non-adsorbed and non-specifically bound protein and PLVs were washed from the columns with Buffer A followed by equilibration with 1 M NaCl in 50 mM Tris-HCl (pH 7.4). The columns were then sequentially washed with the previously listed series

of buffers and the radiolabelled protein eluted was quantified as detailed in section 2.2.5. FIXa studies were all performed at 4°C using solutions that had been pre-chilled to the same temperature.

The procedure used to examine elution of radiolabelled PLVs from FX affinity columns has been set out under section 2.2.5. To control for non-specific binding of radiolabelled proteins to the affinity columns, parallel experiments were also performed using columns comprised of ethanolamine-blocked Affi-Gel 10 and/or the FVIII-LC-specific monoclonal antibody (ESH-8) without added FVIII. The quantity of radiolabelled protein eluted from these columns was not significant and was defined as background. As a further control, studies using the affinity columns were repeated in the absence of added PL. Radiolabelled protein that eluted from these control columns was quantitated and, in FVIII-FX interaction studies, these values were subtracted from the experimental data. Although the absolute quantities of eluted protein varied in each experiment, for example the total quantity of FX eluted from FVIII-affinity columns was 493 ± 660 ng (n=3), the proportion of protein eluted by each buffer remained constant. Thus, the data from these experiments are presented as the proportion of total protein eluted by each treatment (mean ± standard deviation).

3.2.5 ELISA for Detecting FVIII-HC or FVIII-LC: The ELISA procedure for measurement of FVIII-HC or FVIII-LC was adapted from the procedure of Ganz et al. [8]. As outlined in section 2.2.4, microtitre plates were first coated with lipid. However, in the present studies, 10 μg of PS or a PS:PC mixture (40:60, w/w) was used. The plates were blocked overnight at 4°C or 1 hour at 37°C using 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.5% gelatin. All washes and subsequent incubations were performed on a TekTator V shaker (Tekpro, Evanston, IL) at room temperature. In addition, washes and dilutions were performed using Buffer A. FVIII (albumin-free

Monoclate) was applied to each well and the plates were agitated for 90 minutes. The FVIII solution was removed and test wells were then incubated with either the 10 mM EGTA-containing buffer, the 25 μM EDTA-containing buffer or Buffer A for 30 minutes followed by re-equilibration with Buffer A. The presence of FVIII-HC was determined by incubating 90 minutes with a monoclonal chicken-anti-FVIII-HC (IgY), a generous gift of Dr. Duncan Pepper (National Science Laboratory, Scottish National Blood Transfusion Service, Edinburgh, Scotland) followed by washing and a 90 minute incubation with a horseradish peroxidase conjugated rabbit-anti-chicken IgG (Sigma, St. Louis, MO). Ten minutes after addition of orthophenyldiamine (OPD, Sigma) substrate at 0.4 mg/ml in citrate buffer (0.1 M, pH 4.0) containing 0.03% (v/v) hydrogen peroxide, the colour intensity was measured at 492 nm [9]. Similarly, the presence of FVIII-LC was determined using the ESH-8 antibody followed by washing and addition of an alkaline-phosphatase-conjugated anti-murine IgG (Bio-Rad, Richmond, CA). Substrate solution containing paranitrophenylphosphate was prepared and added according to manufacturer's instructions (Protoblot System, Promega Biotech, Madison, WI). Ten minutes later, the colour intensity was assayed at 405 nm [8, 10]. Values obtained from control wells, which were incubated and processed in parallel, but did not contain FVIII, were subtracted from the test results.

3.2.6 Radioligand Binding Studies: Microtitre wells were coated with lipid (PS, 10 μg/well) and blocked, as described above. Background studies established that microtitre wells coated with either PS or the PS:PC mixture yielded similar results. Wells for FX-FVIII and FX-FVIII-LC interaction studies were incubated for 90 minutes with unlabelled FVIII (Monoclate, 1.0 Unit/well). FVIII-LC-containing wells were prepared by removal of FVIII-HC by treatment with the 10 mM EGTA-containing buffer for 30 minutes followed by washing with Buffer A. ^{125}I-FX was then added to all wells,

followed 30 minutes later by the addition of unlabelled FX (1.7 μM=15 μg/well). After an additional 30 minute incubation, the amount of ^{125}I-FX bound to each well was quantified by detection of gamma radiation. As controls, the quantity of ^{125}I-FX bound to PL-coated microtitre plates in the absence of FVIII, was examined in parallel. Saturation binding data were analyzed by the method of Scatchard as previously described [11]. Statistical analyses, using a Students unpaired t-test, were performed using the statistics component of the Sigmaplot computer software program (Jandel Scientific, Corte Madera, CA).

3.3.0 Results

3.3.1 Analysis of the association of FX with FVIII using analytical ligand affinity chromatography: The goal of this series of experiments was to determine which chain(s) of FVIII are involved in binding FX in the presence of PL and to characterize the molecular forces that assemble and maintain the structure of this subunit of the FX activating complex. To this end, FVIII-affinity columns were constructed by immobilizing FVIII on an affinity matrix composed of an antibody specific for FVIII-LC that had been covalently coupled to an Affi-Gel 10 support matrix. In this way, FVIII was uniformly immobilized via FVIII-LC, making possible specific elution of FVIII-HC. Purified ^{125}I-FX that had been preincubated with PLVs, was adsorbed to the column in the presence of calcium, as schematically diagrammed in Figure 3.1a. This was followed by the application of a series of buffers containing defined elution agents: 25 μM EDTA (Figure 3.1b), 10 mM EGTA (Figure 3.1c) and 50% ethylene glycol (Figure 3.1d). The amount of labelled FX that was eluted was quantified at each stage (Table 3.1). For comparison, dissociation of the FX-PL interaction was examined under the same elution conditions. In these experiments, the proportion of radiolabelled PLVs eluted from FX affinity columns was quantified (Table 3.1).

Figure 3.1. Dissociation of FX from FVIII-affinity columns using a series of specifically formulated elution buffers. (a) FVIII was immobilized by an anti-FVIII-LC antibody that had been covalently bound to the support matrix. FX that had been preincubated with PLVs was then adsorbed to the column in the presence of calcium. (see section 3.2.4 for complete details). (b) Elution with the buffer containing 25 μM EDTA dissociated FX from PL [12], causing elution of FX that did not interact with FVIII. As previously explained, this concentration of EDTA did not dissociate the two chains of FVIII. (c) Elution with the buffer containing 10 mM EGTA dissociated FVIII-LC and FVIII-HC. Because FVIII-LC was bound to the immobilized antibody, FVIII-HC was eluted at this stage. (d) Elution with the buffer containing 50% ethylene glycol dissociated FVIII-LC and FX from PL and dissociated FVIII-LC from the anti-FVIII-LC antibody.

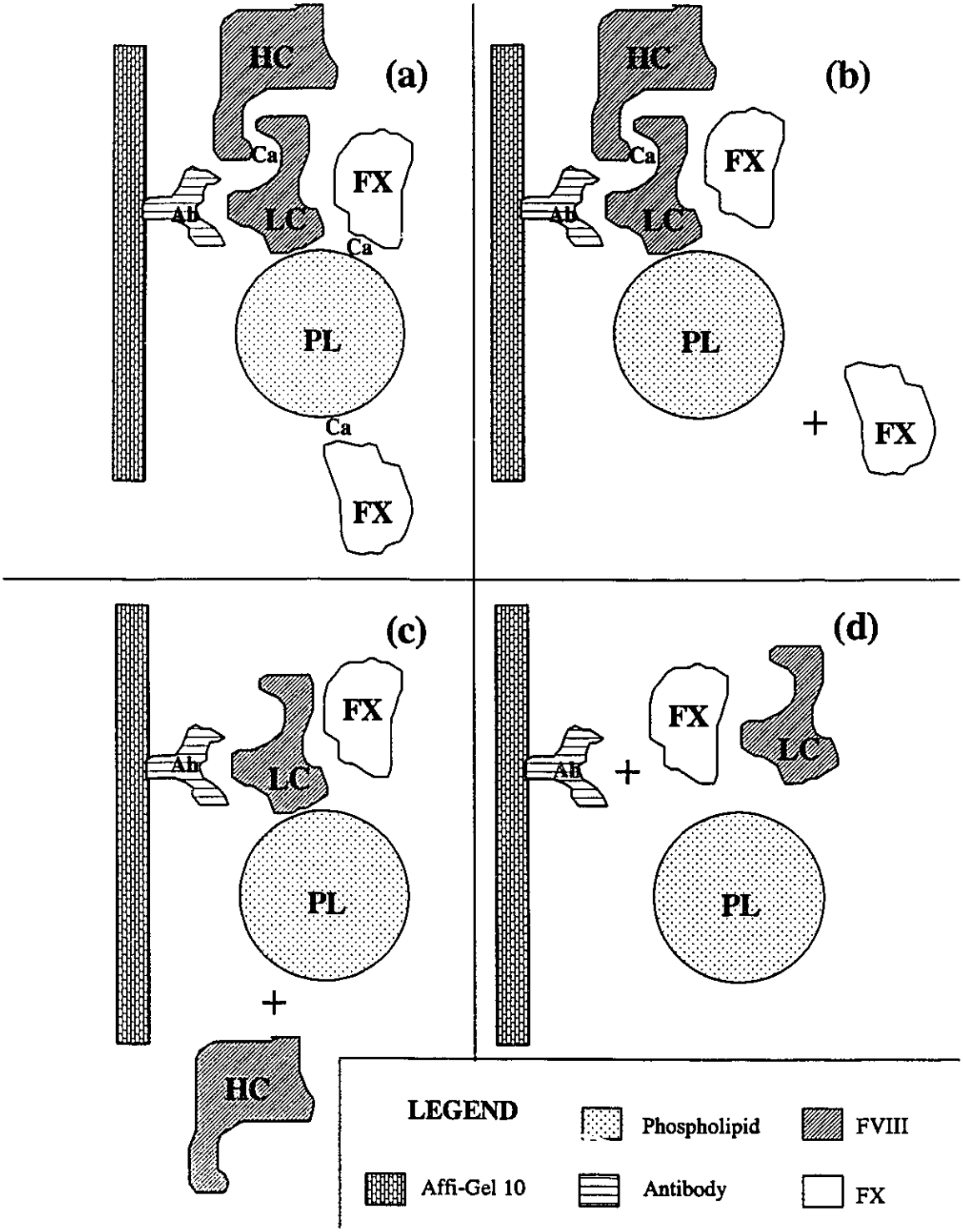

HC
(a)
Ca
FX
Ab
LC
Ca
PL
Ca
FX
HC
(b)
Ca
FX
Ab
LC
PL
+
FX
(c)
FX
Ab
LC
PL
+
HC
(d)
FX
LC
Ab
+
PL
LEGEND
Phospholipid
FVIII
Affi-Gel 10
Antibody
FX

Table 3.1. Dissociation of Phospholipid-Bound FX and Phospholipid Vesicles from Respective FVIII-, FVIII-LC- and FX-Affinity Columns

Order of Application	Elution Agent[a]	% of FX eluted from FVIII (n=3)	% of FX eluted from FVIII-LC (n=5)	% of PL eluted from FX (n=4)
1	25 μM EDTA	41 ± 5[b,c]	67 ± 5[b]	70 ± 3[c]
2	10 mM EGTA	3 ± 3	0[d]	4 ± 1[d]
3	50% Ethylene Glycol	56 ± 3[e,f]	33 ± 5[e,g]	26 ± 2[f,g]

Values reported are mean ± standard deviation
a. Buffers contained the listed components in 50 mM Tris-HCl and 1 M NaCl (pH 7.4).
b-f. Values with the same superscripted letter are significantly different, $p<0.001$.
g. Values are significantly different, $p<0.05$.

In the above series of experiments, the buffer containing 25 μM EDTA was observed to dissociate 70 ± 3% of the total bound PL from FX-affinity columns, whereas 41 ± 5% of the PL-bound ^{125}I-FX was eluted from FVIII-affinity columns (Table 3.1). The proportion of FX dissociated from FVIII, in the presence of PL, was significantly lower than the proportion of FX dissociated from PL in the absence of FVIII ($p<0.001$). Thus, the additional FX must have been retained on the column by interaction with FVIII. As this concentration of EDTA does not dissociate the two chains of FVIII (Figure 3.2), FX eluted by the EDTA containing buffer was comprised of FX that did not interact with FVIII and/or FX that associated with FVIII by simple cation-dependent interactions (Figure 3.1b).

The buffer containing 10 mM EGTA dissociates the two chains of FVIII, but does not dissociate FVIII-LC from PL (Figure 3.2). As FVIII-LC is immobilized on the column by the monoclonal antibody, ESH-8, this buffer causes elution of FVIII-HC

(Figure 3.1c). The buffer containing 10 mM EGTA did not elute a significant amount of FX (3 ± 3%, Table 3.1). If FX bound only to FVIII-HC, then the EGTA containing buffer would have been expected to elute both FVIII-HC and FX. Lack of co-elution signifies that FVIII-HC is not involved in the association of FVIII and FX.

FVIII-LC is dissociated from the anti-FVIII-LC antibody by the ethylene glycol containing buffer (Figure 3.1d). In addition, this buffer eluted 56 ± 3% of bound ^{125}I-FX from FVIII-affinity columns (Table 3.1). In contrast to above, this is significantly greater than the proportion of PL eluted from FX-affinity columns (26 ± 2%, $p<0.001$, Table 3.1), demonstrating that FX interacted with PL-bound FVIII-LC and not with PL alone. Co-elution of FX with FVIII-LC establishes that FVIII-LC is involved in the

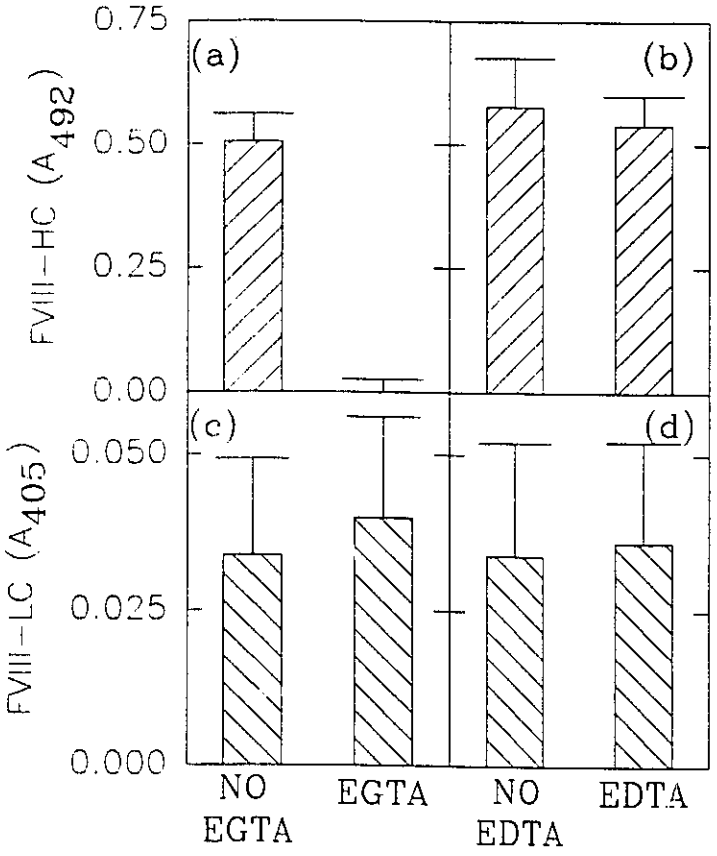


Figure 3.2. Effects of elution buffers containing 25 μM EDTA or 10 mM EGTA on phospholipid-bound FVIII. ELISA plates were coated with PL (10 μg/well) and then incubated with FVIII (6 Units/well). The presence of FVIII-HC was determined after treatment of PL-bound FVIII with elution buffers containing (a) EGTA (n=8) versus no EGTA (n=6), (b) EDTA (n=10) versus no EDTA (n=10). Similarly, the presence of FVIII-LC was detected after treatment of PL-bound FVIII with elution buffers containing (c) EGTA (n=9) versus no EGTA (n=12) and (d) EDTA (n=9) versus no EDTA (n=12). Values reported are mean ± standard deviation

association of FVIII and FX. As electrostatic interactions were previously disrupted, elution of the largest proportion of FX from the FVIII-affinity columns at this stage indicates that the interaction is predominantly hydrophobic in nature.

