STRUCTURAL-FUNCTIONAL STUDIES OF THE NON-ENZYMATIC DOMAINS OF PROTHROMBIN

by

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ABSTRACT

To study the role of the non-enzymatic domains of the clotting protein prothrombin, human prothrombin and several variants were produced by using recombinant DNA techniques and *in vitro* tissue culture expression. The prothrombin variants included the first kringle domain deleted (rΔK1), the second kringle domain deleted (rΔK2), both kringle domains deleted (rΔK1/ΔK2), the second kringle domain substituted with the bovine counterpart (rhBK2), and the second kringle domain substituted with the first kringle (rK1/K1). The recombinant proteins were expressed by using the pNUT-baby hamster kidney cell system under methotrexate selection. The expressed proteins were purified from the media using barium citrate precipitation followed by fast performance liquid chromatography (FPLC). Different gamma-carboxylated recombinant proteins were resolved by pseudo-affinity FPLC using a calcium gradient. The expressed recombinant proteins subjected to several characterization assays including Gla content analysis by capillary electrophoresis-laser induced fluorescence (CE-LIF), calcium and phospholipid binding assays, and activation assays by factor Xa and by the prothrombinase complex using purified systems. Results from the studies have indicated that a reduced Gla content in the protein, by as little as three residues, led to a substantial loss of calcium dependent phospholipid binding, and a reduced clotting ability. Results from the activation assays of the prothrombin variants have demonstrated that the second kringle domain was necessary for the activation of prothrombin by both the protease factor Xa alone and by the prothrombinase complex, implying important interactions of the second kringle with factor Xa and the cofactor Va. In addition, peptides derived from the loops of the second kringle domain were demonstrated to have potent inhibitory activity in the activation of prothrombin by the prothrombinase complex. Taken together the study has demonstrated that the non-enzymatic domains of prothrombin play important roles in the ability of prothrombin to be activated physiologically. The Gla domain is responsible for calcium and phospholipid binding, the kringle domains mediate protein-protein interactions with the protease factor Xa and cofactor Va of the prothrombinase complex.

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LIST OF ABBREVIATIONS

A	absorbance
Ab	antibody
Amp	ampicillin
AP	alkaline phosphatase
APC	activated protein C
APTT	activate partial thromboplastin time
AT	antithrombin (previously called antithrombin III)
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BHK	baby hamster kidney
bis	N,N'-methylenebisacrylamide
bp(s)	basepair(s)
BS	BlueScript
BSA	bovine serum albumin
BSSP-3	brain-specific serine protease-3
Ca^{2+}	calcium (II) ion
CE	capillary electrophoresis
cDNA	complementary deoxyribonucleic acid
CRS	γ-carboxylation recognition site
DFHR	dihydrofolate reductase
dH_2O	deionized water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DODAC	N,N-dimethyl-N,N-di-9-cis-octadecenylammonium chloride
DOPE	1,2-dioleoyl-sn-phosphatidylethanolamine
dsDNA	double stranded DNA
EDTA	ethylene-diamine-tetra-acetic acid

EGF	epidermal growth factor
EOF	electroosmotic flow
EIPCR	enzymatic inverse polymerase chain reaction
ER	endoplasmic reticulum
EtOH	ethanol
F1	prothrombin fragment 1
F2	prothrombin fragment 2
FBP	ferric binding protein
FII	prothrombin (factor II)
FITC	fluorescein isothiocyanate
FPLC	fast performance liquid chromatography
FV(a)	factor V (activated)
FVIII(a)	factor VIII (activated)
FIX(a)	factor IX (activated)
FX(a)	factor X (activated)
Gla	γ-carboxyglutamic acid
Glu	glutamic acid
HEPES	N-2-hydroxyethylpiperazine-N-2-ethansulfonic acid
HGF	hepatic growth factor
HK	high molecular weight kininogen
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
IgG	immunoglobulin G
IPTG	isopropylthiogalactoside
kDa	kilodaltons
Klenow	*E. coli* DNA polymerase (large fragment)
LB	Luria broth
LIF	light induced fluorescence
LOD	limit of detection
mRNA	messenger ribonucleic acid
NBS	newborn bovine serum

NBT	nitro blue tetrazolium
NMR	nuclear magnetic resonance
MT	metallothionein
MTX	methotrexate
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAR-1	protease activated receptor-1
PBS	phosphate-buffered saline
PCPS	phosphatidylcholine:phosphatidylserine
PCR	polymerase chain reaction
PDA	photo diode array
PEG	polyethylene glycol
phFII	human plasma-derived prothrombin
PPACK	D-PheProArgchloromethyl ketone
pre-2	prethrombin-2
PT	prothrombin time
rhFII	human recombinant prothrombin
RT	room temperature
S-2238	D-Phe-L-pipecolyl-L-Arg-p-nitroalilide-dihydrochloride
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
ssDNA	single stranded DNA
SV40	Simian Virus 40
TBST	20 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.5 % Tween-20 buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TF(s)	tissue factor (soluble)
TFPI	tissue factor pathway inhibitor
TM	thrombomodulin
tPA	tissue type plasminogen activator
Tris	tris(hydroxymethyl)aminomethane
uPA	urokinase
UV	ultraviolet

Vh	heavy chain of factor V
Vl	light chain of factor V
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER 1

OVERVIEW

I. THE COAGULATION SYSTEM

Damage to blood vessels leads to blood loss. Fortunately humans do not bleed to death or suffer heavy blood loss because of a physiological process called blood coagulation. Blood coagulation is a host defense mechanism along with other haemostatic responses in the inflammatory and repair systems, and helps to protect, repair and maintain the integrity of the vascular system. Coagulation involves a sequential series of complex interactions between circulating plasma protease zymogens, enzymes and cofactors, platelets, and the vascular endothelial cells. Abnormality in any of these key components can result in serious complications resulting in excessive bleeding (hemorrhage) or unwanted clotting (thrombosis). The system is normally quiescent or is dampened by other regulatory systems to maintain a balanced haemostasis. Within seconds of tissue injury or a triggering event, the coagulation system becomes activated, immediately leading to the formation of a cellular plug from the adhesion and aggregation of circulating platelets, followed by the generation of an insoluble fibrin network.

In 1964, the events of coagulation were elegantly organized into a series of reactions termed the "waterfall" or "cascade" hypothesis by Davie and MacFarlane, respectively (Davie et al., 1964; MacFarlane, 1964). According to these hypotheses, coagulation could be initiated via the "intrinsic" pathway, so named because all the components are present in blood, or by the "extrinsic" pathway in which the subendothelial cell membrane protein tissue factor (also called thromboplastin) is required in addition to circulating components. These earlier works suggested that two independent pathways could initiate the coagulation system; however, recent studies have suggested a revised coagulation theory bringing the components from both pathways together (Rock et al., 1997). The extrinsic pathway initiates coagulation, and the intrinsic components are mainly responsible for the propagation, amplification and regulation of the clotting process.

1. The extrinsic pathway

Historically, it was observed that plasma could be induced to clot when exposed to an exogenous agent called thromboplastin such as a lipoprotein extract from rabbit brain. Thus the term "extrinsic" pathway was coined. This factor has now been identified as tissue factor

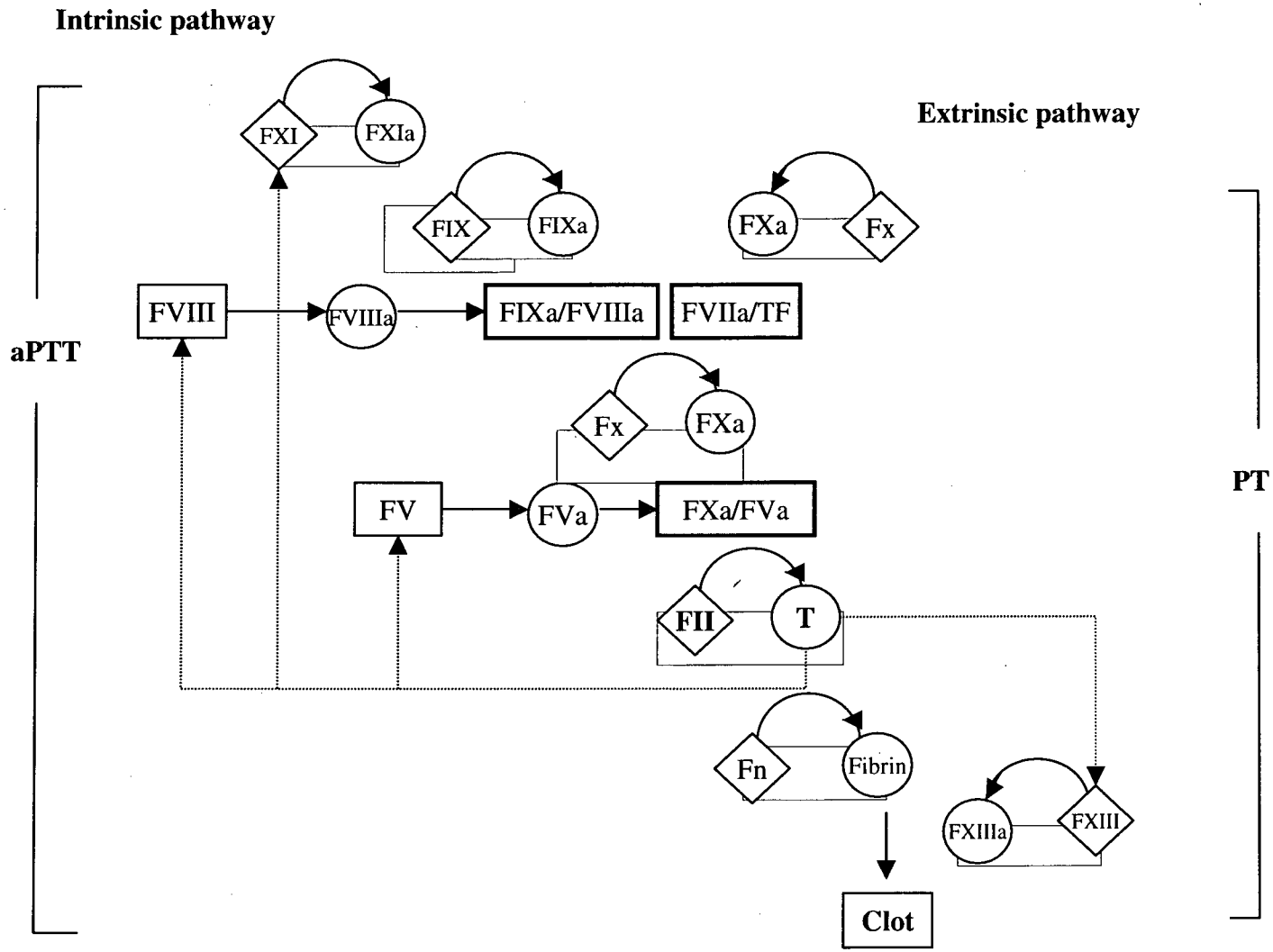
(TF), a glycoprotein constituitively expressed on the surface of fibroblasts within and around blood vessels. Tissue factor, or factor III, is also found on a variety of other stromal and tissue cells located throughout the body to protect against bleeding after tissue injury. The extrinsic pathway can be measured by the clinical assay called the prothrombin time (PT), in which the coagulation process is initiated when small amount of circulating factor VIIa, a blood clotting enzyme, binds to added tissue factor; *in vivo*, the tissue factor would be released as a result of tissue damage or an inflammatory process (Figure 1). The formation of the factor VIIa:TF complex leads to the activation factor X and subsequently to the generation of a fibrin clot through the formation of the protease thrombin (for a review, see Davie et al., 1991).