3.3.2 Analysis of the association of FX with FVIII-LC using analytical ligand affinity chromatography: To further examine whether FVIII-LC was capable of binding the FX-PL complex in the absence of FVIII-HC, the above experiments were repeated in the absence of FVIII-HC. As outlined in Materials and Methods (section 3.2.3), FVIII-LC-affinity matrices were prepared by dissociation of FVIII-HC from FVIII-affinity columns prior to incubation with the FX-PL mixture. The proportions of FX dissociated from FVIII-LC-affinity columns by the previously described series of elution buffers is presented in Table 3.1. The buffer containing 25 μM EDTA eluted 67 ± 5% of the total bound FX from the FVIII-LC-affinity columns. This is significantly greater ($p<0.001$) than the quantity of FX eluted by the EDTA containing buffer in the preceding experiments. It is speculated that the increase in the proportion of FX eluted with the EDTA containing buffer from FVIII-LC-affinity columns is due to divalent cation-dependent binding of FX to newly exposed acidic domains located on the N-terminal region of FVIII-LC, which are likely involved in the divalent cation-dependent association of FVIII-LC and FVIII-HC. Similar to results of FVIII-affinity column experiments, the buffer containing 10 mM EGTA did not specifically elute any FX (Table 3.1).

The ethylene glycol-containing buffer eluted 33 ± 5% of the total bound FX from FVIII-LC-affinity columns. Although this value is significantly lower than the proportion of FX eluted from FVIII-affinity columns ($p<0.001$), once again, this quantity is significantly greater than the proportion of PL eluted from FX-affinity columns (26 ± 2%, $p<0.05$, Table 3.1). These data confirm that FVIII-HC is not

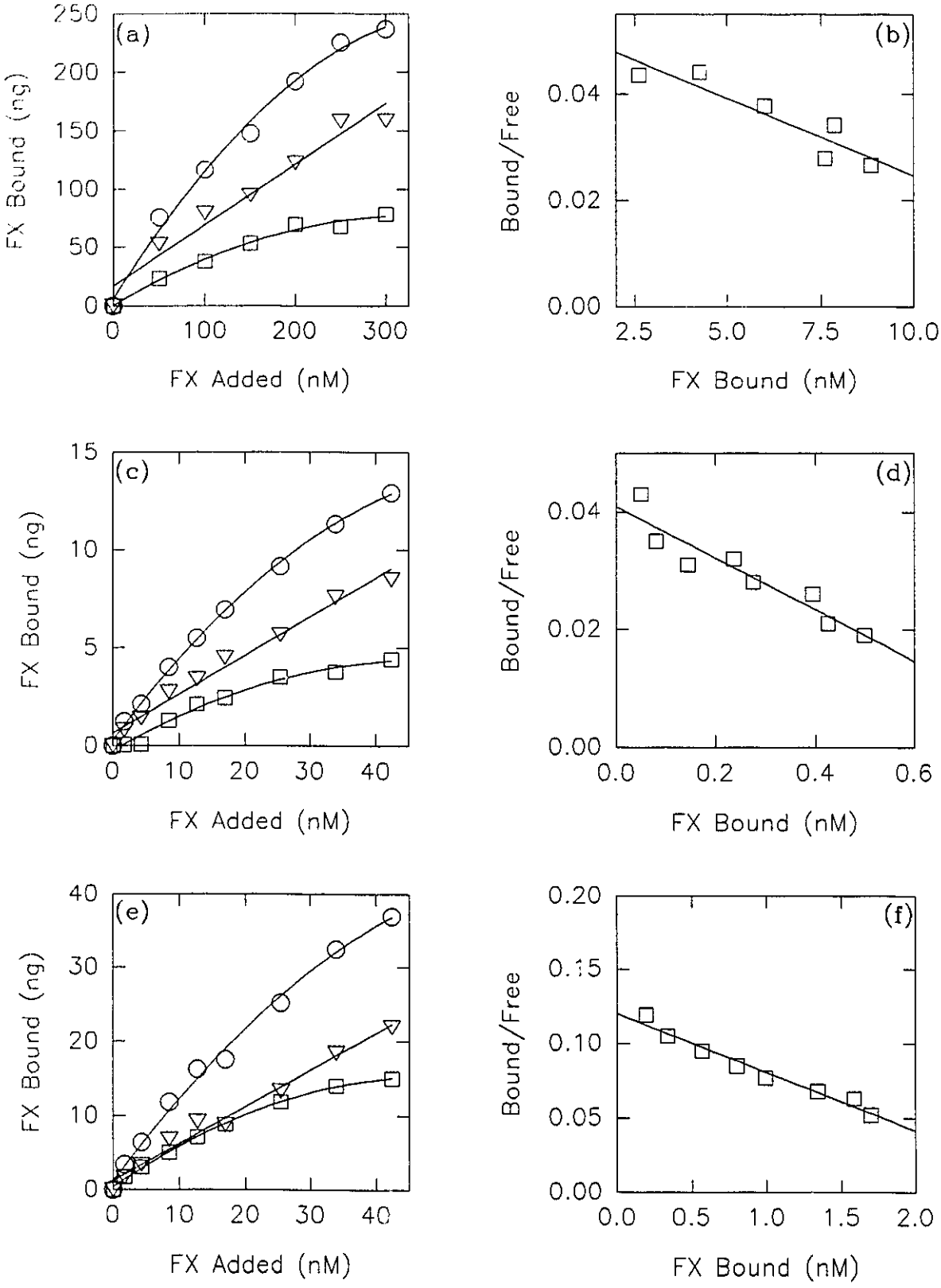
required for the association of FX and FVIII and demonstrate that FX binds to FVIII-LC in the presence of PL. Furthermore, this interaction is predominantly hydrophobic in nature.

3.3.3 Determination of the Affinity of FX Binding to Phospholipid and Phospholipid-Bound FVIII: Data from Table 3.1 indicate that FVIII and FX associate to form a PL-bound complex. To further characterize the association of FVIII and FX in the presence of PL, it was investigated whether FVIII acts as a high affinity binding site for FX on the surface of PL membranes. Radioligand binding studies were used to probe this interaction. This procedure involved coating the wells of microtitre plates with PL. The PL-coated microtitre wells were incubated with FVIII, ^{125}I-FX was then added and the total binding of ^{125}I-FX was determined as a function of increasing concentration of ^{125}I-FX. Non-specific binding was determined by incubating wells, treated in parallel, with unlabelled FX. Specific binding was determined by subtracting the quantity of non-specific binding from the total quantity of ^{125}I-FX bound. As controls, the quantity of ^{125}I-FX bound to PL-coated microtitre plates in the absence of FVIII was examined in parallel.

Data from control experiments show that the amount of FX which specifically bound to PL-coated microtitre plates increased with increasing quantities of added FX, reaching apparent saturation at concentrations of approximately 200 nM (Figure 3.3a). Scatchard transformations of these data were linear, suggesting only one type of binding site (Figure 3.3b). Approximately 2.8 ± 2.2 X 10^{12} molecules of FX were bound per PL-coated microtitre well, at saturation. The K_d was determined to be 234.3 ± 51.9 nM (Table 3.2), which is in agreement with previously published data [13].

Addition of FVIII to the assay mixtures, yielded results that differed from the above studies (Table 3.2). In the presence of FVIII, the quantity of ^{125}I-FX that bound

Figure 3.3. Specific binding of FX to phospholipid, phospholipid-bound FVIII and phospholipid-bound FVIII-LC. Microtitre wells were coated with PL (10 μg/well) and, where indicated, were then incubated with unlabelled FVIII (1.0 Unit/well). FVIII-LC was generated by removal of FVIII-HC by treatment with the 10 mM EGTA containing buffer. ^{125}I-FX was added and binding was determined as described under section 3.2.6. In (a), (c), and (e) nonspecific binding was determined by the addition of unlabelled FX (1.7 μM, 15.0 μg/well). Specific binding (□) was obtained by subtracting nonspecific binding ($\bigtriangledown$) from total binding ($\bigcirc$). (a) Binding of FX to PL-coated microtitre wells, (b) Scatchard plots of the same data. (c) Binding of FX to PL-bound FVIII, (d) the Scatchard transformation of (c). (e) Binding of FX to PL-bound FVIII-LC, (f) data of (e) transformed using Scatchard analysis.


(a)
FX Bound (ng)
FX Added (nM)
0
50
100
150
200
250
0
100
200
300
(b)
Bound/Free
FX Bound (nM)
0.00
0.02
0.04
2.5
5.0
7.5
10.0
(c)
FX Bound (ng)
FX Added (nM)
0
5
10
15
0
10
20
30
40
(d)
Bound/Free
FX Bound (nM)
0.00
0.02
0.04
0.0
0.2
0.4
0.6
(e)
FX Bound (ng)
FX Added (nM)
0
10
20
30
40
0
10
20
30
40
(f)
Bound/Free
FX Bound (nM)
0.00
0.05
0.10
0.15
0.20
0.0
0.5
1.0
1.5
2.0

to PL-coated microtitre plates reached saturation at much lower concentrations (approximately 30 nM, Figure 3.3c). In addition, FVIII significantly lowered the number of molecules of FX bound per well ($2.4 \pm 1.2 \times 10^{11}$, $p<0.001$, Table 3.2). Similar to experiments conducted in the absence of FVIII, Scatchard transformations of the binding data appeared linear indicating a single type of binding site (Figure 3.3d). However, in the presence of FVIII, FX associated with PL-coated microtitre wells with a K_d (23.1 ± 4.4 nM) that was significantly lower than the K_d in the absence of FVIII (234.3 ± 51.9 nM, $p<0.001$). These results indicate that in the presence of FVIII, FX bound to a binding site that had characteristics different from those observed in the FX-PL control studies, above. These data demonstrate that a high affinity binding site for FX forms on the surface of PL membranes in the presence of FVIII.

Table 3.2. Characteristics of FX Binding to Phospholipid, Phospholipid-Bound FVIII or FVIII-LC

	K_d (nM)	molecules FX bound per microtitre well
Phospholipid (n=5)	234.3 ± 51.9^{ab}	$2.8 \pm 2.2 \times 10^{12\ cd}$
Phospholipid-bound FVIII (n=4)	23.1 ± 4.4^{a}	$2.4 \pm 1.2 \times 10^{11\ c}$
Phospholipid-bound FVIII-LC (n=4)	28.5 ± 3.3^{b}	$2.9 \pm 1.1 \times 10^{11\ d}$

Values reported are mean ± standard deviation
a-d. Values with the same superscripted letter are significantly different, $p<0.001$.

3.3.4 Determination of the Affinity of FX Binding to Phospholipid-Bound FVIII-LC: Data from analytical ligand affinity chromatography experiments (Table 3.1) demonstrated that FX bound to FVIII-LC in the presence of PL. Moreover, FVIII-HC was not required for this association. To determine whether FVIII-LC, alone, could act

as a high affinity binding site for FX and to assess whether removal of FVIII-HC influences the affinity of FX for FVIII-LC, additional radioligand binding experiments were carried out. In these experiments, FVIII was first adsorbed to PL-coated microtitre plates. FVIII-HC was then removed by pre-incubation with a buffer containing 10 mM EGTA (Figure 3.2). FVIII-LC remained bound to the PL-coated microtitre plates (Figure 3.2). ^{125}I-FX was then added and the total quantity of ^{125}I-FX bound to the microtitre plates, was assessed as a function of increasing concentration of ^{125}I-FX. Non-specific binding was determined in parallel by adding ^{125}I-FX followed by incubation with unlabelled FX. Specific binding was determined by subtracting the quantity of non-specific binding from the total quantity of ^{125}I-FX bound.

Binding of FX to PL-coated microtitre wells containing FVIII-LC, reached saturation at approximately 35 nM FX (Figure 3.3e). The number of molecules of FX bound to PL-coated microtitre wells containing FVIII-LC was 2.9 ± 1.1 X 10^{11} (Table 3.2). Moreover, it was observed that Scatchard transformations of FX binding to PL-bound FVIII-LC were linear (Figure 3.3f) and exhibited a K_d of 28.5 ± 3.3 nM (Table 3.2). Although the number of molecules bound per well and the K_ds for interaction of FX with PL-bound FVIII-LC were regularly higher than those of the PL-bound FVIII interaction, the observed differences were not significant. The above findings are in agreement with data obtained from ligand affinity chromatographic analyses, which demonstrate that FX binds specifically to FVIII-LC and support the hypothesis that FVIII forms a high affinity binding site for FX on the surface of PL membranes. The similarity in binding parameters derived from experiments which characterized the association of FX with FVIII-LC or native FVIII, further demonstrate that FVIII-HC is not essential for association of FX and FVIII in the presence of PL.

3.3.5 Analysis of the association of FIXa with FVIII using analytical ligand affinity chromatography: It has not been reported whether one or both chains of FVIII are involved in the association with FIXa. To answer this question and to gain insight into the molecular forces that assemble and maintain the structure of the FX activating complex, the FVIII-FIXa association was probed using ligand affinity chromatography. These experiments were conducted in an analogous fashion to the FVIII-FX studies detailed earlier. FVIII- and FIXa-affinity columns were constructed as described under Materials and Methods (section 2.2.3). Purified ^{125}I-FIXa or ^{125}I-FVIII, alone, or that had been pre-incubated with PLVs, was then immobilized on FVIII- or FIXa-affinity columns, as indicated. In all instances, calcium was present in the incubation mixtures and during immobilization. After removal of non-specifically adsorbed protein and PL, the columns were sequentially treated with a series of buffers containing: 25 μM EDTA, 10 mM EGTA and 50% ethylene glycol. Elution of FVIII from FIXa-affinity columns, in the presence of PL, is schematically diagrammed in Figure 3.4. The proportion of radiolabelled protein eluted from the respective columns at each stage is listed in Table 3.3.

3.3.5.1 Elution of ^{125}I-FIXa from FVIII-affinity columns: In the absence of PL, 37.8 ± 13.6% of the bound ^{125}I-FIXa was eluted from FVIII-affinity columns by the buffer containing 25 μM EDTA, while in the presence of PLVs, the proportion of ^{125}I-FIXa eluted by the EDTA containing buffer was 43.5 ± 14.0% (Table 3.3). Elution of FIXa from immobilized FVIII at this stage demonstrates that simple cation-dependent interactions are involved in the association of FIXa and FVIII.

The EGTA-containing buffer elutes FVIII-HC from the FVIII-affinity columns. In the present series of experiments, this buffer also eluted 29.1 ± 10.0% of PL-bound FIXa, while 36.0 ± 7.2% of FIXa was eluted in the absence of PL

Figure 3.4. Dissociation of FVIII from FIXa-affinity columns using a series of specifically formulated elution buffers. (a) FIXa was immobilized by covalently coupling it to the support matrix. FVIII, that had been preincubated with PLVs, was then adsorbed to the column in the presence of calcium (see section 3.2.4 for complete details). (b) Application of the buffer containing 25 μM EDTA eluted FVIII that interacted with FIXa in a simple divalent cation-dependent manner. As previously discussed, this concentration of EDTA did not dissociate the two chains of FVIII. (c) Elution with the buffer containing 10 mM EGTA dissociated FVIII-LC and FVIII-HC. As FVIII-LC was bound, hydrophobically, to the immobilized FIXa, FVIII-HC was eluted at this stage. (d) Elution with the buffer containing 50% ethylene glycol dissociated FVIII-LC from the immobilized FIXa.

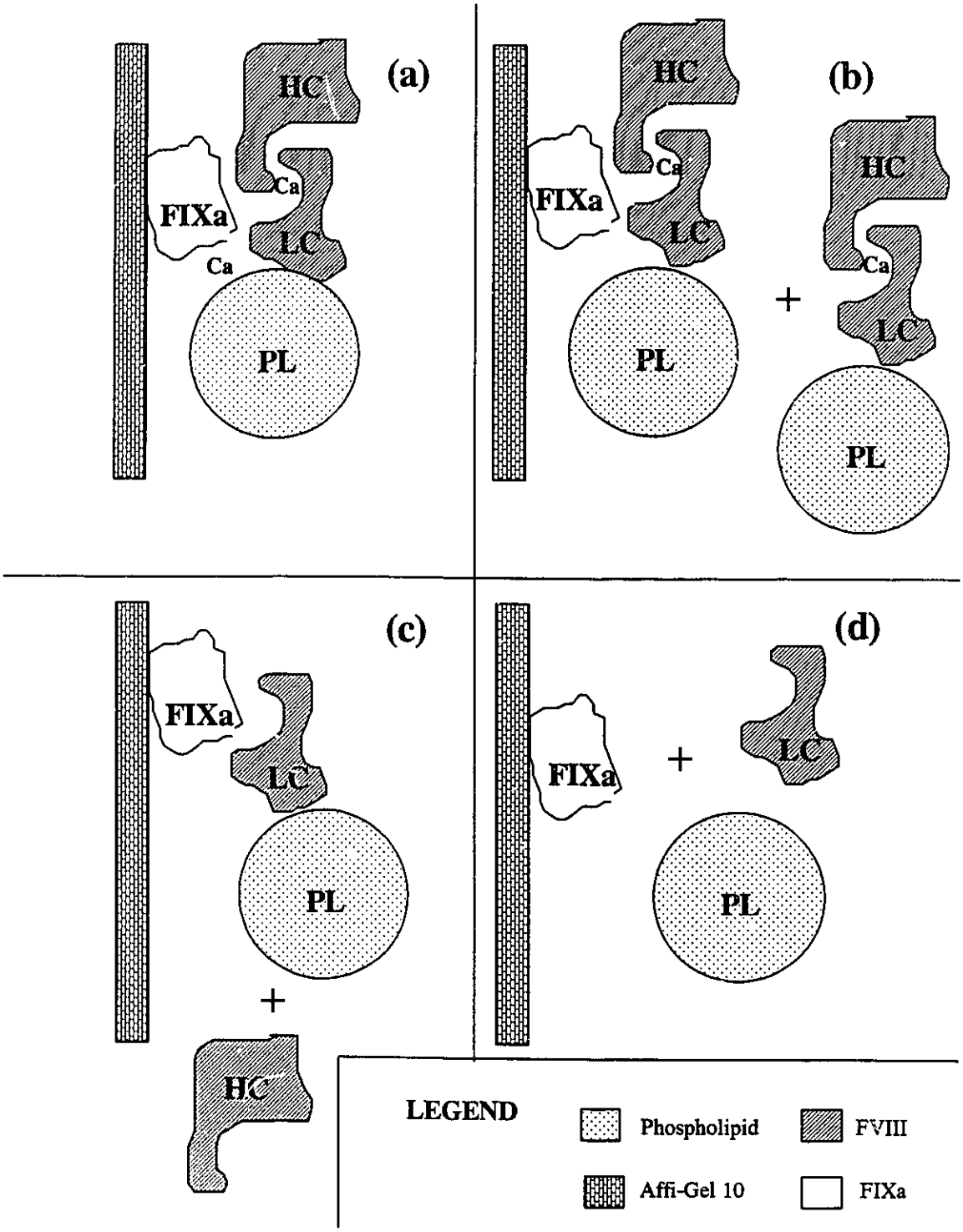

(a)
HC
Ca
FIXa
LC
Ca
PL
(b)
HC
Ca
FIXa
LC
PL
+
HC
Ca
LC
PL
(c)
FIXa
LC
PL
+
HC
(d)
FIXa
+
LC
PL
LEGEND
Phospholipid
FVIII
Affi-Gel 10
FIXa

Table 3.3. Dissociation of FIXa from FVIII-Affinity Columns

Order of Application	Elution Agent[a]	FIXa Eluted from FVIII-Affinity Columns			
		Phospholipid		No Phospholipid	
		(ng)[b]	(%)[b]	(ng)[c]	(%)[c]
1	25 μM EDTA	389 ± 233	43.5 ± 14.0	281 ± 145	37.8 ± 13.6
2	10 mM EGTA	216 ± 49	29.1 ± 10.0	267 ± 145	36.0 ± 7.2
3	50% Ethylene Glycol	213 ± 57	27.4 ± 5.8	174 ± 76	26.2 ± 14.6

a. Buffers contained the listed components plus 50 mM Tris-HCl (pH 7.4) and 1 M NaCl.
b. Mean ± standard deviation, (n=5)
c. Mean ± standard deviation, (n=4)

(Table 3.3). Co-elution of FIXa with FVIII-HC by the EGTA-containing buffer indicates that FIXa interacts with FVIII-HC, or alternatively indicates that FIXa interacts with FVIII-LC in a divalent cation-dependent manner. The types of molecular forces maintaining the FIXa-FVIII-HC association cannot be determined from these data. This has been investigated further by examining the elution pattern of ^{125}I-FVIII from FIXa-affinity columns, described in section 3.3.5.2.

The ethylene glycol-containing buffer dissociated 27.4 ± 5.8% and 26.2 ± 14.6% of FIXa from FVIII-affinity columns in the presence or absence of PL, respectively. These data indicate that FIXa also associates with FVIII-LC and that addition of PL does not influence this interaction. As electrostatic interactions were previously disrupted by the buffers containing high salt and/or chelating agents, elution of FIXa from FVIII-affinity columns by the ethylene glycol containing buffer indicates that the interaction of FIXa with FVIII-LC involves hydrophobic forces.

3.3.5.2 Elution of ^{125}I-FVIII from FIXa-affinity columns: To further examine the association of FIXa with FVIII-HC, the above experiments were repeated, however, radiolabelled FVIII was substituted for radiolabelled FIXa and FIXa-affinity columns were substituted for FVIII-affinity columns. As the two chains of FIXa are held together by a disulphide bond, both chains were retained on the FIXa-affinity columns throughout these experiments.

The EDTA containing buffer eluted 25.9 ± 5.1% of ^{125}I-FVIII from FIXa-affinity columns in the presence of PL and 16.8 ± 3.2% in the absence of PL (Table 3.4). This is in agreement with above results which indicated that simple divalent cation dependent electrostatic interactions were involved in the association of FVIII and FIXa. It was also reported above, that FVIII-LC interacts hydrophobically with FIXa, therefore, FVIII-LC is retained on the FIXa-affinity column after elution with the EGTA-containing buffer. If FIXa interacted with FVIII-HC in a hydrophobic manner, then FVIII-HC would also be retained on the column under these conditions. However, the EGTA-

Table 3.4. Dissociation of FVIII from FIXa-Affinity Columns

Order of Application	Elution Agent[a]	FVIII Eluted from FIXa-Affinity Columns			
		Phospholipid		No Phospholipid	
		(ng)[b]	(%)[b]	(ng)[b]	(%)[b]
1	25 μM EDTA	3.3 ± 2.6	25.9 ± 5.1	1.8 ± 2.12	16.8 ± 3.2
2	10 mM EGTA	4.9 ± 3.5	42.9 ± 9.3	3.1 ± 3.1	38.4 ± 7.0
3	50% Ethylene Glycol	4.5 ± 4.5	31.3 ± 7.3	4.5 ± 5.4	44.8 ± 4.1

a. Buffers contained the listed components plus 50 mM Tris-HCl (pH 7.4) and 1 M NaCl.
b. Mean ± standard deviation, (n=3)

containing buffer caused elution of 42.9 ± 9.3% and 38.4 ± 7.0% of the total bound ^{125}I-FVIII in the presence or absence of PL, respectively (Table 3.4). Thus, FVIII-HC is eluted from FIXa-affinity columns by the EGTA-containing buffer, demonstrating that FIXa interacts with FVIII-HC in a divalent cation-dependent manner.

Subsequent treatment of the FIXa-affinity column with the buffer containing ethylene glycol eluted the remaining proportion of ^{125}I-FVIII (31.3 ± 7.3% in the presence of PL and 44.8 ± 4.1% in the absence of PL, Table 3.4). Electrostatic interactions were previously disrupted, therefore hydrophobic forces are implicated in binding of FVIII and FIXa.

The total quantity of radiolabelled protein eluted from the respective columns, calculated by adding the amount of protein eluted by each elution buffer, is presented in Table 3.5. These data indicate that there was no significant difference in the total quantity of FVIII bound to FIXa-affinity columns in the presence (13 ± 11 ng) or absence (9 ± 11 ng) of added PLVs (Table 3.5). Similarly, there was no significant

Table 3.5. Effect of Phospholipid on the Association of FIXa and FVIII

Immobilized Protein	Protein Eluted	Total Quantity of Protein Eluted (ng) Phospholipid Added	No phospholipid added
FIXa	FVIII	13 ± 11[a]	9 ± 11[a]
FVIII	FIXa	1188 ± 438[b]	1340 ± 324[c]

a. Mean ± standard deviation, (n=3)
b. Mean ± standard deviation, (n=5)
c. Mean ± standard deviation, (n=4)

difference in the quantity of FIXa bound to FVIII-affinity columns in the presence (1188 ± 438 ng) and absence (1340 ± 324 ng) of added PLVs (Table 3.4). Thus, results of

the present study demonstrate that PL is not required in the association of FVIII with FIXa. Furthermore, similar proportions of ^{125}I-FIXa and ^{125}I-FVIII were eluted from their respective affinity columns by each elution buffer in the presence or absence of PL. This indicates that the presence of PL did not alter the forces required to maintain the FVIII-FIXa interaction.

3.3.6 Assessment of the influence of FVIII on binding of FIXa to phospholipid: To further characterize the association of FIXa with PL in the presence of FVIII, it was determined whether FVIII alters the quantity of FIXa bound on the surface of PL membranes. Radioligand binding studies were used to probe this interaction. For these experiments, ^{125}I-FIXa and FVIII were adsorbed to wells of microtitre plates that had been coated with PL. The quantity of ^{125}I-FIXa bound to each PL-coated microtitre well, was assessed by quantification of the gamma radiation. For comparison, the quantity of ^{125}I-FIXa bound to PL-coated microtitre plates in the absence of FVIII, was examined in parallel. Data from these experiments demonstrate that 108.34 ± 3.34 ng of ^{125}I-FIXa bound to PL-coated microtitre wells in the absence of FVIII (Figure 3.5). In the presence of FVIII, the quantity of FIXa bound increased significantly to 126.52 ± 2.92 ng (Figure 3.5, $p<0.05$). Moreover, increasing the quantity of FVIII from 1.5 pmol/well to 3.0 pmol/well increased the quantity of FIXa bound/well to 141.07 ng. These results demonstrate that FVIII facilitates binding of FIXa on the surface of PL membranes.

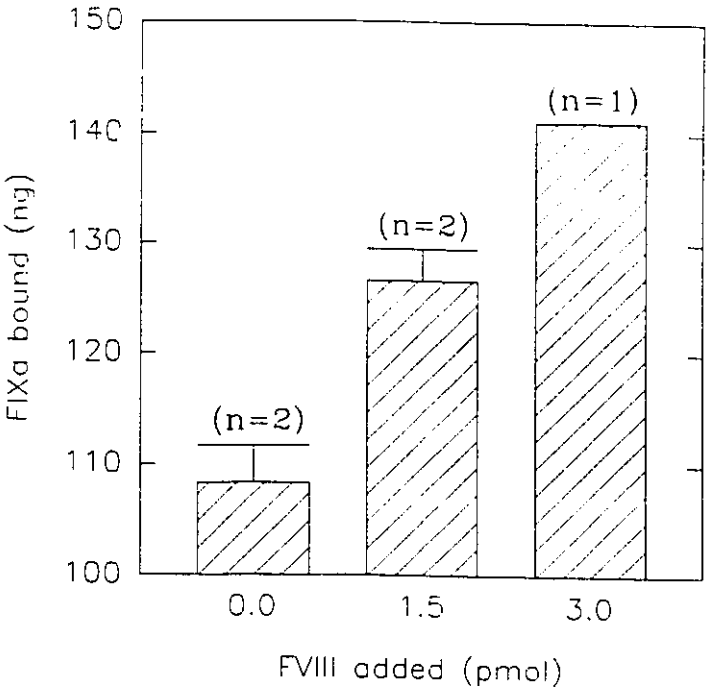


Figure 3.5. Binding of FIXa to phospholipid-coated microtitre plates in the presence and absence of FVIII. The wells of microtitre plates were coated with PL (10 μg/well) and then incubated with FVIII (6 Units/well). The amount of ^{125}I-FIXa bound to PL-coated microtitre wells in the absence of FVIII, in the presence of 1.5 pmol of FVIII or in the presence of 3.0 pmol of FVIII was assessed by quantification of gamma radiation. Values reported are mean ± standard deviation

3.4.0 Discussion

FVIII is believed to form a binding site for FIXa and FX on the surface of PL membranes. This hypothesis, and most of our knowledge of the composition and structure of the FX activating complex, has been based primarily on data derived from studies examining the activation of FX in the absence of one or more of FVIII, FIXa, calcium or PL [3, 14-17]. In the present study, structural aspects of the FVIII-FX-PL and FVIII-FIXa-PL associations were examined in a more direct manner. Based on existing information, we speculate that formation of the FVIII-FX-PL portion of the FX activating complex is the result of at least four specific interactions: i) FVIII-LC-FVIII-HC; ii) FVIII-PL; iii) FX-PL; and iv) FVIII-FX. The first three interactions have previously been investigated and are characterized by specific molecular forces and specific affinities (Table 3.6). In addition, it can be theorized that the FVIII-FIXa-PL portion of the FX activating complex forms in an analogous manner and is governed by similar molecular forces and affinities. The K_d for the FVIII-LC-FVIII-HC interaction

has not been reported in the literature, however, it is predicted to be identical in magnitude to that of the two chains of coagulation FV ($K_d = 10^{-9}$ M [18]), a coagulation protein analogous in structure and function to FVIII [19-21]. Based on these differences in molecular forces and affinities, a series of buffers were formulated which selectively disrupt interactions within the FVIII-FX/FIXa-PL portions of the FX activating complex. These buffers were used in conjunction with analytical ligand affinity chromatography to examine the association of FVIII with FIXa and FX.

Table 3.6. Characteristics of Interactions within the FVIII-FX-Phospholipid Portion of the FX Activating Complex

Interaction	Predominant Molecular Force of Association	Ref.	Dissociation Constant (M)	Ref.
FX-Phospholipid	Divalent cation-dependent	[12, 13]	10^{-7}	[13]
FVIII-LC/HC	Divalent cation-dependent	[6, 7, 22]	10^{-9}	[18*]
FVIII-Phospholipid	Hydrophobic	[12]	10^{-9}	[23]

a. Based on analogies in structure and function [19-21], this value is predicted to be similar to that for FV.

Data obtained using analytical ligand affinity chromatography and radioligand binding studies demonstrated that FX specifically binds to PL-bound FVIII, and that FVIII acts as a binding site for FX on PL. Furthermore, these studies showed that FX binds, hydrophobically, to FVIII-LC. Removal of FVIII-HC prior to addition of FX demonstrated that FVIII-HC was not essential for, and may not be involved in the association of FX with FVIII-LC in the presence of PL. Dissociation of electrostatic interactions by the buffers containing 25 μM EDTA or 10 mM EGTA, followed by elution of the largest proportion of FX from the FVIII-affinity columns by ethylene glycol suggested that the association of FVIII and FX in the presence of PL was predominantly hydrophobic in nature.

The quantity of acidic PLs, which form potential binding sites for FX is theoretically non-saturable on a large surface, such as a PL-coated microtitre well. This is evident by the apparent linear increase in the non-specific component of ^{125}I-FX bound in the radioligand binding experiments (Figures 3.3a, 3.3c, 3.3e). However, as demonstrated by the current study, discrete saturable binding sites for FX form on PL membranes that are characterized by a binding affinity of approximately 10^{-7} M. These sites appear to be composed of clusters of acidic PLs [24, 25]. There is an approximately linear increase in the number of binding sites between 0 and 25% acidic PL [25], thus the number of binding sites is dependent upon the proportion of acidic PLs in the membrane. Addition of a molecule, such as FVIII, which may act as a binding site for FX on the surface of PL membranes, would be expected to increase the affinity of FX binding. In the present study, binding of FX to PL-bound FVIII exhibited a K_d of 10^{-8} M, which was 10-fold lower than that of the FX-PL interaction ($K_d = 10^{-7}$). These data support the hypothesis that FVIII may act as a receptor for FX on the surface of PL membranes.

Data obtained from radioligand binding studies demonstrated that FX bound avidly to FVIII-LC in the absence of FVIII-HC, indicating that FVIII-HC is not required for this interaction. Moreover, the affinity and number of FX molecules bound per microtitre well coated with PL-bound FVIII-LC were not significantly different from the values obtained from experiments examining the binding of FX to native FVIII bound to PL-coated microtitre wells. From this information it can be concluded that FVIII-LC contains a binding site(s) for FX that is of relatively high affinity.