2. The intrinsic pathway

In the intrinsic pathway, so named because all the components are present in blood, the exposure of blood *in vitro* to a negatively charged surface such as glass, kaolin or celite powder leads to clotting, as measured by the activated partial thromboplastin time assay (aPTT). Activation of the pathway involves three components, factor XII, high molecular weight kininogen, and prekallikrein. These three components are grouped together as the "contact system" because they require contact with artificial, negatively charged surfaces for zymogen activation *in vitro*; thus the name contact activation is sometimes referred in place of the intrinsic pathway. This contact complex activates factor XI to XIa that activates factor IX (Christmas factor) to IXa. Factor IXa, in the presence of cofactor VIIIa, calcium, and phospholipid, activates factor X to Xa, and it is at this juncture that the intrinsic pathway shares a common pathway with the extrinsic pathway (Davie et al., 1991). In the common pathway, factor Xa, in the presence of cofactor Va, calcium, and phospholipid, converts prothrombin to the serine protease thrombin. Thrombin proteolytically cleaves plasma fibrinogen to fibrin monomers. These monomers polymerize spontaneously to form a tough, insoluble fibrin network that is subsequently cross-linked by factor XIIIa to strengthen the clot further (Figure 1).

The proposed theory of the coagulation process and the developed clinical assays (the aPTT and PT tests) have proven invaluable for identifying coagulation factors and their deficiencies in the diagnosis of hemorrhagic diseases. For a while, the catastrophic bleeding

Figure 1. The classical coagulation pathways. The activated partial thromboplastin time (aPPT) assay measures the activity of the intrinsic pathway involving the blood clotting proteins: factor XI (FXI), factor IX (FIX), factor X, prothrombin (FII), and the cofactors VIIIa and Va (FVIIIa, FVa). The prothrombin time (PT) assay measures the activity of the extrinsic pathway involving factor VII (FVII), factor X, thrombin and the cofactor tissue factor (TF). The two pathways merge at the formation of thrombin (T), which activates fibrinogen (Fn) to fibrin. Factor XIII (FXIII) cross-links the fibrin network into insoluble clot.


Intrinsic pathway
Extrinsic pathway
FXI
FXIa
FIX
FIXa
FXa
Fx
FVIII
FVIIIa
FIXa/FVIIIa
FVIIa/TF
aPTT
Fx
FXa
FV
FVa
FXa/FVa
PT
FII
T
Fn
Fibrin
FXIIIa
FXIII
Clot

caused by patients with hemophilia A and B, (deficiency in factors VIII and IX of the intrinsic pathway, respectively), led investigators to believe the intrinsic pathway to be the primary pathway. Only recently has there been a resurgence of the role of the extrinsic pathway, particularly from the characterization of tissue factor, leading to recognition of the prominent role of tissue factor in blood clotting. As a consequence, there are two main arguments for a revision of the coagulation process. Firstly, although it has been noted that hemophiliacs A and B do bleed excessively, there is a striking difference between the milder bleeding of factor XI deficiency (hemophilia C) and the severe bleeding of factor IX. This observation suggests that the clotting reactions can bypass factors XII and factor XI in the activation of factor IX *in vivo*. In contrast, patients with factor VII deficiency bleed excessively (Ragni et al., 1981; Triplett et al., 1985). Secondly, patients with deficiency in any one of the "contact" factors have a prolonged aPTT time, but are entirely asymptomatic to bleeding disorders (although John Hageman, after whom factor XII was named, died of pulmonary emboli). Factor XIIa can activate coagulation, but only *in vitro* and furthermore, recent studies have shown other roles of the contact system different from coagulation (Kaplan et al., 1997).

3. The contact system

For many years, there was an extensive search for the physiological equivalent of the negatively charged surface and its role in the pro-coagulant system. Studies of the contact system in recent years have shown that the contact system actually assembles on endothelial cell membranes in a zinc-dependent fashion, but may play an anti-coagulant role (for a review, see Kaplan, 1997). The assembled contact complex leads to the activation of prekallikrein (Fletcher factor) by an endothelial metalloprotease(s), independent of factor XII and its activated form. As a result, the complex assembly leads directly to the formation of kininogen that leads to antithrombin and fibrinolytic activity (Schmaier, 1997). High molecular weight kininogen (HK) has six domains organized into a heavy chain (domains one to three), bradykinin (domain four), and a light chain (domains five and six). The amino terminal end of bradykinin has α-thrombin inhibitory activity, and could thus be a potent inhibitor of thrombin-dependent platelet activation (Kaplan et al., 1997). A mechanism has been postulated that a certain peptide sequence of bradykinin, RPPGF, and HK itself, prevent

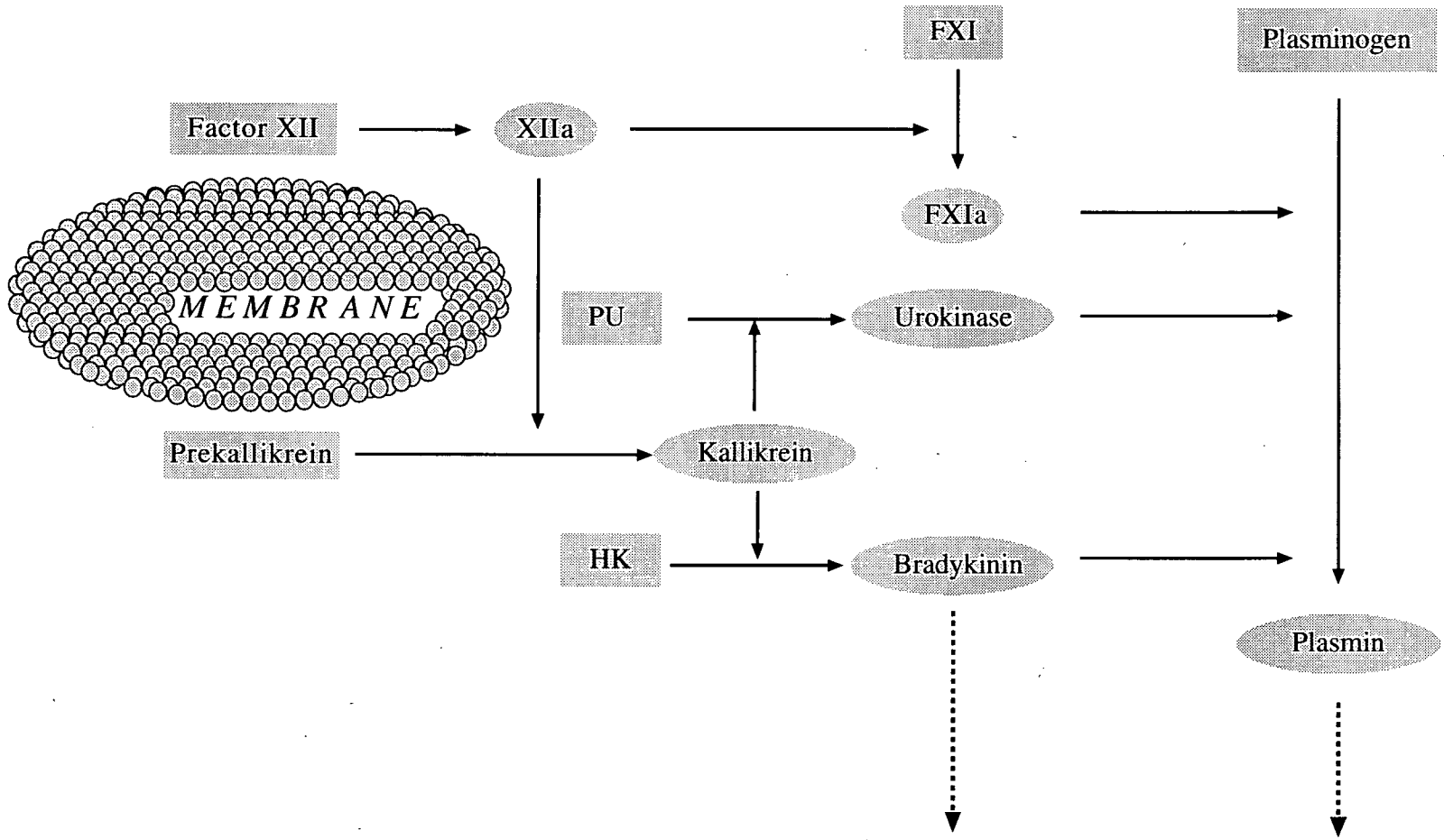
α–thrombin from cleaving the thrombin receptor between the arginine and serine site (Hasan et al., 1996). HK domain two can also inhibit calpain-related platelet aggregation. When α-thrombin activates platelets, cytosolic or internal membrane associated calpain is translocated to the surface. Calpain has proteolytic activity to glycoproteins necessary for platelet aggregation (Puri et al., 1987). In addition, HK and its associated proteins also participate in cellular fibrinolysis.

It has been observed for sometime that kallikrein, factor XIIa, and factor XIa can cleave plasminogen to plasmin, which is the main physiological enzyme responsible for dissolution of the fibrin plug. Recent studies have shown that when HK binds to prekallikrein on endothelial cells, the zymogen prekallikrein becomes activated to kallikrein by a calcium dependent metalloprotease(s), totally independent of factor XIIa (Motta, 1995). Other studies suggest that kallikrein activates the single chain urokinase, a potent plasminogen activator (Hauert et al., 1989). Taken together with the observation that a factor XII-dependent pathway leads to the conversion of plasminogen to plasmin (Iatridis, 1962), these new findings indicate that the contact system not only prevents platelet activation and aggregation, crucial events prior to coagulation, but also plays an important role in the activation of the fibrinolysis system through the direct activation of plasminogen (Figure 2). As such, the contact system is now believed not to play any role in the initiation of coagulation through the intrinsic pathway. A revised theory of coagulation has now been proposed.