The quantity of FX associated with PL-bound FVIII or FVIII-LC in the radioligand binding studies was approximately 10-fold lower than the quantity of FX bound to PL-coated microtitre wells. This suggests that FVIII may compete with FX for binding to PL. However, binding of FVIII requires approximately 170-385 PL

monomers in a membrane comprised of 20% PS [23], whereas binding of FX to membranes was shown to require approximately 5 [24] to 16 [25] PS per molecule of FX [24]. This implies that FVIII may lower the number of FX molecules bound per PL-coated microtitre well by sterically blocking potential PL binding sites for FX. In radioligand binding studies examining the association of FVIII and FX, one unit of FVIII was added to each microtitre well, this corresponds to approximately 4.0 X 10^{11} molecules of FVIII. If 50% of this FVIII bound to the PL-coated microtitre wells, then the stoichiometry of FX binding with FVIII would be 1:1. This further supports FVIII acting as a high affinity binding site for FX on the surface of PL membranes.

FIXa and FX are zymogens of serine proteases. As discussed in chapters 1 and 2, these two proteins are similar in structure. The domains of FIXa and FX possess 48 to 71% sequence homology (excluding the activation peptides) [26] and both vitamin K-dependent proteins have been demonstrated to bind to PL membranes via their GLA-domains in an analogous manner [27, 28]. Based on these similarities, one may predict that FIXa associates with FVIII in a manner similar to FX. However, results of analytical ligand affinity chromatographic analyses and radioligand binding studies in the present study demonstrate that the association of FIXa with FVIII differs from that of the FX-FVIII interaction. In contrast to FX, data from analytical ligand affinity chromatographic analyses demonstrate that binding of FIXa to FVIII involves both FVIII-HC and FVIII-LC. It was observed that FIXa interacts with FVIII-HC in a divalent cation-dependent manner and that the interaction of FIXa with FVIII-LC involves hydrophobic forces.

It has been previously reported that FIXa and FVIIIa interact in a 1:1 stoichiometry on the surface of PL membranes [3]. The apparent K_d of FIXa for PL was lowered from 10^{-6} to 10^{-8} M by the addition of FVIIIa [3]. Moreover, the Kd for the FVIIIa-FIXa interaction on the surface of PLVs was reported to be 3-16 nM [3].

It was concluded from these data that FVIIIa acts as a receptor for FIXa, analogous to FVa acting as a receptor for FXa [3]. However, this hypothesis is based on analyses which did not directly ascertain quantities of FIXa bound to PL in the presence and absence of FVIII. Therefore, the above hypothesis was examined using microtitre binding experiments. In the absence of FVIII 108.34 ± 3.34 ng of ^{125}I-FIXa bound to PL coated microtitre wells (Figure 3.5). The presence of FVIII significantly increased the quantity of ^{125}I-FIXa bound to 126.52 ± 2.92 ng (Figure 3.5, $p<0.05$). In addition, increasing the quantity of FVIII from 1.5 pmol/well to 3.0 pmol/well also increased the quantity of FIXa bound/well to 141.07 ng (Figure 3.5). Thus, data presented in this report demonstrate that FVIII increases the quantity of FIXa bound to PL membranes. This is in agreement with earlier reports which suggest that FVIII acts as a receptor for FIXa.

In summary, FVIII was found to act as a binding protein for both FIXa and FX on the surface of PL membranes. FX was observed to interact hydrophobically with FVIII-LC. Moreover, in the absence of FVIII-HC, FX bound to FVIII-LC with an affinity that was not significantly different from binding to native FVIII, indicating that FVIII-HC was not involved in the association of FVIII and FX. Similar to FX, FIXa bound to FVIII-LC in a hydrophobic manner. However, the current work also provides evidence that FIXa interacted with FVIII-HC in a divalent cation-dependent manner.

3.5.0 References

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CHAPTER 4

BINDING OF HUMAN BLOOD COAGULATION FACTORS IXa, X AND Xa TO STIMULATED HUMAN PLATELETS

CHAPTER 4: Binding of Human Blood Coagulation Factors IXa, X and Xa to Stimulated Human Platelets

4.1.0 Introduction

Propagation of the blood coagulation pathway has been demonstrated to require the presence of PL membranes which in vivo is believed to be supplied by the plasma membrane of stimulated platelets. Membrane PL appears to form a focal point for the formation of multi-protein enzyme complexes of the blood coagulation pathway, such as the FX activating complex. Data from studies performed using synthetic model membrane systems, described in Chapters 2 and 3, indicated that FX bound specifically to PL membranes. Additional results presented in Chapter 3 suggested that FVIII may act as a receptor for FX on the surface of these model PL membranes. The current chapter focuses on studies involved in characterizing the association of FX with a physiological surface, namely the plasma membrane of platelets. The influence of FVIII on this interaction was concurrently examined.

Platelets are highly specialized anucleate cells, derived from megakaryocytes. The platelet plasma membrane is composed of 35 to 40% protein (w/w) and 50% lipid (w/w) [1]. Eight to 10% (w/w) of the membrane is composed of carbohydrate, which is isolated in membrane glycoproteins and glycolipids [1]. The structure and function of most of these glycoproteins and glycolipids remain undefined. Platelets play an essential role in thrombosis and haemostasis. Not only do they aggregate to form a mechanical barrier to blood loss at the site of a vascular injury, but when stimulated, they also secrete various substances that promote coagulation and fibrinolysis. Platelets exhibit a host of other functions. They provide a blood-borne defence mechanism whereby tissue damage causes platelets in the vicinity to secrete agents that increase vascular permeability and mediate inflammation [2]. Platelets bind to bacteria and may

opsonize them for neutrophil phagocytosis [3]. Furthermore, platelets promote chemotaxis of leukocytes to the site of tissue damage through activation of component C5 of the complement pathway [4]. Upon stimulation, they secrete platelet-derived growth factor which penetrates the subendothelium and stimulates the smooth muscle cells in the vessel wall to migrate and proliferate [5] as required during vessel repair. They may down-regulate their own production through secretion of an alpha granule platelet factor that inhibits megakaryocyte colony formation [6]. The production of platelets is up-regulated by thrombopoietin, a humoral growth factor [7] which may also be secreted by the kidney, liver and spleen.

4.1.1 Platelets in Thrombogenesis

Thrombogenesis involves formation of a platelet plug which is maintained and stabilized by a fibrin network. Platelet plug formation involves a series of overlapping events which include: 1) adhesion of platelets to the site of vessel injury; 2) platelet spreading on the exposed subendothelial surface; 3) secretion of platelet granules; 4) recruitment of additional platelets through aggregation; 5) acceleration of coagulation; and 6) platelet-dependent clot retraction. The mechanisms mediating these events have not been completely elucidated. Evidence suggests that adhesion of platelets to collagen of the subendothelium involves a vWF "bridge". vWF is secreted by endothelium and also circulates in blood as a non-covalent complex with FVIII. It is also secreted from alpha granules by stimulated platelets. Binding of vWF to collagen induces a conformational change in vWF which allows it to also bind to a platelet surface receptor, GPIb.

When platelets are exposed to the subendothelial surface they become stimulated and undergo a shape change which allows them to spread along the exposed subendothelium. Stimulation results in disappearance of the marginal band of

microtubules that normally maintains the discoid shape of the platelet, centralization of storage granules, formation of pseudopodia, and phosphorylation of intracellular proteins. Cytoplasmic calcium levels have also been observed to increase as well as liberation of arachidonic acid from membrane PLs by phospholipase A_2. Known agonists include thrombin, ADP, collagen, and arachidonic acid. Specific glycoprotein receptors for some of these stimulating agents have been identified on the platelet surface (Table 4.1), however, it is not known whether receptors exist for all agonists. Furthermore, information about the mechanism of stimulus-response coupling in this system is still fragmentary. Secretion, also called the release reaction, occurs upon stimulation and results in the discharge of intracellular granule contents (Table 4.2), exposure of latent surface receptors for plasma proteins, such as GPIIb/IIIa for vWF and fibrinogen, and alterations in lipid structure of the plasma membrane (Table 4.3) leading to acceleration of coagulation.

Platelet aggregation is triggered by a variety of agents such as ADP, thrombin and collagen. Aggregation, in turn, induces the release reaction, and establishes a positive feedback mechanism for the stimulation and aggregation which results in formation of a platelet plug at the site of a vascular injury. How such a great variety of stimulative, adhesive and aggregative agents can induce similar effects, has not been clearly defined. The final step in thrombus formation is clot retraction which is the result of actin and myosin-dependent contraction of platelet pseudopodia and the fibrin network.

4.1.2 Platelets in Coagulation

Stimulated platelets express procoagulant activity, while resting platelets do not. Furthermore, stimulated platelets have been demonstrated to bind a number of different coagulation proteins (Table 4.4). The ability of activated platelets to promote

Table 4.1. Examples of Platelet Membrane Glycoproteins that Act as Receptors.

Platelet Glycoprotein Complex	number per platelet	Subunits	molecular weight	Function
Ia/IIa[a]	2,000[8]	Ia: IIa:	167,000[9] 148,000	collagen receptor[8]
Ib/IX	25,000[10]	Ib: α Ib: ß IX:	143,000[9] 23,000[9] 17,000[13]	Ib: membrane-bound form of glycocalicin[11] intracellularly bound to actin (unstim. platelet)[12] IX: ND[b] Ib/IX: vWF receptor[14] deficient in Bernard-Soulier Syndrome[15] thrombin receptor[14]
IIb/IIIa[a]	50,000[16]	IIb: α IIb: ß IIIa:	125,000[17] 23,000[17] 110,000[17]	IIb: ND IIIa: ND IIb/IIIa: fibrinogen receptor[18] deficient in Glanzmann's Thrombasthenia[19] vWF, fibronectin and thrombospondin receptor[18] intracellularly bound to actin (stim. platelets)[20]
IV (IIIb, CD36)	12,000		88,000[21]	thrombospondin[22] and collagen[23] receptor
V	ND		82,000	thrombin receptor/substrate[9]
PADGEM (GMP-140)	13,000[24]		140,000[25]	neutrophil and monocyte receptor[26]
64	200		64,000	adrenalin receptor
180	240		180,000	PAF receptor

a. members of the integrin family
b. ND, not determined

Table 4.2. Contents of Platelet Granules

Platelet Granule	Granule Contents	
Dense	ATP-ADP, serotonin, PPi, Pi, Calcium	
Lysosomal	lysosomal enzymes	
alpha	plasma proteins:	Fibrinogen, fibronectin, albumin, FV, plasminogen, HMWK, vWF, C1-inhibitor, factor D+ß1H globulin of the complement system, plasminogen activator inhibitor, α_1-antitrypsin, α_2-macroglobulin, α_2-antiplasmin, antithrombin III, protein S, immunoglobulin G
	platelet-specific proteins:	platelet basic protein, ß-thromboglobulin, platelet-derived growth factor, platelet factor 4
	Additional factors:	thrombospondin, vascular permeability factor, bactericidal factor, chemotactic factor, inhibitor for megakaryocyte colony formation

Table 4.3. Phospholipid composition of the outer leaf of the platelet plasma membrane[a].

Phospholipid	Whole Platelet[28]	Unstimulated[27]	Stimulation Agonist	
			thrombin[27]	collagen[27]
PS	10	2.4	4.9	6.0
PE	27	9.5	15.5	17.0
PC	38	31.0	39.3	41.4
SM	19	57.1	40.2	35.6
PI	5			
% total PL[b]		21	32	34
FX activation[c]		2	3	18

a. Values are expressed as percentage of the hydrolysed fraction.
b. Hydrolysed fraction as percent of total PL.
c. nM Xa generated per minute in the presence of platelets treated as indicated.

Table 4.4. Binding of Coagulation Factors to Stimulated Platelets.

Coagulation Factor	Dissociation Constant	Binding Sites per Platelet	Platelet Receptor or Binding Protein
High molecular weight kininogen	HMWK: 20 nM HMWKa: ND	HMWK: 24,000[29]	ND
Prekallikrein	Prekallikrein: ND Kallikrein: ND		
XIII	XIII: NA XIIIa: ND	XIII: no binding[30] XIIIa: 12,000 ± 1,600[30]	 ND
XII	XII: ND XIIa: ND XIIf: ND		
XI	XI: 10 nM XIa: 1 nM	XI: 1500[31] XIa: 130-550[32]	ND ND
X	X: NA Xa: 0.03 nM[35] to 0.34 nM[34]	X: no binding[33,34] Xa: 200[35] Xa: 420[34]	 FVa FVa
IX	IX: 2.68 nM IXa: 2.57 nM	IX: 306[36] IXa: 515[36]	ND ND
VIII	VIII: 2.9[37]-6.6[38] nM VIIIa: ND	VIII: 650[38]	M_r=80,000 -90,000 polypeptide[39]
VII	VII: ND VIIa: ND	VII: ND VIIa: ND	Tissue factor?? Tissue factor??
V	V/Va: 3 nM Va: 0.3 nM	V/Va: 3,500[40] Va: 900 [40,41]	ND ND
II	II: ND IIa: 1.8-2 nM	IIa: 300-400 [42]	IIa: GPIb
I	I: 1000 nM I: varies with agonist Ia: ND	I: 40,000-80,000 I: varies with agonist	I: GPIIb/IIIa I: thrombospondin

ND=Not Determined
NA=Not Applicable

coagulation has been ascribed to changes in the distribution of their membrane lipids (the platelet factor 3 effect) which causes increased binding of coagulation factors. There is additional evidence which demonstrates that certain membrane glycoproteins form binding sites for specific coagulation factors (Table 4.1). Thus, the increase in the rate of coagulation upon platelet stimulation may be due to expression of membrane proteins that function as adhesion molecules (e.g. GPIb), as aggregation stimulators (e.g. GPIIb/IIIa) or as binding proteins for coagulation proteins (e.g. GPIIb/IIIa, GPIb, GPV). Alternatively, the increase may be due to the release of platelet granule contents which increases the local concentration of clotting factors, such as high molecular weight kininogen, FV and fibrinogen. The mechanism(s) by which platelet stimulation promotes reactions in the coagulation pathway, such as FX activation, have not been fully delineated.

4.1.2.1 Changes in the Platelet Plasma Membrane Lipid Profile: In the unactivated state, the outer leaf of the platelet plasma membrane contains very few charged PLs [43] (Table 4.3). However, rapid changes in the lipid profile occur during the initial phase of platelet stimulation. Activation results in hydrolysis of membrane PLs such as PI [44] by phospholipase A_2 and PC by phospholipase C. In addition, activation leads to "flipping" of PLs from one side of the bilayer to the other. One result of PL "flipping" is increased exposure of PS on the outer surface of the platelet plasma membrane [27] (Table 4.3). The proportion of PS exposed does not appear to be influenced by the stimulating agent [27]. As discussed earlier, PS exposure is required for binding of FVIII, FIXa and FX to the PL surface. Thus, increased exposure of PS may promote binding of these proteins to the platelet surface. Furthermore, changes in membrane lipid composition and fluidity have been demonstrated to alter membrane protein function [45-47]. Therefore, modification of

the lipid profile could result in transformation of cryptic membrane glycoproteins into coagulation factor receptors or binding proteins. Altering the lipid profile of each half of the bilayer may also result in changes in the lateral pressure between enzymes and substrates and produce altered mobility of receptors, thus influencing the activity of membrane-bound proteins and signal transduction [48, 49].

4.1.2.2 Changes in the Platelet Plasma Membrane Protein Profile: Expression of fluid phase and latent membrane proteins during platelet stimulation results from extrusion of the open canalicular system (OCS) and granule membrane fusion. Stimulation also results in secretion of a number of granule proteins. The release of the contents of alpha-granules and dense bodies is essential for expression of the procoagulant properties of platelets. This was demonstrated by the prolonged bleeding times in patients with grey platelet syndrome (lack of alpha granules) [50] or Hermansky-Pudlak syndrome (lack of dense bodies) [51].

Many platelet membrane proteins such as GPIb, GPIIb and GPIIIa, interact with the platelet cytoskeleton [52, 53]. It has been reported that GPIIb/IIIa associates with the cytoskeleton upon platelet stimulation [54]. Conversely, platelet stimulation was found to cause dissociation of GPIb from the platelet cytoskeleton [54]. These data imply that reorganization of the platelet cytoskeleton upon stimulation may alter the distribution or function of proteins that associate with both the cytoskeleton and membrane.