4. The revised coagulation pathway or the tissue factor pathway

Recent rediscoveries of tissue factor, its physiological inhibitor, new information about the activation of factor XI, and more understanding about the role of the contact system, have led to the proposal of a revised coagulation theory termed the tissue factor pathway. Clotting is initiated by the exposure of subendothelial tissue factor to circulating factor VIIa. The VIIa:TF complex proteolytically activates limited amounts of factors X and factor IX. While some factor Xa continues to act as a pro-coagulant through prothrombin activation, other Xa binds to tissue factor pathway inhibitor (TFPI) and leads to feedback inhibition of the VIIa:TF complex. To sustain the coagulation process, additional Xa production must occur through the action of IXa in concert with its cofactor VIIIa.

Figure 2. The contact system and fibrinolysis. The contact system is comprised of factor XII (FXII), high molecular weight kininogen (HK), and prekallikrein. The contact system activates factor XI (FXI) to factor XIa (FXIa), pro-urokinase (PU) to urokinase, prekallikrein to bradykinin thereby activating plasminogen to plasmin. Plasmin is the major protease responsible for fibrinolysis. Bradykinin also activates components of the inflammation system.

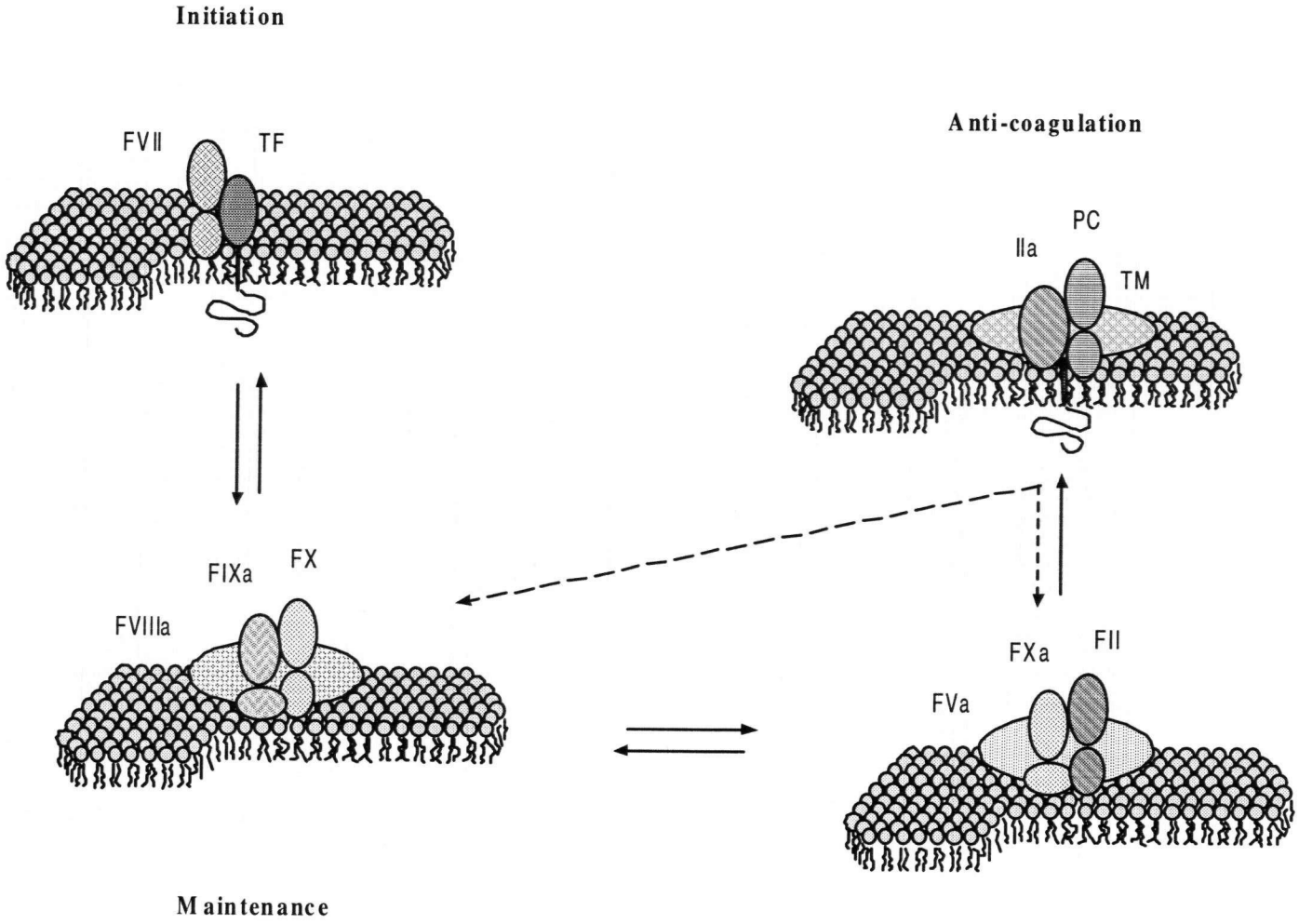

FXI
Plasminogen
Factor XII
XIIa
FXIa
MEMBRANE
PU
Urokinase
Prekallikrein
Kallikrein
HK
Bradykinin
Plasmin
CONTACT SYSTEM
INFLAMMATION
FIBRINOLYSIS

Thrombin-mediated activation of factor XI also leads to the generation of IXa to supplement the cascade. In essence, the tissue factor coagulation pathway is composed of three distinct, interactive phases: TF-dependent initiation, positive feedback maintenance of the coagulation cascade, and finally negative feedback regulation of the coagulation cascade (Figure 3).

4.1. Tissue factor dependent initiation of coagulation

The formation of circulating factor VIIa and exposed tissue factor is the first crucial step in the initiation of clotting. Tissue factor belongs to the cytokine receptor family, with 219 amino acid residues in the extracellular domain, 23 transmembrane residues, and 21 intracellular domain residues. The crystal structures of a factor VIIa-soluble tissue factor (sTF) complex (a truncated form of tissue factor without the transmembrane domain and the cytosolic domain) has been solved (Banner et al., 1996). The complex structure is shaped like a question mark with the factor VIIa catalytic domain and the amino-terminal end of TF on the top rim, and factor VIIa light chain embracing the curved stem of sTF. The Gla domain of factor VIIa extends to the carboxy terminal end of sTF and would probably anchor it to the phospholipid membrane through calcium ions. The two EGF domains of factor VIIa provide the most and the strongest hydrophobic contact to sTF. The interaction in the complex is an allosteric one resulting in a change in the conformation of the protease domain of factor VIIa, now ready for substrate contact and proteolytic activity. The factor VIIa:TF complex leads to the activation of more factor VIIa, and most importantly factor Xa and factor IXa leading to the propagation of the coagulation cascade. However, questions remain in the triggering event for the very first activation of circulating factor VII to its active form VIIa. It has been shown that a minute quantity of factor VIIa always exists in plasma and this could trigger initial VIIa:TF-catalyzed generation of Xa and IXa when exposed to extravascular TF (Wildgoose et al., 1992). In fact, assays have been developed to measure the level of factor VIIa in plasma by using variant forms of TF, such as the soluble form (Fong et al., ; Morrissey et al., 1993; Neuenschwander et al., 1992). In addition, other studies have shown that factor VII can also be activated by a putative membrane-associated serine protease called hepsin, in a calcium dependent manner (Kazama et al., 1995). Thus once the formation of the first few VIIa:TF complexes are established, more factor VII:TF complexes are activated providing an initial burst of active VIIa leading to activation of factor IX and

Figure 3. The revised coagulation pathway and the coagulation complexes. Initiation of coagulation is through the tissue factor:factor VII complex (TF:FVII). Propagation of coagulation is maintained by the tenase complex of factor IXa:factor X:cofactor VIIIa (FIXa:FX:FVIIIa) and by the prothrombinase complex of factor Xa:prothrombin:cofactor Va (FXa:FII:FVa). The anti-coagulation complex is composed of thrombin:activated protein C:cofactor thrombomodulin. All complexes require negatively charged phospholipid membrane surface and calcium ions at physiological concentration.


Initiation
FVII
TF
Anti-coagulation
PC
IIa
TM
FIXa
FX
FVIIIa
FXa
FII
FVa
Maintenance

factor X. The formation of factor Xa is the next important step in the cascade as factor Xa is the protease responsible for the activation of prothrombin to its active enzyme thrombin. Thrombin is the primary protease responsible for the formation of the fibrin network, as well as the maintenance and magnification of the subsequent clotting events.

4.2. Maintenance of coagulation

There are two main complexes involved in the amplification of the clotting cascade, the tenase complex and the prothrombinase complex. Each complex is made up of a protease, its substrate, calcium ions, phospholipid membrane, and a cofactor. In the tenase complex, the enzyme factor IXa, in the presence of its cofactor, factor VIIIa, converts the substrate factor X into factor Xa. The protease Xa, in the presence of the cofactor, factor Va, in turn activates prothrombin to thrombin (Figure 3). Thrombin cleaves fibrinogen to form the fibrin clot. The cofactors play very crucial roles in the amplification of the protease activity in these complexes, thus ensuring a rapid response once initiation is activated.

There are several positive feedback mechanisms for the sustained process of coagulation. Firstly, factor Xa has been shown to activate factor VIIa (Radcliffe et al., 1976). Due to several regulatory mechanisms (that will be discussed below), factor VIIa:TF activity can not be prolonged, thus the factor Xa-dependent activation of factor VIIa is probably significant. Secondly, thrombin plays the primary role for the amplification and maintenance of the clotting cascade mainly through the two main coagulation complexes mentioned above. In the presence of cofactors HK and or prothrombin, thrombin has been shown to activate factor XI on negatively charged surfaces and on platelet surfaces (Baglia et al., 1998; Naito et al., 1991). Factor XIa is the main activator of factor IXa in the tenase complex, leading to more Xa formation, and subsequently, to more thrombin formation. Thrombin has been characterized to be the main activator of the essential cofactors, factor VIII and factor V (Butenas et al., 1997). In addition, thrombin is also responsible for the activation of factor XIII to XIIIa, the agent instrumental in the cross-linking of fibrous strands in the fibrin network. The formation of thrombin, then, is the most important step for the dynamic magnification and prolonged sustenance of the coagulation process once initiation is activated.

Negatively charged surfaces play a crucial role at all times during coagulation. Endothelial- and nonvascular cell surfaces, and activated platelet surfaces have been characterized as the main providers for this function. The tenase and prothrombinase complexes absolutely require a membrane surface for optimal activity, as well the initial formation the factor VIIa:TF complex. Receptors for factors VIIIa, Va, and thrombin have been found on activated platelet surfaces (Nesheim et al., 1988b; Sims et al., 1988). Thrombin is a major contributor to platelet activation and aggregation through its proteolytic activity on the thrombin receptor (Vu et al., 1991). Activated and aggregated platelets play an integral part in the formation of the cellular plug at the site of injury, as well as stimulating the repair and inflammation responses.

The coagulation process is, therefore, sustained and amplified so that within minutes of tissue injury, a platelet-fibrin network is formed to prevent bleeding and to maintain vascular integrity. On the other hand, the cascade must also be vigorously regulated if clotting is to be a localized event, so that unwanted or spontaneous thrombosis is thoroughly circumvented.

4.3. Regulation of coagulation

There are three main inhibitory mechanisms in the regulation of the clotting cascade: (1) inhibition of the tissue factor pathway, (2) plasma serine protease inhibitors, and (3) the protein C/protein S anticoagulation pathway (Kalafatis et al., 1997). Other negative feedback mechanisms also help to control and regulate the events of blood clotting (for a review, see Kalafatis, 1997).

4.3a. Tissue factor pathway inhibitor

Tissue factor pathway inhibitor (TFPI) is a 276 amino acid protein containing three Kunitz-type inhibitory domains (Wun et al., 1988). The first domain inhibits the factor VIIa:TF complex. The second domain binds to and inhibits factor Xa directly, and in a factor VIIa:TF-dependent fashion, produces feedback inhibition of the factor VIIa:TF complex. The third domain may be important in lipoprotein binding (for a review, see Rapaport, 1995 and Broze, 1995). At physiological concentration, the formation of the quaternary complex of factor VIIa:TF/factor Xa:TFPI not only leads to the inhibition of factor Xa, but also

effectively abolishes the factor VIIa:TF catalytic activity (Papaport et al., 1995). TFPI exists in three pools, the majority bound to lipoproteins, some in platelet, and the remainder as truncated forms circulating in plasma or bound to endothelium of small blood vessels. Upon stimulation by thrombin and other agonists, platelets aggregated at the site of wound release its TFPI pool during coagulation so that the concentration of TFPI reaches a level almost threefold the normal venous concentration. However, the potential contribution of endothelial cells to the local increase in FPI concentration can not be excluded (Broze, 1995). TFPI has also been shown to inhibit the coagulation initiation complex, factor VIIa:TF, independent of factor Xa. Thus TFPI not only dampens coagulation initiation, but also may play an important role in the regulation of basal coagulation resulted from small traces of circulating clotting proteases.

4.3b. Serine protease inhibitors

The second regulatory system of the coagulation system involves the plasma serine protease inhibitors, commonly known as serpins. Antithrombin III now known as antithrombin (AT) is a major serpin and is found at a high concentration in plasma. Its target enzymes, the clotting serine proteases, are inhibited through the formation of 1:1 enzyme inhibitor complex, in which the active site of the protease is blocked. It is a reversible, yet essentially permanent inhibition. The main inhibitory effects of AT are on thrombin, factor Xa, and factor IXa. Other serpins are found at lower concentration in plasma and also lower effectiveness, and include heparin cofactor II, α-2 macroglobulin, and α–1 protease inhibitor (Rock et al., 1997). Serpins play a significant role in localizing the events of clotting at the site of injury by scavenging any clotting proteases swept way from the growing clot by the flowing blood. The formation of the AT:thrombin complex is greatly accelerated by heparin, forms of which (for example, heparan sulfate, a glycosaminoglycan) are located in the microvasculature on the surface of endothelial cells. Antithrombin, in the presence of heparin, has been shown *in vitro* to rapidly inhibit factor VIIa:TF complex as well (Lawson et al., 1993; Rao et al., 1993; van 't Veer et al., 1997). Thus antithrombin may also play an important role in regulating TF-induced coagulation, through inhibiting the activation of VII by factor Xa, factor IXa, or other proteases (Broze et al., 1993; Lawson et al., 1993; Rao et al., 1993).

4.3c. The protein C/protein S anti-coagulation system

The protein C/protein S anti-coagulation system is mostly responsible for the inactivation of the clotting cascade, preventing further activation of more pro-coagulant proteases and unchecked clot growth (Esmon, 1989). Proteins C and S, like factors II, VII, IX and X, are vitamin K-dependent plasma glycoproteins. To perform its anti-coagulant function, protein C is converted to a serine protease, activated protein C (APC), by thrombin bound to vascular thrombomodulin in the presence of calcium ions. Thrombomodulin (TM), an endothelial membrane protein, binds to thrombin and changes thrombin catalytic activity from a pro-coagulant to an anti-coagulant function. Instead of activating the cofactors V and VIII, thrombin changes its substrate specificity to protein C and in a negative feedback manner leads to the direct inactivation of those cofactors by protein C. The interaction between thrombin and thrombomodulin rapidly accelerates protein C activation as much as 20,000 fold (Esmon et al., 1981). Activated protein C then complexes with protein S on the surfaces of platelets and endothelial cells. These complexes catalyze the proteolytic inactivation of factors Va and factor VIIIa, which are critical components in the amplification of the coagulation process. Deficiencies in the anti-coagulant factors, protein C, S and thrombomodulin lead to complicated clinical disorders. For example, infants born without protein C often die with massive thrombotic complications. Recently, an inherited defect in the factor V gene (Arg^{506}-Gln) was identified. This mutation results in a protein product that is resistant to cleavage by activated protein C, and has been characterized as a major risk related to venous thrombosis (Bertina et al., 1994). In fact, the incidence of APC resistance accounts for about 35-40 % cases of familial thrombosis in humans, while deficiencies of protein S, antithrombin III, and thrombomodulin together are responsible for only 5-10 % (Davie, 1995).

In summary, although it is desirable to have rapid clotting to prevent bleeding, it is critical to control and modulate the process. Once started, initiation must be dampened, the clot has to be localized and the amplification of the cascade must be held in check. Tissue factor pathway inhibitor, the serpins, and the protein C/protein S anticoagulation system together are major players in this regulation. Recently, factor Va has been implicated in tissue plasminogen cofactor activity, thus linking the activation of procoagulant to the fibrinolytic system (Pryzdial et al., 1995). In addition, the factor VIIa:TF complex has been

shown to activate factor V directly to a form that is resistant to thrombin activation, but with increased sensitivity to activated protein C inactivation (Safa et al., 1999). This could be a potential regulatory pathway where the factor VIIa:TF complex modulates the coagulation process in the extravascular space. Other potential regulatory mechanisms have yet to be investigated, weaving more threads to the complex tapestry of the system. After a clot has formed, other haemostatic mechanisms, such as the fibrinolysis, repair, and inflammatory systems, take over to complete the task of preserving haemostasis.

II. PROTHROMBIN

Prothrombin, a plasma glycoprotein with molecular weight 72,000 Daltons, is the zymogen for the blood clotting enzyme thrombin (Degen et al., 1998). Thrombin functions during blood coagulation to cleave plasma fibrinogen to form insoluble fibrin polymers. Thrombin also cleaves a number of other protein substrates including factor V, factor VIII, protein C, factor XI, factor XIII, as well as prothrombin itself. In addition, thrombin activates platelets and initiates a variety of cellular responses. The formation of thrombin, therefore, has important consequences on a variety of homeostatic events, and prothrombin activation *in situ* must be highly regulated to prevent undesirable clotting.

1. Genomic organization

The structure and organization of the genes encoding for the blood coagulation proteins have been characterized using advances in recombinant DNA techniques. Analysis indicates that the genomic evolutions occurred via gene (exon) duplication, gene modification, and gene shuffling (Patthy, 1985). In many cases, the exons may be considered to encode modules coding for a homologous domain in a protein. The backbones of these homologous domains are nearly identical, with substitution of amino acid side chains on the outside surface giving each module its own unique property. The human and bovine prothrombin coding sequences (Degen et al., 1983; MacGillivray et al., 1984) and genes (Degen et al., 1987; Irwin et al., 1985; Irwin et al., 1988) show remarkable identity with other vitamin K-dependent proteins (Figure 4). The prothrombin gene is located on chromosome