As indicated above, there are multiple ways by which stimulation influences the procoagulant activity of platelets. However, in most instances, the component(s) of platelets that form binding sites for each coagulation factor have not yet been isolated or defined (Table 4.4). Identifying the component with which a coagulation factor

associates, will facilitate delineating the mechanisms of binding. As a first step in identifying the platelet binding component(s) for factors of the FX activating complex, model PL membrane systems were examined. In Chapters 2 and 3 it was reported that FX bound specifically to PL membranes. Additionally, results described in Chapter 3 showed that FVIII could serve as a high affinity binding protein for FX on the surface of PL membranes. To ascertain whether these results could be generalized to a physiological surface, binding of FX to platelets was characterized and the affect of FVIII on binding of FX to platelets examined.

4.2.0 Materials and Methods

4.2.1 Proteins: The coagulation proteins used in this series of experiments were the same as those listed in sections 2.2.1. and 3.2.1. Bovine serum albumin (essentially fatty acid free, ELISA and RIA grade), human thrombin (3000 NIH units/mg thrombin protein) (Sigma, St. Louis, MO) and collagen type 1 (Diagnostic Stago, Ansieres, France) were purchased. Radiolabelling of FIXa (specific activity 80,000-700,000 CPM/μg), FX (specific activity 70,000-350,000 CPM/μg) and FXa (specific activity 300,000-400,000 CPM/μg) and protein quantification were carried out as described in section 2.2.1.

4.2.2 Platelets: Approximately 250 mls of blood were collected from volunteer donors (Canadian Red Cross Blood Transfusion Service, Ottawa Centre, Ottawa, Ontario) into CLX system blood packs (Miles Canada, Inc., Etobicoke, Ontario) and was centrifuged within 10 minutes from the end of collection at 600g for 7 minutes. The platelet-containing plasma was aseptically removed and immediately centrifuged at 2000g for 6 minutes. Platelet-depleted plasma was aseptically drained from the platelet pellet until approximately 50 mls of plasma remained. After incubating, motionless, for 45

minutes, the platelets were resuspended in the remaining plasma by gently rotating, end-over-end, for an additional 45 minutes. Platelet concentrates were prepared and stored at 22°C. Gel-filtered platelets were prepared by a procedure adapted from Sinha et al. [55], in which filtration columns were constructed using 50 mls of Sepharose CL-2B (Pharmacia LKB, Uppsala, Sweden) contained in a 60 ml plastic syringe (Becton Dickinson & Co., Rutherford, NJ). To prevent loss of gel, the syringes were plugged with rayon fibres (Curity rayon balls, Kendall Hospital products division, Toronto, Ontario). Prior to platelet filtration, the columns were washed with two column volumes of Buffer I: 137 mM NaCl, 27 mM KCl, 11.9 mM $NaHCO_3$, 0.36 mM $NaH_2PO_4.H_2O$, 5.55 mM D-glucose, 25.2 mM HEPES (pH 7.4) and then with two column volumes of Buffer I containing 1% bovine serum albumin (BSA). Four to ten millilitres of platelet concentrate prepared from citrated human whole blood (Blood Transfusion Service, Canadian Red Cross Ottawa Centre, Ottawa, Ontario) were applied to the Sepharose CL-2B and eluted by gravity using a modified Tyrodes buffer (Buffer I containing 4 mM $CaCl_2.2H_2O$, 1 mM $MgCl_2.6H_2O$, and 2% BSA). Fractions (1.5 ml) were collected and the number of platelets quantified (Coulter T540, Hialeah, FL). Appropriate fractions were pooled to give the desired final platelet concentration and/or diluted with the above elution buffer. The ability of the gel-filtered platelets to aggregate when stimulated by thrombin (0.16 U/ml) or collagen (62.5 μg/ml) was assessed using a model 400 Lumi Aggro-meter (Chrono-log Corporation, Havertown, PA). D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK, Calbiochem, La Jolla, CA) was used as a thrombin inhibitor. The ability of PPACK to inhibit thrombin, but not collagen-induced platelet aggregation was established by adding 1.31 ng of PPACK (final concentration of 5 nM) prior to addition of thrombin or collagen in the above platelet aggregation assay. In all experiments, only fresh platelets (prepared between 2.5 and 7.0 hours post-phlebotomy) that aggregated in response to exposure with thrombin or collagen were

used. Where specified, platelet plasma membranes were isolated by the method of Barber and Jamieson [56].

4.2.3 Equilibrium Binding Studies: Unless stated otherwise, all binding studies were performed within 4.5 hours from phlebotomy. Gel-filtered platelets (7.5 X 10^8 platelets/ml) were added immediately after addition of PPACK (10 nM) and collagen (60 μg/ml) or thrombin (0.1 Units/ml), to 1.5 ml microfuge tubes (Sarstedt, St. Laurent, Quebec) containing the indicated coagulation protein(s). The solutions were incubated for 20 minutes at 37°C before being layered onto 1 ml of 20% sucrose, dissolved in modified Tyrodes solution (described above), contained in 1.5 ml conical microcentrifuge tubes. After centrifuging at 14,000 g for 3 minutes in an Eppendorf 5415 centrifuge (Fisher Scientific, Ottawa, Ontario), the supernatants were carefully aspirated and reserved while the tips of the microfuge tubes containing the platelet pellets were amputated. The amount of radiolabelled protein contained in the supernatants and pellets were quantified using a Beckman 5500 gamma counter (Beckman Instruments (Canada) Inc., Toronto, Ontario). Non-specific binding was determined by addition of 20- to 100- fold excess unlabelled FX at the same time as, or 5 minutes after addition of radio-labelled FX. In addition, all binding experiments were done at least in duplicate on a minimum of 4 separate occasions using platelets obtained from different individuals using different lot numbers of purified human FIXa, FX and FXa. Saturation binding data were analyzed by the method of Scatchard [57].

4.2.4 Assays to Determine the Effect of Short-Term Platelet Storage on Binding of Coagulation Factors: Two to three units of platelet concentrates were aseptically pooled and stored in standard plastic blood packs (described in section 4.2.2) at 22°C, gently rotating end-over-end. At appropriate time intervals, 5 to 10 ml aliquots were

removed and gel-filtered as detailed above. The capacity of these platelets to specifically bind radiolabelled FXa (45.5 nM), FX (33.9 nM) or FX in the presence of FVIII (11.4 U/ml) was determined as outlined in section 4.2.3. Times indicated in the text and Figures correspond to the time from the end of phlebotomy until addition of the gel-filtered platelets to the equilibrium binding assay mixtures.

4.2.5 Electrophoresis, Autoradiography and Western Blotting: Platelet pellets obtained from the current binding studies were solubilized in a laurylsulfate-containing reducing buffer (125 mM Tris, 4.6% Sodium dodecyl sulfate, 20% glycerol, 2% 2-mercaptoethanol, 0.02% Bromophenol Blue, pH 6.8). Proteins, from the indicated sources, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the discontinuous buffer system of Laemmli [58] as previously described [59]. The resultant gels were silver-stained [60], dried and then incubated with X-ray film (Kodak XAR5 film, Picker, Brampton, Ont.) to produce autoradiograms. The resolved proteins were electroblotted onto nitrocellulose sheets, as outlined elsewhere [61]. The presence of FVIII-antigen on western blots was probed by addition of a monoclonal human anti-FVIII IgG (ESH-8, American Diagnostica Inc., Greenwich, CT) followed by washing and addition of an alkaline phosphatase-conjugated anti-murine IgG (Bio-Rad, Richmond, CA). FVIII was visualized by a change in colour intensity caused by action of IgG-conjugated alkaline phosphatase on its substrate (Section 3.2.5).

4.2.6 Statistical Analysis: Statistical analyses were performed as described in section 3.2.3.

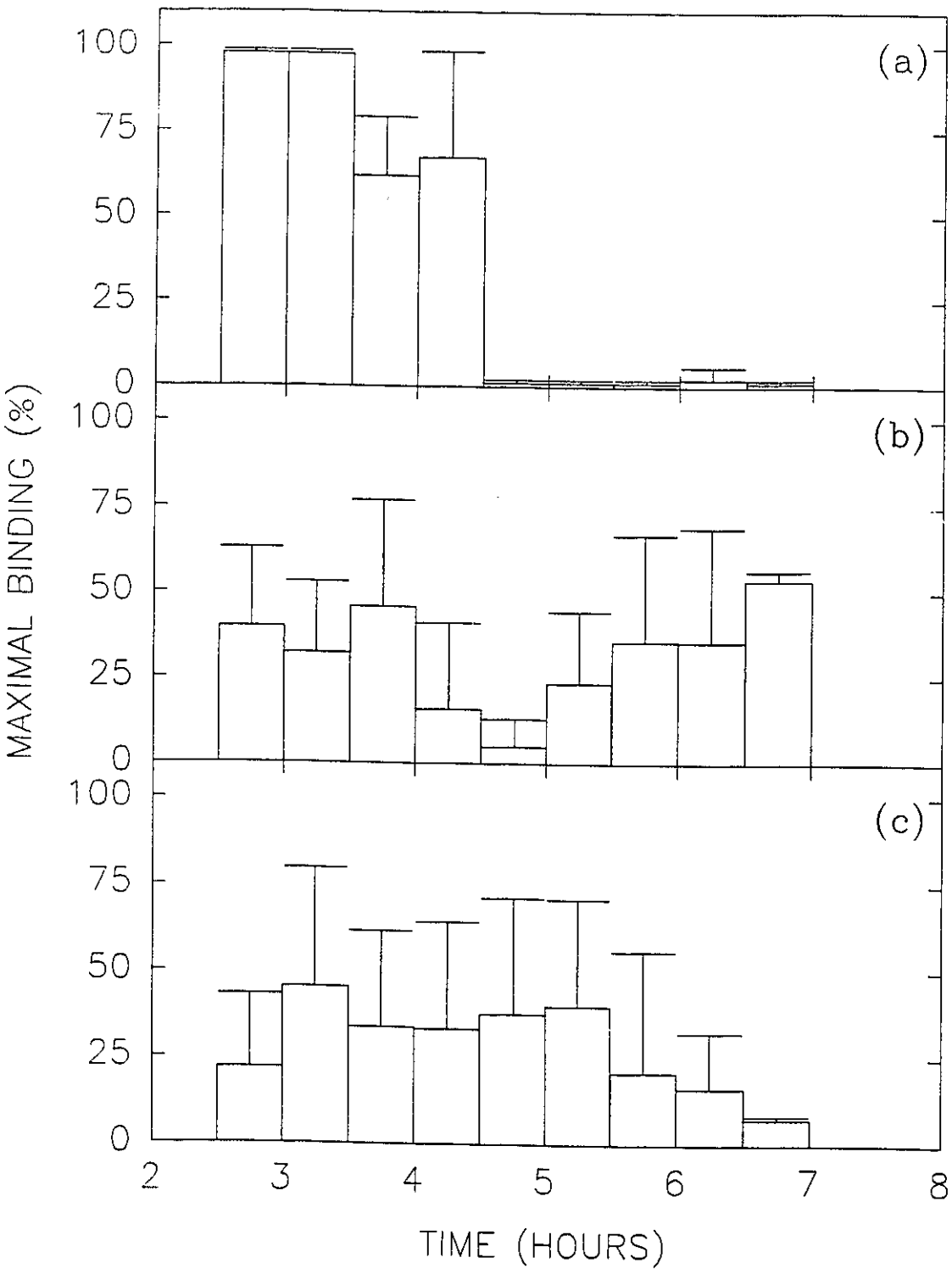
4.3.0 Results

4.3.1 Binding of FX to Platelets: In Chapters 2 and 3, binding of FX to the surface of PL membranes was examined using model membrane systems, namely PL vesicles or PL-coated microtitre wells. Binding of FX was specific and exhibited a Kd of 234.3 ± 51.9 nM. To determine whether FX also interacts in a specific manner with the surface of a physiological membrane, such as the plasma membrane of platelets, the association of FX with stimulated platelets was investigated.

Platelet concentrates were prepared as described in Section 4.2.2 and were stored at room temperature, with gentle agitation. At intervals between 2.5 and 7 hours post-phlebotomy, aliquots of the platelet concentrates were removed and gel-filtered. The ability of the platelets to bind FX was determined immediately after gel filtration. Prior to addition of FX, the platelets were stimulated with collagen. The total quantity of radiolabelled ligand that associated with platelets was determined by subjecting the reaction mixture to centrifugation through 20% sucrose to separate the bound from unbound ligand. Non-specific binding was determined in parallel by addition of a 100-fold excess of unlabelled FX or FXa to the reaction mixture prior to separation of bound and unbound ligand. Specific binding was calculated by subtracting non-specific binding from the total quantity of FX or FXa bound. The data demonstrated that specific binding of FX to collagen-stimulated platelets occurred within the first 4.5 hours from phlebotomy. However, as indicated in Figure 4.1a, platelets stored for longer periods (4.5 to 7 hours) before testing, became refractory to binding of FX.

4.3.2 Determination of the Affinity of FX Binding to Platelets: The above studies demonstrated that FX bound in a specific manner to stimulated platelets within 4.5 hours from phlebotomy. To further characterize this interaction, equilibrium-binding studies were undertaken utilizing platelets prepared within four hours following phlebotomy.

Figure 4.1. Binding of FX and FXa to Platelets as a Function of Time. Binding of ^{125}I-FX to platelets that were collagen-stimulated at the indicated times from phlebotomy was determined as described under Materials and Methods (section 4.2.4). Maximal binding was calculated using the greatest quantity of FX or FXa bound in each series of experiments. Values reported are mean ± standard deviation. (a) FX (n=4); (b) FX in the presence of FVIII (n=7); FXa (n=7).


100
75
50
25
0
(a)
100
75
50
25
0
(b)
100
75
50
25
0
(c)
MAXIMAL BINDING (%)
2
3
4
5
6
7
8
TIME (HOURS)

As a negative control, the association of FX with unstimulated platelets was examined. In agreement with results of others [33, 34], FX did not bind specifically to unstimulated platelets. However, when platelets were stimulated with collagen, a progressive uptake of FX was observed as the concentration of FX increased, reaching apparent saturation at concentrations of approximately 25 nM (Figure 4.2a). Scatchard transformations generated binding isotherms that appeared linear, indicating that FX bound to a single class of binding site. A K_d of 35.7 ± 5.2 nM was calculated for the association of FX and approximately 202 ± 180 molecules of FX were bound per platelet at saturation (Figure 4.2 and Table 4.5).

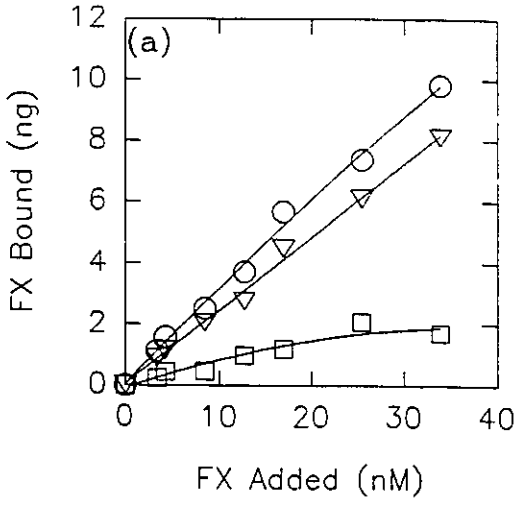
Table 4.5. Characteristics of FX, FXa and FIXa Binding to Stimulated Platelets

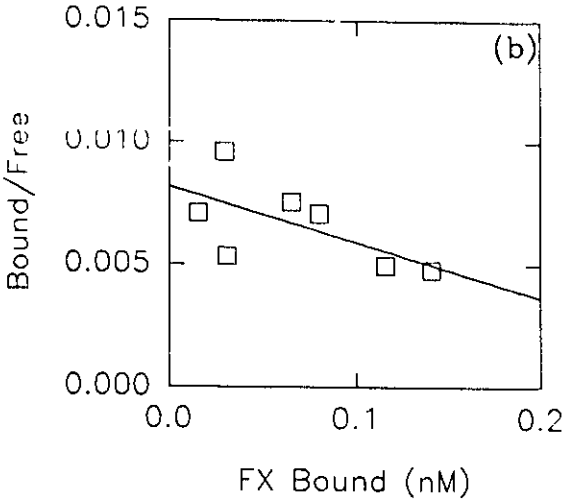
Factor	K_d (nM)	molecules FX bound per platelet
FX in the absence of FVIII (n=4)	35.7 ± 5.2[a]	202 ± 180[b]
FX in the presence of FVIII (n=6)	51.4 ± 14.4[c]	202 ± 82[d]
FXa (n=5)	307.8 ± 180[a,c]	2268 ± 1105[b,d]
FIXa (n=4)	2.89 ± 0.09	262 ± 39

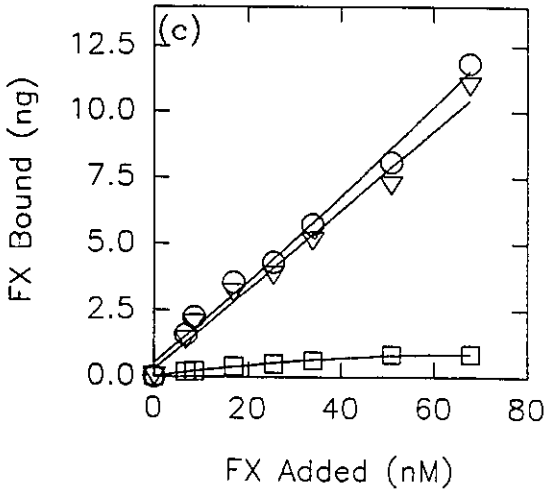
Values reported are mean ± standard deviation
a. Values are significantly different from each other, $p<0.02$.
b. Values are significantly different from each other, $p<0.01$.
c. Values are significantly different from each other, $p<0.02$.
d. Values are significantly different from each other, $p<0.01$.