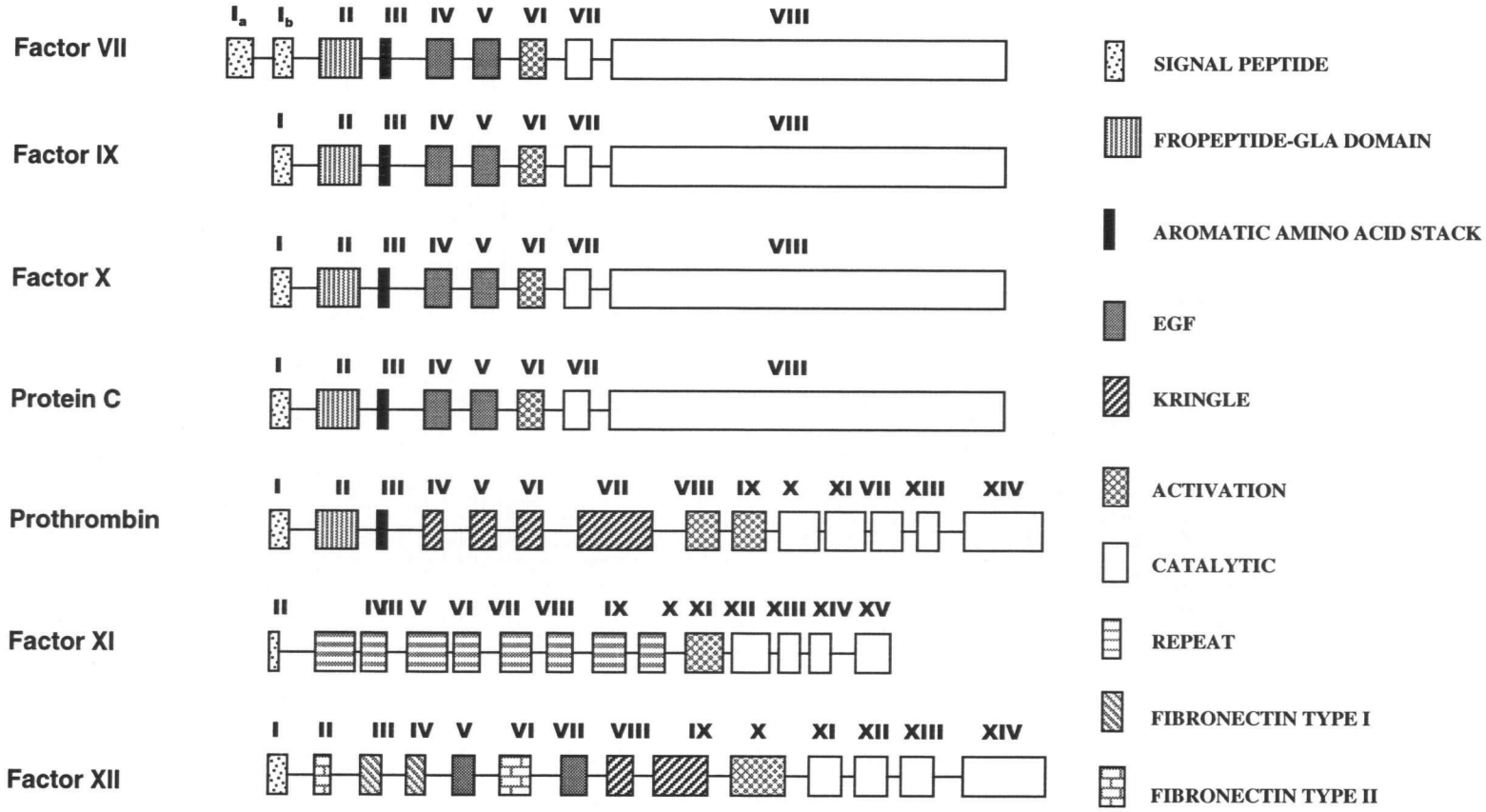


Figure 4. Genomic organization of coagulation proteins. The numbered boxes represent the exons of the proteins. The lines in between the boxes are the intronic sequences.

11 (11p11-q12) and consists of 14 exons spreading over 21 kilobases of DNA, interupted by 13 introns ranging in size from 84 to 9447 base pairs (for a review, see Degen, 1998). The exon organization is as follows: exon I encodes the signal peptide domain; exon II encodes the propeptide and the Gla domain; exon III encodes the amino acid stack domain; exons IV-VII code for the two kringle domains; exons VIII-IX encode the α-chain of thrombin; and lastly exons X-XIV encode for the catalytic protease domain (Figure 4). The prothrombin gene does not have an obvious TATA- promoter box, but it has been characterized to have a weak promoter immediately before the initiation transcription site, together with a liver-specific enhancer sequence located 860-940 nucleotides away from the gene (Bancroft et al., 1992; Chow et al., 1991). The upstream regulatory sequence contains a binding motif for hepatocyte nuclear factor 1 (HNF-1) and may be important to the liver-specific transcriptional activity of the prothrombin gene.

2. Biosynthesis and post-translational modifications

Similar to most clotting proteins, prothrombin is mainly synthesized and secreted by the liver into the blood stream. The synthesis of prothrombin occurs at a very early stage of embryogenesis as demonstrated by recent mouse transgenic studies (Sun et al., 1998; Xue et al., 1998). In rabbit fetal liver, the levels of prothrombin mRNA is greater than in the adult rabbit liver, while the protein levels are comparable (Cohen et al., 1997). The rate of synthesis of prothrombin is maintained from embryogenesis through to adulthood. Similar to other clotting proteins, several post-translational modifications occur to prothrombin before it is secreted in a biologically active form. The modifications include signal peptide and propeptide processing, γ-carboxylation (vitaminK-dependent), glysosylation (N-linked), and disulphide bond formation and protein folding. All of these events occur after protein synthesis by the ribosomal system. Vitamin K-dependent γ-carboxylation of prothrombin occurs mainly in the endoplasmic reticulum (Bristol et al., 1996), resulting in 10 γ-carboxyglutamic acids (Gla) in the N-terminal region of the protein termed the Gla domain. Gla exists in several clotting proteins and calcium binding proteins, and plays a significant role in the metal-binding properties of these molecules. The vitamin K-dependent carboxylase is a microsomal membrane enzyme that catalyzes the carboxylation of some glutamic acid residues to Gla. The cDNAs of the human (Wu et al., 1991) and the bovine

carboxylase (Rehemtulla et al., 1993) have been characterized to encode for a single-chain polypeptide of 758 amino acids long. The N-terminal one-third of the protein has been implicated in glutamate binding and catalysis, as well as γ-carboxylation recognition site (CRS) binding, while vitamin K-epoxidase activity is located in the C terminal end (Roth et al., 1995).

While prothrombin does not undergo β-hydroxylation, other vitamin K-dependent proteins such as factors IX, X, VII, proteins C and S, have either erythro-β-hydroxyaspartic acid or erythro-β-hydroxyasparagine in the EGF domains (Furie et al., 1988). The function of β-hydroxylation remains to be determined, although postulations have been made involving the metal-binding properties of these proteins. Prothrombin has three N-linked glycosylation sites at Asn-77, Asn-101, and Asn-373. The first two carbohydrates lie in the first kringle domain, and the third is in the catalytic thrombin domain. Any role of the carbohydrates in prothrombin is unclear, although glycosylation in tissue plasminogen activator may be involved in secretion efficiency, plasma clearance, as well as in altering its enzymatic activity (Berg et al., 1993). The consensus N-glycosylation site in proteins consists of a Asn-X-Ser/Thr motif (Marshall, 1974). In baby hamster kidney (BHK) cells, at least one glycosylation event is absolutely required for the production and secretion of recombinant prothrombin (Hewitt et al., 1999). Thus the carbohydrate moieties probably play an important role in the synthesis and secretion of prothrombin, although the exact mechanism is still unknown.

Once prothrombin is modified and folded correctly with the proper disulfide linkages, the pro-peptide is cleaved and the mature protein is released into plasma circulation. Two contiguous basic amino acids (Lys-Arg) in the pro-peptide adjacent to the cleavage site play important role for this important processing event. The protease responsible is furin, a membrane associated, calcium-dependent serine endopeptidase homologous to the bacterial subtilisins (Barr et al., 1991; Hosaka et al., 1991). Furin catalyzes the processing of prothrombin and other proteins using a motif of Arg-X-Lys/Arg-Arg (present in prothrombin). Furin is synthesized as a zymogen and after autoproteolytic activation concentrates in the trans Golgi network (Leduc et al., 1992; Molloy et al., 1994). After propeptide cleavage in the Golgi complex by furin, prothrombin is now secreted into the circulation as a fully functional, biologically active protein.

3. Protein organization

Human prothrombin has 579 amino acid residues distributed into several characteristic regions of protein structure. Similar to most secreted proteins, prothrombin has an N-terminal hydrophobic sequence, the signal peptide, responsible for the biosynthesis and secretion of the protein. The signal peptide is cleaved by a signal peptidase in the cisternal space of the Golgi complex (Suttie, 1985; Vermeer, 1990). In prothrombin, and also in other vitamin K-dependent clotting proteins, the signal peptide is followed by a 17-25 residue sequence, termed the propeptide, immediately preceding the N-terminus of the protein. This propeptide is removed by proteolytic cleavage prior to secretion. At its amino terminal region, prothrombin contains 10 residues of γ-carboxyglutamic acid localized to a 44-amino acid region termed the Gla domain. Prothrombin binds to negatively charged phospholipid surfaces as a consequence of Ca^{2+} binding and a Ca^{2+}-dependent conformational change in the Gla domain. Additional distinct regions of the protein structure in prothrombin include two triple disulfide loops of approximately 90 amino acids termed kringles and a serine protease segment in the carboxy terminus region (Figure 5). Several proteolytic fragments of prothrombin can be isolated including prothrombin fragment 1 (consisting of the Gla domain and the first kringle), fragment 2 (consisting of the second kringle), and prethrombin 2, containing the serine protease domain (Degen et al., 1998).

3.1. Pro peptide domain

The propeptide domain of the vitamin K-dependent clotting proteins is critical for the post-translational γ-carboxylation and protein processing. After processing, the peptide is cleaved and the mature protein is released into plasma. The zymogens of the vitamin K-dependent proteins contain 10-12 glutamic acid (Glu) residues that must be modified to γ-carboxyglutamic acids (Gla) residues in order to generate a fully functional mature protein. Carboxylation of the Glu residues takes place in the rough endoplasmic reticulum (Bristol et al., 1996) and is carried out by the vitamin K-dependent carboxylase (Carlisle et al., 1980). Determination of the propeptide sequence of factor IX and the observation of a propeptide mutation associated with incomplete γ-carboxylation led to the hypothesis that the propeptide region is involved in the γ-carboxylation of the protein. The precursor forms of the entire

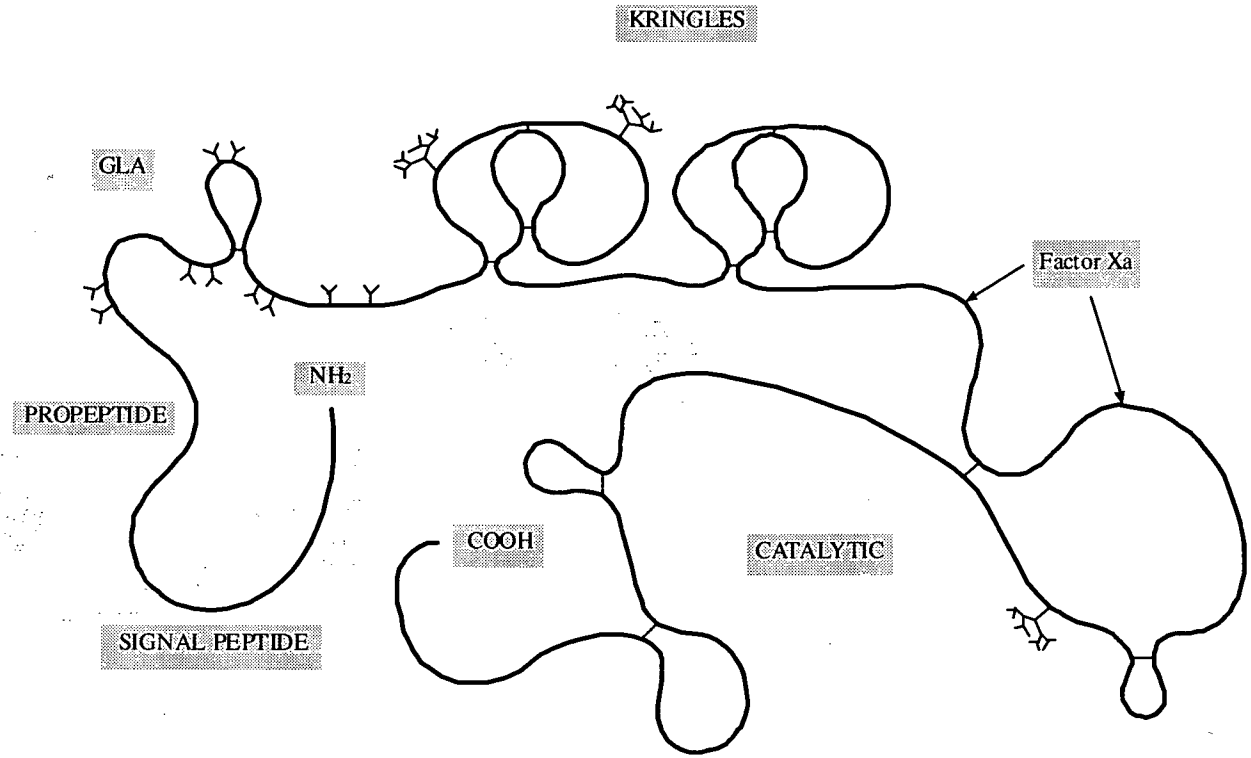


Figure 5. Domain organization of prothrombin. The Y represents the gamma-carboxyglutamic acids, while the $\overset{\diagdown\ \diagup}{\diagup\ \diagdown}$ represents the carbohydrates. Disulfide linkages (small lines) connect the polypeptide backbone (dark line). Factor Xa cleavages site are shown (arrows).

vitamin K dependent plasma proteins have amino- terminal propeptide extensions, residues -18 to -1, that share significant sequence identity (Figure 6). Both phenylalanine at –16 and alanine at –10 are completely conserved. Other hydrophobic amino acids at positions –17, -7, and –6 are also well conserved, while basic amino acids at positions –4, -3, -2, and –1 are found. Secondary structural analysis of the propeptide sequence of the vitamin K dependent proteins (Chou et al., 1978) suggests the presence of an amphipathic α-helical structure (as a result of the spaces of the hydrophobic and hydrophilic residues in the sequence). Residues -13 to -3 form an amphipathic α-helix as indicated by two-dimensional NMR spectroscopy and circular dichroism (Sandford et al., 1991).

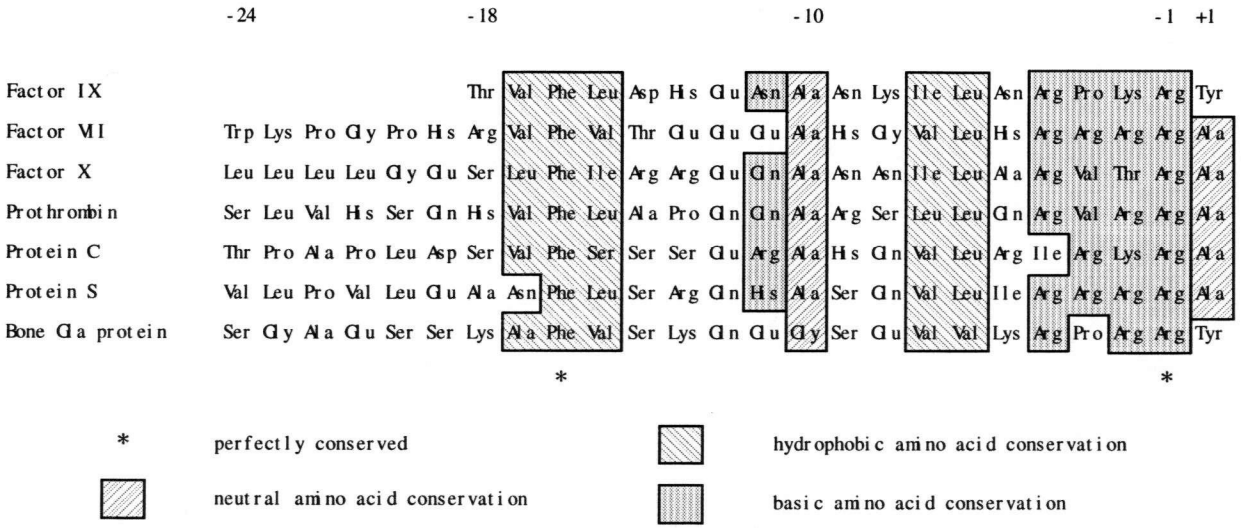
3.1a. The gamma-carboxy recognition site (γ-CRS)

A number of mutagenic studies to elucidate the essential functional residues of the propeptide have been carried out using *in vitro* and *in vivo* processes. It has been shown that factor IX lacking the propeptide region could not be carboxylated (Jorgensen et al., 1987b). A similar result was also found in a study with protein C (Foster et al., 1987). Furthermore, amino acid substitutions of alanine for phenylalanine at –16 and glutamic for alanine at –10 in the propeptide (factor IX) result in severely impaired carboxylation (Rabiet et al., 1987). Together, these findings suggest that the propeptide contains a recognition element, termed the γ-CRS (gamma-carboxylation recognition site), important for the post-translational carboxylation modification (Figure 6). Other mutagenic studies of specific amino acids at various positions in the propeptide region of prothrombin demonstrate that residues –18 to –15, and –10 are crucial for carboxylation and might be involved in the γ-CRS formation (Huber et al., 1990). Residues Ala-14, Ser-8, Arg-4, and Arg-1 appear to be non-essential for the carboxylation process (Ratcliffe et al., 1993).

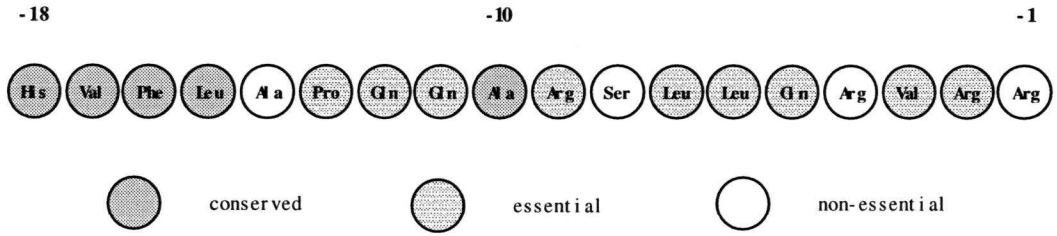
In a different approach, synthetic peptides including the γ-CRS have been subjected to *in vitro* vitamin K- dependent γ-carboxylation using the bovine liver carboxylase (Hubbard et al., 1989). Two 28-residue peptides, proPT28 (pro-prothrombin -18 to +10) and proFIX28 (proFactor IX -18 to +10), were carboxylated with a K_m of 3 μM. The observed V_{max} of proPT28 showed that it was a better substrate than pro-FactorIX. Other peptides that were based on naturally occurring mutant such as FIX Cambridge (Arg^{-1} to Ser) and FIX San

Figure 6. Propeptide sequence identity of coagulation proteins. The propeptide sequences of factor IX, VII, X and prothrombin, bone gla protein, and protein C and S are aligned showing highly conserved amino acid residues (panel A). The conserved residues, the essential residues for carboxylation and the non-essential residues of the propeptide sequence of prothrombin are also shown (panel B).

(A)


-24
-18
-10
-1
+1
Factor IX
Factor VII
Factor X
Prothrombin
Protein C
Protein S
Bone Gla protein
Thr Val Phe Leu Asp His Glu Asn Ala Asn Lys Ile Leu Asn Arg Pro Lys Arg Tyr
Trp Lys Pro Gly Pro His Arg Val Phe Val Thr Glu Glu Glu Ala His Gly Val Leu His Arg Arg Arg Arg Ala
Leu Leu Leu Leu Gly Glu Ser Leu Phe Ile Arg Arg Glu Gln Ala Asn Asn Ile Leu Ala Arg Val Thr Arg Ala
Ser Leu Val His Ser Gln His Val Phe Leu Ala Pro Gln Gln Ala Arg Ser Leu Leu Gln Arg Val Arg Arg Ala
Thr Pro Ala Pro Leu Asp Ser Val Phe Ser Ser Ser Glu Arg Ala His Gln Val Leu Arg Ile Arg Lys Arg Ala
Val Leu Pro Val Leu Glu Ala Asn Phe Leu Ser Arg Gln His Ala Ser Gln Val Leu Ile Arg Arg Arg Arg Ala
Ser Gly Ala Glu Ser Ser Lys Ala Phe Val Ser Lys Gln Glu Gly Ser Glu Val Val Lys Arg Pro Arg Arg Tyr
*
*
* perfectly conserved
neutral amino acid conservation
hydrophobic amino acid conservation
basic amino acid conservation

(B)


-18
-10
-1
His Val Phe Leu Ala Pro Gln Gln Ala Arg Ser Leu Leu Gln Arg Val Arg Arg
conserved
essential
non-essential

Dimas Oxford 3 (Arg^{-4} to Gln) also showed equivalent carboxylation. These mutations are found in hemophiliacs, where the factor IX is characterized by incomplete carboxylation and impaired propeptide cleavage (Giannelli, 1998). These findings suggest that residues -4 and -1 are not involved in the γ-CRS but may interfere with the processing of the zymogen. This approach was the first to utilize synthetic peptides to study the carboxylation process; however, non-specific carboxylation and a lack of the assay to differentiate partial or full carboxylation complicate its usage. The γ-CRS, responsible for downstream carboxylation on a protein, has been demonstrated recently in an experiment where the prothrombin propeptide was attached to only the thrombin domain, leading to the carboxylation of eight adjacent glutamic residues within the first 40 amino acids in the N-terminus of thrombin (Furie et al., 1997). Thus this is direct evidence that the propeptide region itself alone may be sufficient to designate a protein as a substrate for the vitamin K-dependent carboxylase leading to the synthesis of γ-carboxyglutamic acids. Recently, the factor IX Gla-domain has been shown to have a ≥230 fold higher Km for the carboxylase than factor IX Gla domain covalently attached to the propeptide region (Stanley et al., 1998), and thus the Gla domain may play a role in the carboxylation process through specific interaction(s) with the carboxylase enzyme.