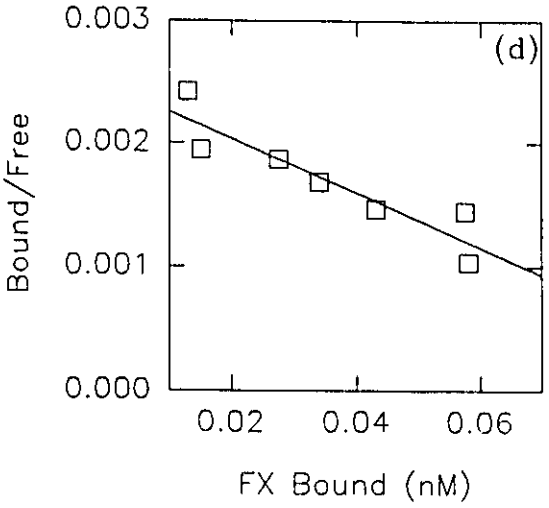
4.3.3 Influence of FVIII on Binding of FX to Platelets: In the studies outlined in Chapter 3, it was observed that FVIII enhanced binding of FX to the surface of PL membranes. It was, therefore, of interest to test whether FVIII may also influence the association of FX with platelets. For comparison, these experiments were conducted in parallel with the FX-platelet binding studies described in Section 4.3.1.

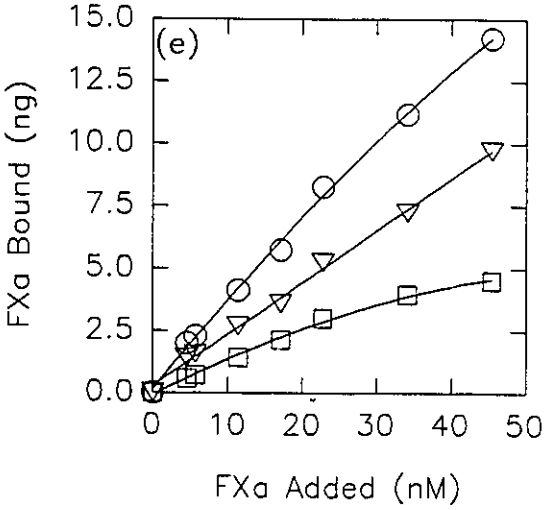
Figure 4.2. Binding of FX or FXa to Platelets. Binding of ^{125}I-FX to gel-filtered platelets as a function of concentration was determined as described under section 4.2.3. Binding isotherms from representative experiments are presented and were linearized by transforming the data from the left panel according to the method of Scatchard [57]. Specific binding (□) was obtained by subtracting non-specific binding (▽) from total oinding (○). (a) FX; (c) FX in the presence of FVIII (11.4 U/ml); and (e) FXa. The corresponding Scatchard plots of the above data are shown in figures (b), (d), and (f), respectively.

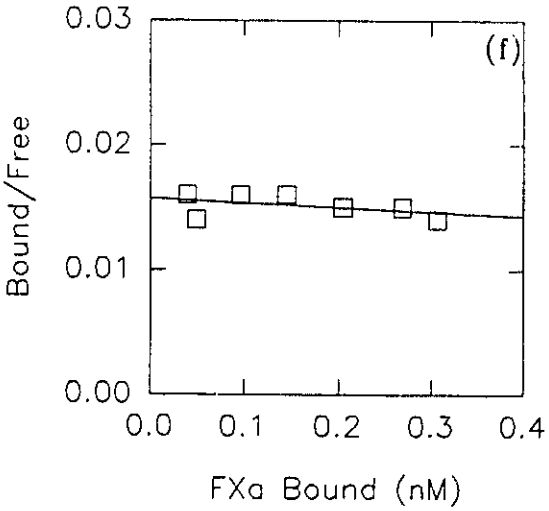

(a)
FX Bound (ng)
FX Added (nM)
0
2
4
6
8
10
12
0
10
20
30
40


(b)
Bound/Free
FX Bound (nM)
0.000
0.005
0.010
0.015
0.0
0.1
0.2


(c)
FX Bound (ng)
FX Added (nM)
0.0
2.5
5.0
7.5
10.0
12.5
0
20
40
60
80


(d)
Bound/Free
FX Bound (nM)
0.000
0.001
0.002
0.003
0.02
0.04
0.06


(e)
FXa Bound (ng)
FXa Added (nM)
0.0
2.5
5.0
7.5
10.0
12.5
15.0
0
10
20
30
40
50


(f)
Bound/Free
FXa Bound (nM)
0.00
0.01
0.02
0.03
0.0
0.1
0.2
0.3
0.4

The quantity of ^{125}I-FX specifically bound to the stimulated platelets was calculated by subtracting the quantity of non-specific binding, determined in the presence of FVIII and a 100-fold excess of unlabelled FX, from the total quantity of ^{125}I-FX bound in the presence of FVIII. These investigations used platelets that were gel-filtered and stimulated with collagen between two to seven hours post-phlebotomy.

Data from these studies demonstrated that, in the presence of FVIII, specific binding of FX occurred at all times examined (Figure 4.1b). Moreover, the quantity of FX bound at time periods upto 4.5 hours post-phlebotomy was not significantly different than the quantity of FX bound after 4.5 hours (Figure 4.1b). This is in contrast to data obtained with FX alone, which showed that binding to platelets was refractory in the time interval from 4.5 to 7 hours from collection (Figure 4.1a). Taken together, results of experiments which examined binding of FX to stimulated platelets in the absence or presence of FVIII, indicated that within 4.5 hours from phlebotomy, FX was able to bind to stimulated platelets independently of FVIII and that after this time, binding of FX to stimulated platelets was dependent upon the presence of FVIII. The requirement of FVIII to induce enhanced binding of FX to platelets that were stored for at least 4.5 hours before stimulation, suggests that after this time, FVIII may function as a binding protein for FX. These results are in agreement with data from Chapter 3 which showed that the association of FX with PL-coated microtitre wells was accentuated by the addition of exogenous FVIII. Thus binding of FX to stimulated platelets appears to occur by two mechanisms: FVIII-independent and FVIII-dependent, at least one of which hinges upon the time from phlebotomy.

4.3.4 Equilibrium Binding Studies Examining Binding of FX to Stimulated Platelets in the Presence of FVIII: Data from studies described in sections 4.3.1 and 4.3.3 indicated that prior to 4.5 hours from phlebotomy, binding of FX to stimulated platelets

may occur independently of FVIII. Equilibrium binding studies were, therefore, undertaken to characterize in further detail the affect FVIII has on the binding of FX to platelets. Platelets in these studies were used within four hours following phlebotomy and, as detailed in section 4.2.3, were stimulated with collagen immediately before incubation with ^{125}I-FX in the presence of a constant level of FVIII. Non-specific binding was determined in parallel by addition of a 20- to 100-fold excess of unlabelled FX to the reaction mixture prior to separation of bound and unbound ligand.

In the presence of FVIII, the pattern of FX binding exhibited a progressive increase in the quantity of bound FX as the concentration of added FX was increased, reaching apparent saturation at a concentration of approximately 35 nM FX (Figure 4.2b). Scatchard transformations of the binding data were linear, indicating that FX bound to a single type of binding site. From the Scatchard analyses, it was calculated that, in the presence of FVIII, 202 ± 82 molecules of FX bound per platelet with a K_d of 51.4 ± 14.4 nM (Figure 4.2 and Table 4.5). This K_d value was not significantly different from that observed for FX in the absence of FVIII (Table 4.5). Moreover, the addition of FVIII did not significantly influence the quantity of FX bound per platelet (Table 4.5). These results are in agreement with the data documented in sections 4.3.1 and 4.3.3 which suggest that FX binds independently of FVIII to stimulated platelets within 4.5 hours from phlebotomy.

4.3.5 Determination of the Adsorption of Endogenous FVIII to Gel-Filtered Platelets: In an earlier study, the presence of FVIII had been reported to increase the quantity of FX bound to collagen-stimulated platelets [62]. To rule out the possibility that platelets used in our experiments contained FVIII adsorbed during preparation from platelet rich plasma, platelets filtered under the current conditions (section 4.2.2), were analyzed to determine whether they contained FVIII. Proteins present in the preparation

of gel-filtered platelets or fresh plasma were subjected to electrophoresis and the resolved proteins were subsequently electroblotted onto nitrocellulose sheets, as described in Materials and Methods (section 4.2.5). This was followed by immunoblotting with a FVIII-LC specific antibody, ESH 8, to probe for the presence of FVIII. Results from these studies are shown in Figure 4.3. The protein content of 20 μl of plasma (which includes approximately 0.02 Units of FVIII and approximately 8 X 10^6 platelets) was resolved in lane (a), and the protein content of gel-filtered platelets (approximately 4 X 10^7 platelets) was resolved in lane (b). A band with a M_r of 80,000 was observed in lane (a). This M_r corresponds to that of FVIII-LC. To increase the probability of detecting adsorbed FVIII, a five-fold excess of gel-filtered platelets were resolved in lane (b), nonetheless, no bands were observed. Thus, within the detection limits of the technique (5-100 pg), these data are evidence that platelets filtered under the described conditions were free of FVIII. It appears unlikely, therefore, that the observed binding of FX was due to adsorption of FVIII to platelets during the separation of platelets from other blood components.

4.3.6 Characterization of Platelet-Bound FX: The specific association of FX with platelets (section 4.3.1 and 4.3.2) is a novel observation that has not been previously described. However, a number of authors have shown that the activated form of FX, FXa, does associate specifically with stimulated platelets [34, 35]. Thus, experiments were performed to rule out the possibility that the observed binding of FX to stimulated platelets may have been due to activation of FX to FXa. Binding

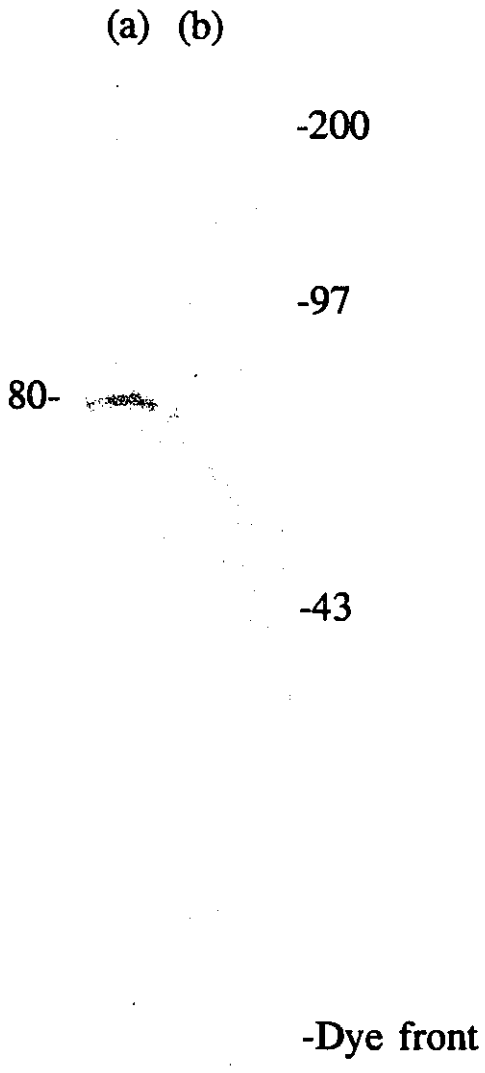


Figure 4.3. Determination of the Presence of Endogenous FVIII by Immunoblotting. The presence of FVIII was determined using western blotting as described under section 4.2.5. Numbers beside the lanes represent the migration distances of molecular weight standards as indicated ($X10^3$). Lane a: 20 μl of plasma, containing approximately 0.02 Units of FVIII and approximately 8 X 10^6 platelets; Lane b: approximately 4 X 10^7 gel-filtered platelets.

experiments utilizing FXa in place of FX were carried out in parallel to assess whether FX was activated to FXa under the current experimental conditions (Section 4.2.3). Data presented in Figure 4.1c show that FXa binds to collagen stimulated platelets but, unlike FX, binding of FXa was not affected by storage of platelets over the time intervals examined. Distinct differences in the binding patterns suggest that the observed association of FX with stimulated platelets was not due to activation of FX to FXa. To further investigate whether the specific binding of FX to platelets was the result of activation of FX to FXa, studies were performed to calculate the binding parameters which characterize the FXa-platelet interaction. The quantity of FXa that bound to stimulated platelets progressively increased as the concentration of FXa increased, reaching apparent saturation at approximately 300 nM. By transforming these binding data using Scatchard analyses, it was observed that FXa bound to stimulated platelets with a K_d of 307.8 ± 180.0 nM and that under saturating conditions 2268 ± 1105 molecules of FXa bound per platelet (Figure 4.2b and Table 4.5). Similar results were obtained whether collagen or thrombin was used as the platelet stimulating agent. Examination of the data reveal that the quantity of FXa bound per platelet (2268 ± 1105 molecules) was significantly greater than the quantity of FX bound per platelet (202 ± 180 molecules, $p<0.01$, Table 4.5). In contrast, FX bound to platelets with an affinity that was significantly greater than that of FXa (a K_d of 35.7 1 ± 5.2 nM versus 307.8 ± 180.0 nM, respectively, $p<0.02$, Table 4.5). These data confirm that it is unlikely that FX was converted to FXa during the platelet binding assay.

Data have been published that characterize the association of FIXa with stimulated platelets [36]. Therefore, as a positive control for the equilibrium binding experiments, we have re-examined this interaction. Results of these studies

demonstrated that 262 ± 39 molecules of FIXa bound per platelet with a K_d of 2.89 ± 0.09 nM (Table 4.5) which is consistent with previous results [36].

Electrophoretic analyses of platelet-bound FX were performed to further confirm that FX was not converted to FXa during the course of the binding experiments. Platelets containing bound ^{125}I-FX were recovered from equilibrium binding experiments and were solubilized as detailed in Materials and Methods (section 4.2.5). Platelet-bound ^{125}I-FX was separated from other platelet proteins using reducing SDS-PAGE. As controls, aliquots of purified ^{125}I-FX and ^{125}I-FXa were resolved in parallel. Following electrophoresis, the SDS-PAGE gels were dried and autoradiographed (Figure 4.4). The heavy chain of purified FX was detected as a band of M_r 42,000, while the light chain had a M_r of 18,000. The heavy-chain-derived fragment of FXa was isolated as a doublet with M_r values of 27,000 and 25,000. Purified ^{125}I-FXa also exhibited a light chain with a M_r of 18,000 and an activation peptide with a M_r of 11,000. Autoradiographs of ^{125}I-FX that had been bound to collagen-stimulated platelets demonstrated a band with M_r 42,000 and a weaker band with M_r 18,000. Collectively, these data show that FX was not activated to FXa during the binding procedure since the electrophoretic pattern of radio-labelled bands observed in the lane containing stimulated platelets correspond to that of FX and not FXa (Figure 4.4).

4.4.0 Discussion

Data from investigations examining the association of FX with PL membranes were presented in chapters 2 and 3 and demonstrated that binding was specific. In addition, studies by others have shown that binding of FX is dependent upon the

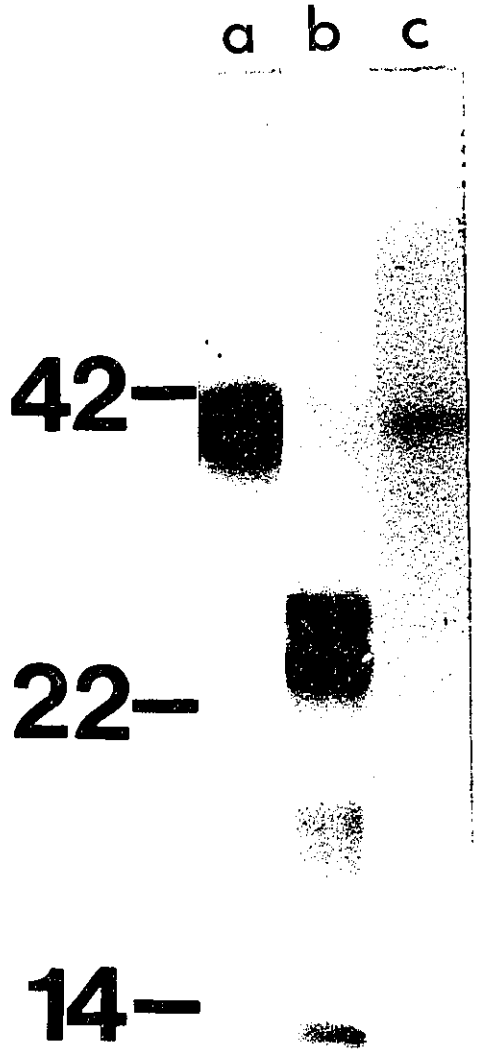


Figure 4.4. Characterization of Platelet-Bound FX by Autoradiography. Platelets were incubated with ^{125}I-FX (33.9 nM) according to the FX equilibrium binding protocol. The resultant platelet pellets were solubilized in SDS-PAGE reducing buffer. Autoradiographs of FX, FXa and platelet-bound FX, were produced as outlined under Materials and Methods (section 4.2.5). Numbers beside the lanes represent the migration distances of molecular weight standards as indicated (X10^3). Lane a, ^{125}I-FX; Lane b, ^{125}I-FXa; Lane c, platelet-bound ^{125}I-FX.

acidic PL content of the membrane [63]. Unstimulated platelets have very few charged PLs exposed on their surface [43] and do not bind coagulation factors, such as FX [33, 34]. However, upon stimulation there is an increase in the quantity of acidic phospholipids exposed, such as PS [27]. The increased exposure of acidic PLs may provide binding sites for FX on the surface of stimulated platelets. This possibility was investigated in experiments reported in the current chapter.

The data demonstrated that FX bound specifically to collagen stimulated platelets. However, this binding was only observed when the platelets were isolated and tested within 4.5 hours from phlebotomy (Figure 4.1a). Binding of FX sharply declined to platelets that were stored for longer periods of time (Figure 4.1a). Further characterization of the association of FX with platelets used within 4.5 hours from phlebotomy, was under taken to determine the affinity and capacity of binding. Results of equilibrium binding studies demonstrated that FX bound to collagen stimulated platelets with a dissociation constant of 35.7 ± 5.2 nM, and at saturating concentrations of FX, 202 ± 180 molecules were specifically bound per platelet (Table 4.5). Transformation of the data using the technique of Scatchard generated linear binding isotherms, suggesting a single class of binding site. At present, it cannot be deduced what platelet membrane component(s) constitute(s) the FX binding sites on these cells.

The molecular mechanism by which FVIII exerts its cofactor function in the activation of FX to FXa has not been fully defined. Data from investigations, described in Chapter 3, showed that FVIII served as a high affinity binding protein for FX on the surface of PL membranes. It is possible that FVIII may also act as a binding protein for FX on the surface of stimulated platelets, thereby localizing FX to the platelet membrane and increasing the probability of FX activation. Studies detailed in the current chapter also examined binding of purified coagulation FX to human platelets in the presence of FVIII. Studies utilizing platelets within four hours from phlebotomy

indicated that, in the presence of FVIII, 202 ± 82 molecules of FX bound per platelet with a K_d of 51.4 ± 14.4 nM (Table 4.5). These data demonstrated that addition of FVIII did not significantly alter the number of molecules of FX bound per platelet or influence the affinity of binding during this time. After 4.5 hours from phlebotomy, the presence of FVIII restored the ability of collagen stimulated platelets to bind FX (Figure 4.1b). Thus, consistent with the data of chapter 3, FVIII could play a role as a binding protein for FX on the surface of platelets that had been prepared and stored for more than 4.5 hours before use.

From the studies detailed herein, it was found that FX can bind to stimulated platelets by more than one mechanism. Data obtained from FX-platelet binding studies, discussed above, demonstrated that platelets, which were prepared and used within 4.5 hours from collection, bound FX without addition of exogenous FVIII. Further binding studies performed in the presence of FVIII indicated that the addition of exogenous FVIII to the reaction mixture was required to obtain binding of FX to platelets that were prepared and stored for more than 4.5 hours before use. The original mechanistic hypothesis proposed that FVIII served as a binding protein for FX on the surface of PL-containing membranes. This hypothesis is supported by data derived from studies using simplified model systems, specifically PS and PS:PC membranes (Chapter 3), and is supported by results derived from present studies which used a physiological surface, namely the platelet plasma membrane. In addition, the existing data provide the basis for an alternative mechanism for binding. The ability of FX to bind independently of FVIII to platelets that had been stored for less than 4.5 hours, reveals that a heretofore unrecognized component of the platelet membrane may serve as a binding site for FX. This unidentified platelet component may represent a specialized zone of membrane lipids or, alternatively, a platelet membrane protein.

In Chapter 3, it was found that the K_d for binding of FX to synthetic PL

membranes was 234.3 ± 51.9 nM (Table 3.2). This K_d is significantly higher ($P<0.001$) than the K_d values observed for binding of FX to platelets in the presence (51.4 ± 14.4 nM) or absence (35.7 ± 5.2 nM) of FVIII (Table 4.5). If FX bound to the PL milieux of platelet membranes, then affinity constants of similar magnitude would have been expected. It has been shown that binding of FVIII, FIXa and FX to PL membranes requires the presence of acidic PLs, such as PS [64]. Therefore, if a specialized zone of membrane lipid constituted the platelet binding site for FX, one would expect to observe competition for binding between FVIII, FIXa and FX. Recently, it was demonstrated that both FVIII [37, 39] and FIXa [36] bound specifically to stimulated platelets. Results of the current report indicate that FX does not compete with FVIII for binding to stimulated platelets, as FVIII did not significantly increase or decrease the number of FX binding sites. Moreover, minimal (35%) competition between FIXa and FX (300 to 440-fold excess) for FIXa platelet binding sites has been reported [36]. Taken together, the above information indicates that FX likely bound to a non-PL component of the platelet membrane. Although addition of exogenous FVIII restored the ability of platelets to bind FX after 4.5 hours from phlebotomy (Figures 4.1a and 4.1b), it did not alter the number of molecules of FX bound per platelet. This may indicate that FVIII could bind to the same binding site as FX. Investigations are in progress in our laboratory to identify the putative platelet binding component for FX and to determine the molecular basis for the loss of FX binding activity during platelet storage.

Changes in platelet structure and function which occur during preparation and storage have been well documented in the literature [65-71]. For example, during storage, lesions occur which are the result of alterations in the platelet protein and lipid profiles. These changes may be caused by stimulation [65] or the action of proteases [68, 69] which may be the result of interactions with the collection and/or storage

media. In addition, modification of environmental parameters during storage, such as temperature [68], pH [66] and pO_2 [72], have been reported and may contribute to the induced platelet lesions. Some alterations have been linked to specific changes in platelet function [65, 73]. The observed decline in specific binding of FX after 4.5 hours post phlebotomy (Figure 4.1a) may be explained by changes in the platelet membrane caused by an as yet undefined platelet storage lesion.

Studies presented in section 4.3.3, which examined the influence of FVIII on the binding of FX to collagen stimulated platelets, demonstrated that FVIII was able to enhance binding of FX to platelets that had been stored from 4.5 to 7 hours from phlebotomy. These results confirm those obtained in chapter 3 which demonstrated that FVIII augmented binding of FX to PL membranes. It is possible that specific binding of FX to gel-filtered platelets that had been stored for shorter periods of time may have been due to the presence of endogenous FVIII that adsorbed to platelets during the isolation procedure. To investigate this possibility, electroblots of gel-filtered platelets were probed with an antibody directed towards FVIII-LC. As a control, an aliquot of platelet rich plasma was prepared and electrophoresed in parallel with a sample of gel-filtered platelets. The platelet rich plasma (20 μl) contained approximately 0.02 Units of FVIII and approximately 8 X 10^6 platelets. To increase the probability of detecting FVIII, the sample of gel-filtered platelets was adjusted to contain approximately 4 X 10^7 platelets, a quantity which was five-fold greater than the control. A band with a M_r of 80,000 was observed in the lane containing the platelet rich plasma which corresponds to the M_r of FVIII-LC. No bands were observed in the lane containing the gel-filtered platelets. Thus, considering the detection limit of the technique (5-100 pg), gel-filtered platelets were free of FVIII and specific binding of FX to platelets utilized within 2.5 to 4.5 hours from phlebotomy, was not likely due to the presence of FVIII.

It can be argued that the observed binding of FX may be due to activation of FX

to FXa during the assay procedure. Control studies using FXa were performed in parallel with the above, to determine whether activation of FX had occurred. Binding of FXa to stimulated platelets occurred at all times examined and the quantity of binding at each time interval did not differ significantly, whereas the pattern of binding of FX demonstrated a sharp decline after 4.5 hours from phlebotomy (Figure 4.1). Thus, FX and FXa demonstrated different binding patterns to platelets that had been stored for 2.5 to 7 hours before use (section 4.2.1 and section 4.3.6). Moreover, binding constants calculated from equilibrium binding studies, in which the association of FX or FXa with stimulated platelets was examined, were significantly different (Table 4.5). In addition, ^{125}I-FX bound to stimulated platelets exhibited an electrophoretic pattern consistent with that of purified ^{125}I-FX, not ^{125}I-FXa (Figure 4.4). These data, taken together, indicated that it was unlikely that FX was activated to FXa during the binding procedure.

Binding of FXa to platelets has also been examined by Dahlback and Stenflo [34] using bovine FXa and platelets and Miletich et al. [33] using human components. Their studies demonstrated that FXa bound to stimulated platelets with K_d values ranging from 0.03 nM [35] to 0.34 nM [34]. The number of molecules of FXa bound per platelet was reported to range from 200 [35] to 420 [34]. These values differ from those obtained in the current study (2268 ± 1105 molecules of FXa bound per stimulated platelet with a K_d of 307.8 ± 180.0 nM, Table 4.5). In addition, Miletich et al. [33] and Dahlback and Stenflo [34] did not observe specific binding of FX to stimulated platelets. However, there are a number of differences between the present and previous studies which may account for the conflicting results. These include the source of platelets, the agonist used to stimulate the platelets and the method used to separate bound from unbound ligand. The time from phlebotomy and platelet storage conditions may also have varied from those described herein. Without further information, it cannot be determined how or which procedural difference(s) account for the divergent

results observed between the previous and current studies.

Data reported by other investigators [33, 34] revealed that specific binding of FX to unstimulated platelets was not observed. As a negative control for the current studies, binding of FX to unstimulated platelets was also examined. In agreement with the earlier results, association of FX with unstimulated platelets was not observed.As a positive control for the equilibrium binding procedure, the binding of FIXa to platelets was re-examined. The K_d published by Ahmad et al. [36] (Table 4.4) for the interaction of FIXa with stimulated platelets is consistent (within two standard deviations) with the K_d of 2.9 ± 0.1 nM obtained in the present report (Table 4.5). In the current study, 262 ± 39 molecules of FIXa were observed to bind per platelet. The previously reported value exceeds the current value by almost two-fold (Table 4.2). Separation of platelet-bound and unbound ligand by centrifuging through 20% sucrose has been demonstrated to be more efficient than oil-based procedures [74]. Thus, results of present and past studies [36, 74] are consistent with a better separation of unbound and bound ^{125}I-FX by the sucrose centrifugation procedure.

In summary, current results demonstrated that FX bound specifically to collagen stimulated platelets. However, this binding was only observed when platelets had been isolated and used within 4.5 hours from phlebotomy, after this time specific binding of FX was not observed unless exogenous FVIII was added to the reaction mixture. Further characterization of the association of FX with platelets was accomplished using equilibrium binding techniques. These studies demonstrated that FX bound to collagen stimulated platelets with a K_d of 35.7 ± 5.2 nM. The presence of exogenous FVIII did not significantly influence the affinity of FX binding to platelets (K_d=51.4 ± 14.4 nM) nor did FVIII significantly alter the quantity of FX bound per platelet (202 ± 82 molecules in the presence of FVIII versus 202 ± 180 molecules in the absence of FVIII).

It is unlikely that the observed specific binding of FX to stimulated platelets was due to the presence of endogenous FVIII or activation of FX to FXa since gel-filtered platelets were found to be devoid of adsorbed FVIII and FX was shown not to be activated to FXa during the binding procedure. Binding of FX to platelets occurred with a significantly higher affinity than binding of FX to synthetic membranes comprised of purified phospholipids (a K_d of 35.7 $\pm$ 5.2 nM versus a Kd of 234.3 $\pm$ 51.9 nM, respectively, $p<0.001$). These data suggest that binding of FX to the platelet surface may have occurred by a mechanism which differs from that involved in binding of FX to synthetic phospholipid membranes. Further research should be directed towards the identification of this specific FX-binding platelet membrane component.

4.5.0 References

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CHAPTER 5

CONCLUSIONS AND GENERAL DISCUSSION

Chapter 5: Conclusions and General Discussion

5.1.0 Conclusions and General Discussion

Upon commencement of the studies described in the previous chapters, the most widely accepted model of the FX activating complex pictured membrane-bound FIXa and FX interacting with each other as well as with membrane-bound FVIII (Figure 1.2). This model was based on information obtained from investigations in which the FX activating complex was reconstituted in vitro from purified components [1]. The data presented herein, are in general agreement with this model, however, the current studies have extended the knowledge of the structure of the FX activating complex. Figure 5.1 presents a revised model which incorporates the novel findings of the thesis. These results and their significance are described in further detail below.

Data obtained from studies examining the association of FVIII, FIXa and FX with PL (Chapter 2) demonstrated that hydrophobic forces played a heretofore unrecognized role in the association of PLV with FIXa or FX. These results demonstrate that binding of FIXa and FX to PL membranes involves more than formation of divalent cation bridges.