3.1b. The propeptidase recognition site

The propeptide is usually cleaved from the secreted protein after complete γ-carboxylation by one or more propeptidases. Propeptidases are subtillisin-like enzymes that are similar to those that cleave secretory proteins. The consensus for the propeptidase recognition site and cleavage is **Arg**$^{-4}$-X-Lys/Arg-**Arg**$^{-1}$ immediately preceding the mature protein (Hosaka et al., 1991) (Figure 6). Naturally occurring mutations at the basic amino acids position, specifically the Arg at –4 and –1, often lead to secretion of dysfunctional proteins with the propeptide domain still attached. Similar to factor IX Cambridge and San Dimas mentioned above, a naturally occurring Arg^{-1} to His mutation in human protein C has been characterized to lead to aberrant propeptide processing and secretion of a dysfunctional protein C with complete carboxylation (Lind et al., 1997). These mutations have been found in mainly factor IX (Arg^{-4} to Gln/Leu/Trp; Lys^{-2} to Asn; Arg^{-1} to Ser) and protein C (Arg^{-1} to

Cys/His/Ser) (Lind, 1997). An Arg^{-1} to His mutation has been recently found in protein S (Gandrille et al., 1995).

3.2. Gla domain

The N-terminal region of the prothrombin is called the Gla region (residues 1-44) because of the presence of 10 γ-carboxyglutamic acid residues (Figure 7). Administration of an anticoagulant, warfarin, or a vitamin K deficient state reduces and/or abolishes the formation of Gla residues leading to a non-functional molecule (Suttie, 1985; Vermeer, 1990). The abnormal prothrombin was termed Des-γ-carboxy prothrombin or PIVKA-II (prothrombin induced by vitamin K antagonist or vitamin K deficient states). The dysfunctional molecule was eventually found to have deficient metal and phospholipid binding properties. In the beginning, relatively little was known about the precise metal ion binding sites in fragment 1 (F1) of prothrombin, a fragment containing the Gla domain derived from limited proteolysis. Loss of as few as two Gla residues resulted in considerable loss of protein function (Borowski et al., 1985; Malhotra et al., 1985; Wright et al., 1984). This has greatly restricted the ability for modification of Gla residues as an approach to understanding the metal ion binding function of fragment 1. By using equilibrium dialysis studies on various tryptic peptides of prothrombin F1, peptide 1-45 (the Gla domain) of bovine prothrombin was shown to bind three calcium ions in a positive, cooperative manner (Pollock et al., 1988). Extensive studies have since been pursued to investigate this Ca^{2+}-dependent property, namely the ability to bind calcium ions. With the advance of the crystal structure of bovine prothrombin F1 (Soriano-Garcia et al., 1989; Tulinsky et al., 1988), some details of the structure of fragment 1 were understood. However, there are still some shortcomings even with the X-ray crystal structure. The first 35 residues of the Gla domain are disordered in the apo structure and the carbohydrate residues of the first kringle region are also disordered. As well, the intrinsic differences between the structure in crystal forms and in solution posed innate problems as described in the following section. Hence, many investigators had tried to utilize various methodologies in solving the aqueous secondary structure of prothrombin (F1) and its calcium binding properties. Such methods include the use of conformation specific antibody immunoassays, circular dichroism, light scattering, fluorescence spectroscopy,

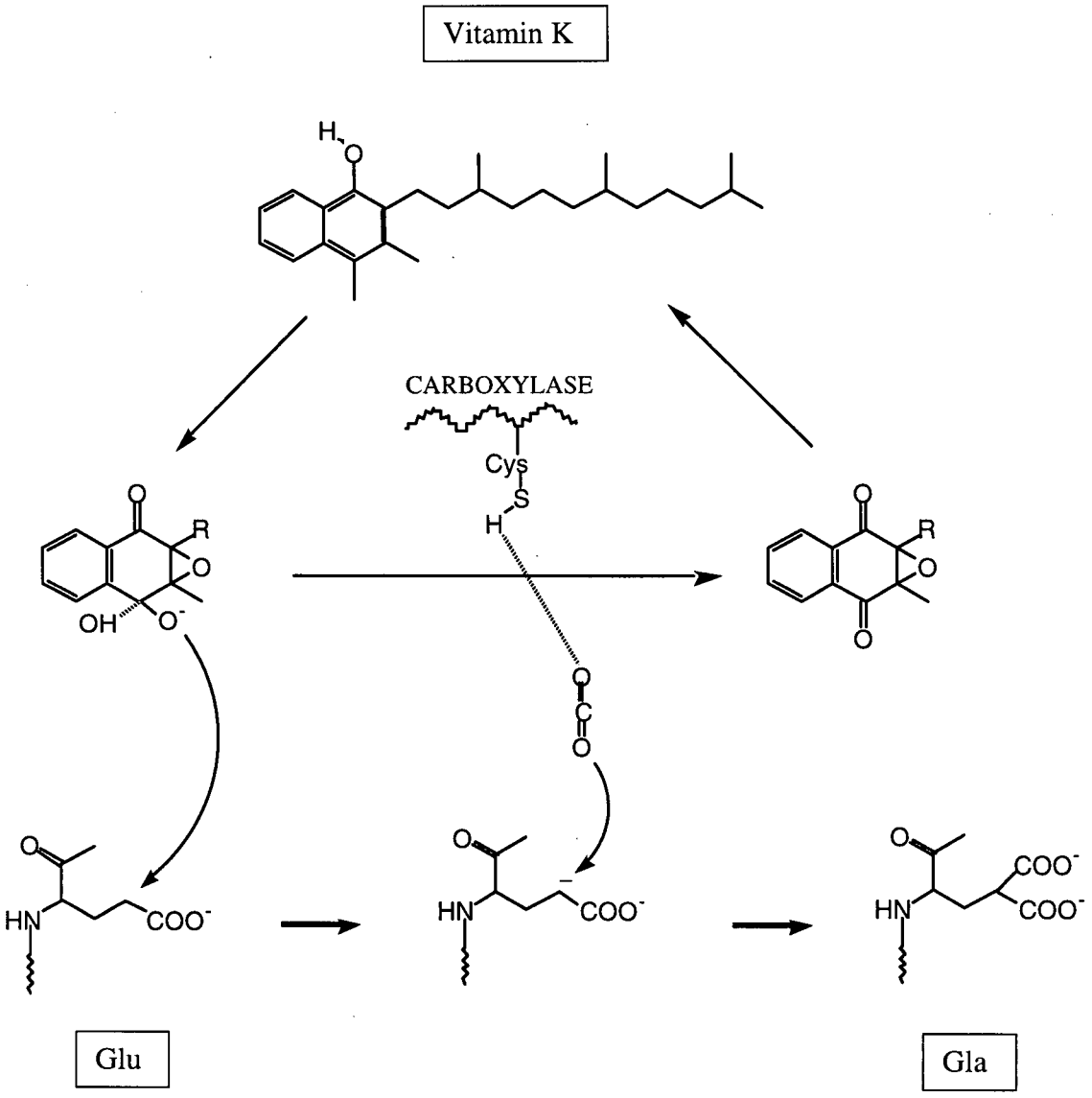


Figure 7. Mechanism of vitamin K-dependent carboxylase catalyzed formation of Gla. Glutamic acid (Glu) is converted to gamma-carboxyglutamic acid by the enzyme carboxylase using the cofactor vitamin K. Vitamin K is converted to an epoxide in the process but is subsequently reduced to its original form by the same carboxylase enzyme.

NMR spectroscopy, infrared spectroscopy, equilibrium dialysis, and recombinant DNA techniques, specifically site-directed mutagenesis (see following sections for references).

Based on the X-ray data, the overall structure of fragment 1 of bovine prothrombin is a discoid (oval) shape. The N-terminal end consists of two short α-helixes in a horseshoe shape, and the C-terminal end has 3 turns of α-helixes. A cluster of three hydrophobic residues, the conserved aromatic "stack" (Phe-40, Trp-41, and Tyr-44) is adjacent to the disulfide bond of Cys-18 and Cys-23. The Gla residues at positions 6,7,16,26, and 29 form a negatively charged channel with an array of 5 calcium ions. Three other Gla residues at 14, 19, and 20 form another small negatively charged cluster with 2 more calcium ions adjacent to the hexapeptide disulfide loop (Cys18-Cys23). Alanine-1 is involved in a hydrogen bond pair with Gla-16 and Gla-26, forming the horseshoe shape. Alanine-1 plays a crucial role in the secondary structure of the calcium-binding Gla domain of prothrombin as shown in the chemical modification experiments below.

It has been shown that addition of divalent cations to prothrombin F1 quenches the intrinsic fluorescence of tryptophan residues in the protein (Nelsestuen, 1976; Nelsestuen et al., 1976b; Prendergast et al., 1977). This quenching is the result of a conformational shift in the protein secondary structure that is necessary for membrane binding. This quenching property has been exploited in several studies to investigate the structural-functional relationships of the Gla domains of clotting factors. The Gla domains in vitamin K-dependent plasma proteins demonstrate significant sequence similarity (Figure 8). The calcium-induced physical properties of these Gla domains are quite similar and chemical modification experiments have also yielded similar results (Schwalbe et al., 1989). Chemical modifications of prothrombin fragment 1, such as acetylation and methylation (Welsch et al., 1988b; Welsch et al., 1988), and phenylation (Weber et al., 1992), result in an inactive peptide with reduced phospholipid binding compared to the native peptide. Calcium plays a protective role as in its presence, the membrane binding is only slightly reduced. Consequently, conformational features modulated by this calcium binding process mediate the phospholipid binding property. In addition, the amino terminal alanine functions in a calcium specific event that is essential to phospholipid binding. The positive cooperativity of Ca^{2+} binding is abolished when this alanine is modified or when two or three amino acids are removed from the amino terminus. Thus the amino terminal of fragment 1 is an integral

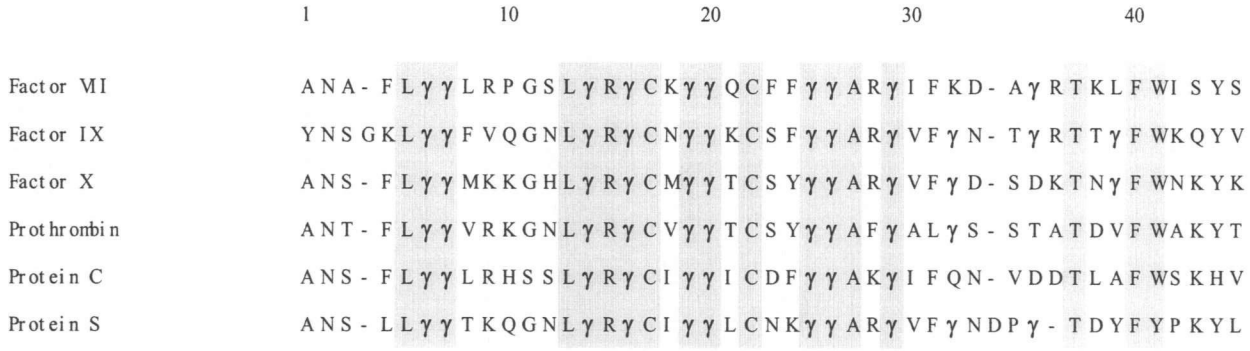


Figure 8. Sequence identity of Gla domains of coagulant proteins.