It is generally agreed that FVIII participates in multiple interactions within the FX-activating complex. FVIII not only interacts with PL, but also associates with FIXa and FX (Figure 1.2). As a first step to identifying the sequences of FVIII that are essential to maintain the structure and function of the FX activating complex, we have examined which chain(s) of FVIII contain binding domains for FIXa and FX and have defined the molecular forces that govern these interactions (Chapter 3). Both chains of FVIII (FVIII-LC and FVIII-HC), were found to participate in the binding of FVIII to FIXa. Binding of FVIII-LC to FIXa was observed to be mediated by hydrophobic forces, whereas divalent cation-dependent forces mediated binding of FVIII-HC to FIXa. Ligand affinity chromatographic studies performed in the presence of PL,

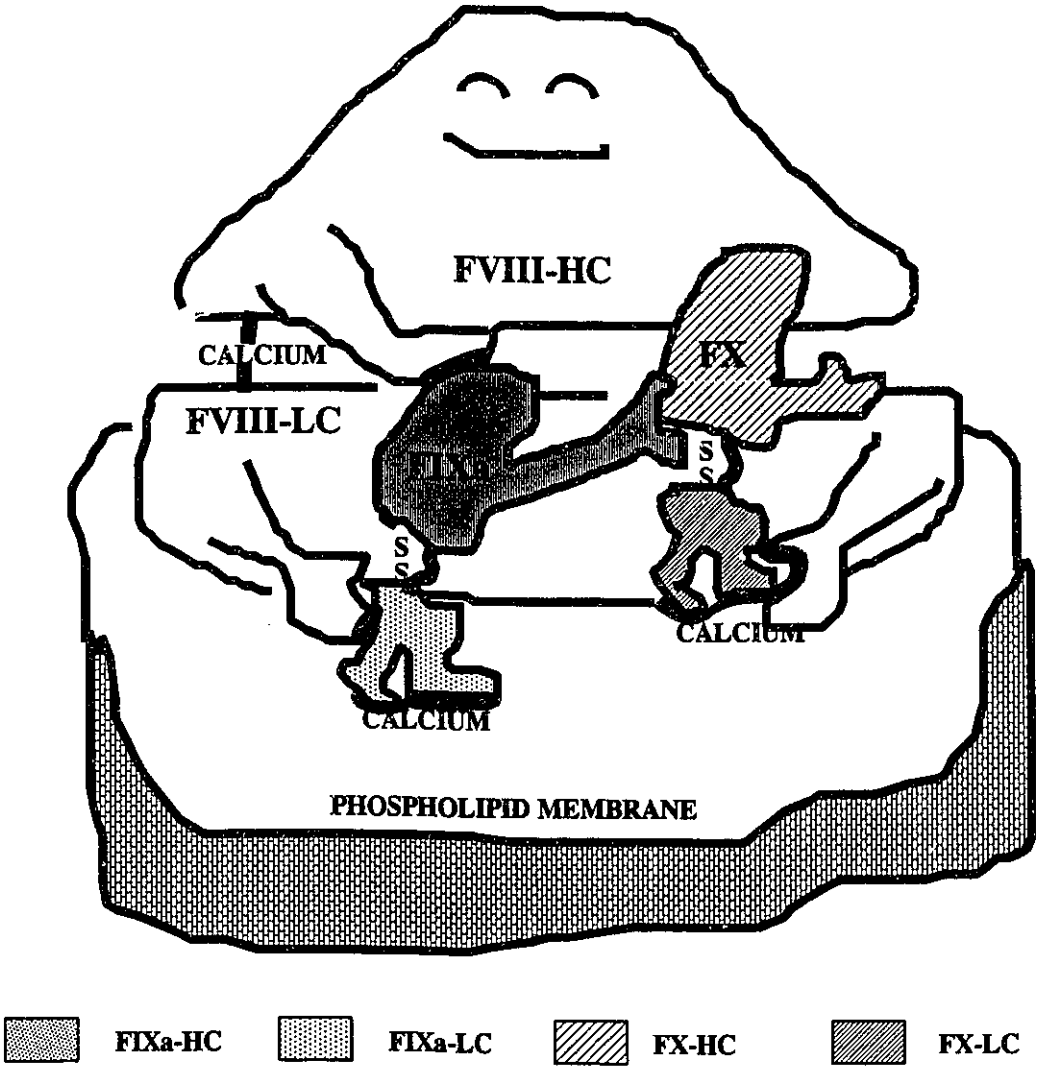

Figure 5.1. Schematic model of the structure of the FX activating complex which incorporates novel findings of the current study. In the present model, FVIII-LC interacts with both FIXa and FX, however, FVIII-HC interacts only with FIXa. Previously it was reported that FVIII interacts with both FIXa-LC [2] and FIXa-HC [3]. The catalytic subunit of FIXa (FIXa-HC) must bind to FX-HC, as the activation peptide, located within FX-HC, must be removed for the serine protease domain of FX to function. Whether FVIII binds to FX-LC or FX-HC remains to be defined. It is speculated that FVIII binds to FX-LC. Hydrophobic associations are shown in red, while electrostatic interactions are shown in blue.

demonstrated that FX bound hydrophobically to FVIII-LC, however, FVIII-HC was not involved in this association. These results agreed with those of radioligand binding studies in which it was found that the affinity of FX for PL-bound FVIII-LC (28.5 ± 3.3 nM) was not significantly different from that for PL-bound native FVIII (23.1 ± 4.4 nM). Binding of FX to PL was observed to occur with a K_d of 234.3 ± 51.9 nM. Thus, FX bound to PL membranes with a significantly greater affinity in the presence of FVIII or FVIII-LC ($p<0.001$), demonstrating that FVIII serves as a high affinity binding protein for FX on the surface of PL membranes. The mechanism by which FVIII exerts its cofactor effect has not been delineated. These data suggest that the cofactor effect of FVIII is the culmination of a number of discrete interactions with other components of the FX activating complex.

The above observations led to the hypothesis that FVIII may act as a binding protein for FX on physiological membranes, such as stimulated platelets. Binding in this manner would localize FX to the platelet membrane, thereby increasing the probability of FX activation. Data of Chapter 4 demonstrated that FX bound to stimulated platelets by two mechanisms: one which relied upon FVIII and one which was FVIII-independent. Furthermore, the mechanism of binding depended on the duration of storage. During the first 4.5 hours, the affinity (K_d of 35.7 ± 5.2 nM) and quantity (202 ± 180 molecules of FX per platelet) of FX bound to stimulated platelets was not significantly altered by the presence of FVIII (K_d of 51.4 ± 14.4 nM, 202 ± 82 molecules FX per platelet). After this time, binding of FX to platelets was FVIII-dependent, indicating that FVIII acted as a binding protein for FX.

The conclusions derived from Chapter 4 indicate that an as yet to be identified, component of platelet membranes forms a very labile, high affinity binding site for FX. The existence of binding proteins for coagulation factors has only recently been

proposed. Prior to commencing the current work, it was believed that the multi-protein enzyme complexes involved in the coagulation cascade formed on the PL component of cellular membranes. The PL binding theory was substantiated by the ability of synthetic PLV to support coagulation. The role and importance of receptors or binding proteins in the process of coagulation is unknown. However, it has been reported that a defect in blood coagulation observed in Scott syndrome may be due to a deficiency in the platelet receptor for FVIII [4]. The model presented in Figure 5.1 was therefore revised to depict the involvement of platelet membrane proteins in formation of the FX activating complex (Figure 5.2).

Thrombus formation at the site of vascular injury requires activation of the coagulation cascade and stimulation of platelets. For progression of the coagulation cascade and thus initiation of thrombus formation, thrombin must be generated to promote activation of FVIII as well as other coagulation proteins. To date, the exact mechanism by which thrombin is formed in the initial phase of coagulation has not been delineated. In contrast, the methods by which platelets are stimulated have been well documented. Stimulation induces numerous changes in platelet structure including reorganization of the platelet cytoskeleton. Many trans-membrane proteins are connected intracellularly to the cytoskeleton. Thus, cytoskeletal reorganization not only results in pseudopodia extension and retraction, but also redistribution of membrane proteins such as that involved in pino- and exocytosis and capping [5]. It is speculated that stimulated platelets are intimately involved in the initial production of thrombin. Specifically, it is possible that platelet receptors or binding proteins for FVIII, FIXa and FX are required in the initial stages of coagulation to promote rapid formation of thrombin which in turn enables rapid amplification of the coagulation cascade reactions.

Coagulation proteins, such as those of the FX activating complex, may bind

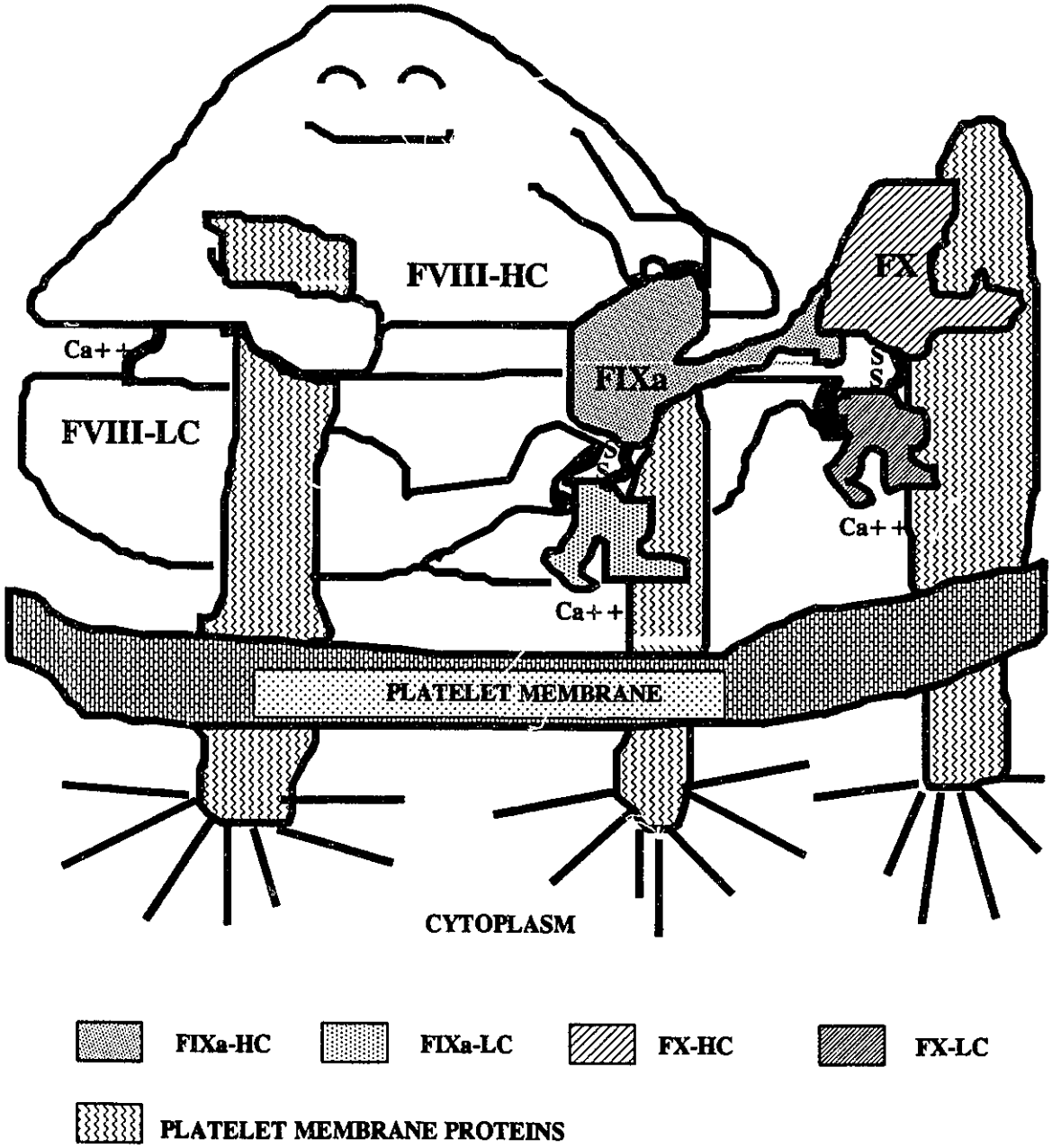

Figure 5.2. Schematic model of the FX activating complex as it may form on the surface of platelets. Data from the present and previous studies indicate that FVIII [6], FIXa [7] and FX binds to proteins exposed on the surface of stimulated platelets. Hydrophobic associations are shown in red, while electrostatic interactions are shown in blue.

directly to their respective receptors or binding proteins on unstimulated platelets or bind to stimulated platelets in a biphasic reaction. In an initial phase, the proteins are rapidly concentrated on the surface of platelets by interaction with PL and in a second phase, they diffuse on the membrane surface until they encounter high affinity specific platelet membrane receptors. In vitro, activation of FX by FIXa and FVIII in the presence of synthetic PLV has been demonstrated to occur. Similar activation of FX by platelet-bound FVIII and FIX likely occur in vivo. It is conceivable that stimulation causes expression of the receptors and that reorganization of the platelet cytoskeleton induced by activation causes the receptors for FVIII, FIXa and FX to be brought together on the membrane surface. Assuming that the receptors are occupied by FVIII, FIX and FX, then production of FXa and subsequent production of thrombin by FXa, FV(a) and prothrombin (the prothrombinase complex of blood coagulation) will occur, thus providing a secure and rapid process of thrombin production.

An inherited or congenital lack of receptors may not cause a clinically obvious disease state, as thrombin generation could still occur, albeit after a time lag, resulting in delayed clotting times. This slower, second wave of thrombin formation, which likely represents the major source of production, is dependent upon PL-bound FVIII, FIX and FX diffusing on the membrane surface of stimulated platelets to form FXa-generating multi-protein enzymatic complexes as described by the "classical" model of coagulation. Further research should clarify these issues.

5.2.0 References

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APPENDIX A

CURRICULUM VITAE

NAME: Judith Sharron Atkins

DATE OF BIRTH: May 19, 1961
PLACE OF BIRTH: California, USA
CITIZENSHIP: Canadian

SUMMARY OF EDUCATION

University of Toronto (Honours)	1980-1984	Bachelor of Science Specialties: Biochemistry & Nutrition
University of Alberta	1984-1987	Master of Science

Department of Home Economics (Nutrition & Metabolism)
Supervisor: Dr. M.T. Clandinin
Thesis Title: Carnitine Dependent Fatty Acid Transport

University of Ottawa	1987-1992	Doctor of Philosophy

Department of Biochemistry
Supervisor: Dr. P.R. Ganz
Thesis Title: Structural Studies of the Factor X Activating Complex of the Intrinsic Pathway of Blood Coagulation

Ontario Cancer Institute	1992-	Fellowship

Department of Medicine
Supervisors: Drs. Minden and McCulloch

SCHOLARSHIPS AND AWARDS

(a) Ontario Scholar
Sponsored by the Government of Ontario. Awarded for study at a post secondary institution in June of 1980 for the 1980-81 academic year.

(b) MacLachlan Sisters Memorial Scholarship
Sponsored by University College, University of Toronto. Awarded for studies at University of Toronto in July of 1982 for the 1982-83 academic year.

(c) Graduate Research Assistantship
Sponsored by the University of Alberta. Awarded for graduate studies at the University of Alberta in December 1986 for January to April of 1987.

(d) The Canadian Red Cross Research Award
Sponsored by the Canadian Red Cross. Awarded for graduate research at the Canadian Red Cross (Ottawa Centre) in May of 1987 for June 1987 to May 1988.

(e) The Canadian Red Cross Research Award
Sponsored by the Canadian Red Cross. Awarded for graduate research at the Canadian Red Cross (Ottawa Centre) in May of 1988 for June 1988 to May 1989.

(f) David Rae Memorial Fellowship
Sponsored by the Leukaemia Research Fund. Awarded for Post-doctoral research at the Ontario Cancer Institute in June of 1991.

TEACHING AND RESEARCH EXPERIENCE

(a) Graduate Teaching Assistant
Academic years 1989/90, 1988/89 and 1987/88. University of Ottawa, Department of Biochemistry. Courses: The second year introductory biochemistry laboratory course for biochemistry students and the third year introductory biochemistry laboratory course for biology students.

(b) Graduate Teaching Assistant
September to December, 1989. University of Ottawa, Department of Biochemistry. Course: The fourth year metabolism laboratory course.

(c) Graduate Teaching Assistant
September to December, 1986. University of Alberta, Department of Home Economics. Course: Third year nutrition.

(d) Summer Research Student
May to August, 1984. The Canadian Red Cross (Ottawa Centre). Project Title: DEHP Incorporation into Red Blood Cell Plasma Membrane and the Effects of DEHP on Arachidonic Acid Metabolism.

(e) BIO110 Multi-Media Laboratory Supervisor
Academic years 1983/84, 1982/83 and 1981/82. The University of Toronto, Department of Biology. Course: First year introductory biology.

PUBLICATIONS

Thesis:

(a) Atkins JS, (1992). Structural Studies of the Factor X Activating Complex of the Intrinsic Pathway of Blood Coagulation, University of Ottawa Press.

(b) Atkins JS, (1987). Carnitine Dependent Fatty Acid Transport, University of Alberta Press.

Papers and abstracts:

(1) Atkins JS, Rock G, Ganz PR, (1988). Factor VIIIa and Factor X Interactions in the Presence of Phospholipid. Blood 72:289.

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(9) Ganz PR, Palmer DS, Rowe D, Atkins JS, Rose D, Dudani A, (1992). Human FVIII: A Calcium Metalloprotein. (in preparation)

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