Amino acid sequences of the Gla domains of human vitamin K-dependent coagulation proteins. γ is the single letter abbreviation for gamma carboxyglutamic acid. Conserved residues are in shaded areas.

feature of the polymeric Ca^{2+}-Gla domain. Disruption of this matrix renders the peptide inactive (in phospholipid interaction). This domain matrix constitutes significant salt bridges confirmed from the crystal structure, and cooperativity is lost if the important residues are destroyed (salt bridges disturbed).

Mutagenic studies of bovine prothrombin and protein C have demonstrated that mutations to aspartic acid of Gla residues 6, 7, 16, 26, and 29 in the calcium-binding channel lead to complete loss of biological activity and phospholipid binding properties of the protein (Ratcliffe et al., 1993; Zhang et al., 1992). Mutations in Gla-14, 19, and others have little if any effect. The calcium binding property of the Gla domain is a cooperative one, as mentioned above. Co-operative binding of the first 2 to 4 calcium ions leads to a conformational change of the peptide as measured by fluorescence quenching of Trp-41. The calcium binding results in the formation of a linear channel by the N-terminus exposing the three hydrophobic residues, Phe-5, Leu-6, and Val-9, a prerequisite for the interaction with anionic phospholipid membrane (Zhang et al., 1994). When leucine-5 of protein C (a conserved amino acid in all vitamin K-dependent clotting factors) was mutated to Glu, normal calcium binding resulted, but there was a total loss of anionic phospholipid binding. Other amino acid residues in the Gla domain also play significant roles in the interaction with membrane surfaces besides the hydrophobic cluster. For example, cis-proline-22 may be important for membrane-binding conformation (Evans et al., 1996); and glycine^{12}-Arg, a naturally occurring mutation in hemophilia B (factor IX), leads to less than 1 % of biological activity but an antigen level of ~ 45 %. Side chains other than Gla residues are seldom implicated as calcium ligand(s), and more likely these groups are required for some other functions such as maintaining proper protein folding. Thus the calcium interaction seems to require substantially more structure in protein folding than is provided for by simple placement of Gla residues in a primary protein sequence.

3.3. Kringle domains

A kringle is a unique polypeptide module composed of approximately 90 amino acids. The kringle has a unique secondary structure formed by three pairs of disulfide bonds, resembling the Danish roll that it was named after. The first reported kringle was found in the amino acid sequence of prothrombin fragment 1 (Magnusson et al., 1975). Since then,

the kringle module has been found in other serine proteases, such as urokinase (uPA), tissue-type plasminogen activator (tPA), plasminogen, and factor XII (Thery et al., 1996). Kringle domains have also been found in some other proteins that have biological functions different from the clotting proteases. The plasma protein apolipoprotein(a), involved in the transfer of lipids in blood, possesses 38 kringles (McLean et al., 1987). The hepatic growth factor (HGF) has four kringles and is known to be responsible in the regeneration of liver cells (Miyazawa et al., 1989; Nakamura et al., 1989). A novel hyaluronan-binding protein from plasma has been characterized and contains one kringle domain in its structure, as well as a serine-protease like domain (Choi-Miura et al., 1996). Angiostatin, an angiogenesis inhibitor of endothelial cells, is a 38 kDa peptide that has also been found to contain one kringle (Cao et al., 1996). A novel brain-specific serine protease, BSSP-3, belonging to a class of scavenger-receptor-cysteine-rich proteins contains a kringle-like domain (Yamamura et al., 1997).

The kringle domains of coagulation proteins (Figure 9) are quite similar and this led to the idea that all of the kringle domains originate from a single ancestral gene (Patthy et al., 1984). The phylogenetic tree of kringle evolution has been suggested by many since (Castellino et al., 1987; Ikeo et al., 1995; Lawn et al., 1997), and is consistent with the first single kringle gene duplication event about 500 million years ago. An ancestral gene for plasminogen having five kringles emerged approximately 300 million years ago, and duplicated into two genes about 220 million years after. From this gene duplication event, one remains as plasminogen now, the other underwent dynamic and drastic evolution. Particularly, three kringles were deleted and only two remained in the second plasminogen-type-duplicated gene, probable ancestors of prothrombin, factor XII, tPA and uPA. Repeated duplication of one of the two kringles in this gene probably led to the present apolipoprotein(a) gene, an event taking place about 5 million years ago (Ikeo et al., 1995).

Since the kringle domains are usually found in the non-catalytic portion of the proteases, they are thought to play a significant role as docking modules in protein-protein interaction of the enzyme-substrate complex, or macromolecule assembly. There seems to be two types of kringle in plasma proteins based on their biological properties and functions: one with lysine binding properties and one without. The first and fourth kringle domains of plasminogen (Hochschwender et al., 1981; Lerch et al., 1980; Trexler et al., 1982), and the

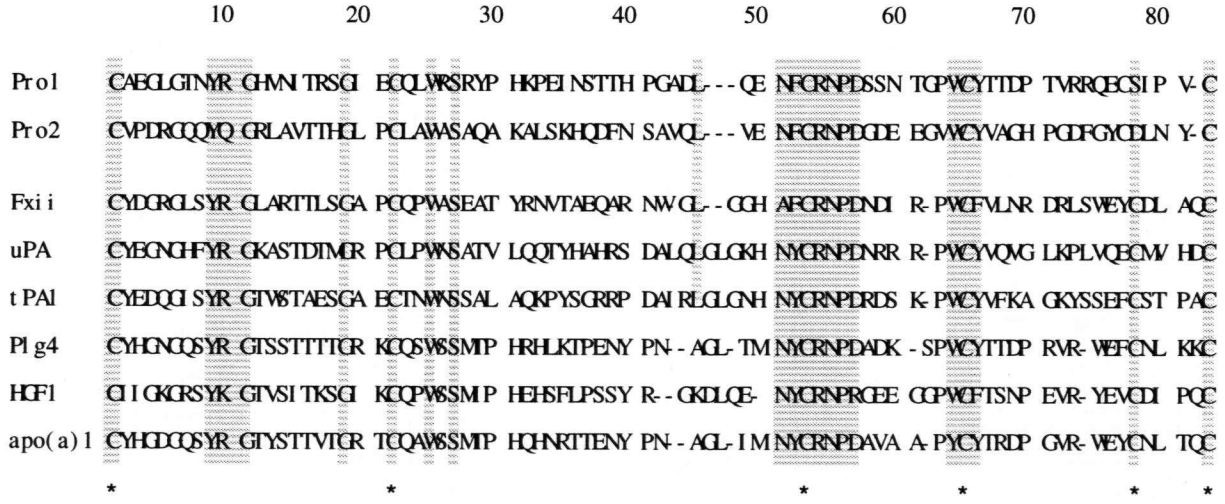


Figure 9. Sequence identity of the kringle domains of plasma proteins. The amino acid sequences are aligned for the first kringle and the second kringle of human prothrombin (Pro1 and Pro2), for factor XII (Fxii), for urokinase (uPA), for the first kringle of tissue type plasminogen activator (tPA1), for the fourth kringle of plasminogen (Plg4), for the first kringle of hepatic growth factor (HGF1), and for the first kringle of apolipoprotein(a) (apo(a)1). Conserved residues are shown in boxed areas and the conserved cysteins for the tri- sulfide linkages are marked (*).

second kringle of tissue-type plasminogen activator (van Zonneveld et al., 1986; Verheijen et al., 1986) bind to fibrin, lysine, or ω–amino-carboxylic acids in general. In addition, kringle 5 of plasminogen is believed to facilitate its interaction with platelet thrombospondin (Depoli et al., 1989). The lone kringle domain of urokinase is thought to help in the binding to heparin and dextran sulfate (Stephens et al., 1992). Because of the association of apolipoprotein(a) with atherosclerosis, it is postulated that the multiple of kringle domains are involved in the binding of the protein to exposed macromolecules at the site of vascular damage (Lawn et al., 1992). None of the kringles in prothrombin possesses any lysine-binding properties, and their roles are still unclear. The importance of the kringle domain(s) in other proteins, such as the hepatocyte growth factor, angiostatin, and BSSP-3 receptor is not known. The kringles may act as docking modules for the interaction between these proteins and their respective receptors, and vice versa.

3.3a. The first kringle

In prothrombin, the kringle 1 domain (residues 41 to 155) is located at the N-terminal region of the proteolytic fragment 1 (F1). Crystal structures of both the apo form and the calcium-bound form of fragment 1 have been determined (Soriano-Garcia et al., 1989; Tulinsky et al., 1988). The two kringle structures are indistinguishable with the carbohydrate remaining disordered in calcium-fragment I. Little is known about the biological function of the two kringles of prothrombin. Because kringle 1 is a part of fragment 1 (F1) that is responsible for calcium and phospholipid binding (mainly through the Gla domain), it has been postulated that kringle 1 may also play some role in this property. In fact, kringle 1 has been demonstrated to have calcium protection in acetylation experiments. Chemical modification studies of prothrombin F1 (Sugo et al., 1990; Welsch et al., 1988b; Welsch et al., 1988) have demonstrated that the peptide contained two acetylation sites that were protected from derivatization by calcium. The first acetylation site was the amino terminal residue. Extensive characterization of the acetylated peptide by sequencing, NMR and mass spectrometry analysis indicated that the second radio labeled acetate was associated with the side chain of Asn-101. The postulated site of acetylation was the β-amide nitrogen of Asn-101, the amino acid that was one of the two carbohydrate-linked amino acids of prothrombin fragment 1 (the other is Asn -77). Asn -101 is in the kringle region of fragment 1. The fact

that metal ions protected a site on the kringle region of prothrombin F1 from acetylation suggests that metal ion binding somehow influenced this region of the protein. This was supported by the same fluorescence intensity characteristics displayed by both the native proteins containing metal ions and the acetylated apoprotein. The first kringle of the protein was critical to the metal ion induced fluorescence quenching event, perhaps very closely placed to Trp-90 and Trp-126. Acetylation of Asn-101 may disrupt the conformation of this critical region of the protein. Further studies would be needed to clarify this role and to show the relationship between Asn-101 and the calcium and membrane binding events.

Kringle 1 of prothrombin may also play a role in the activation of the protein by the prothrombinase complex. Inhibition of the prothrombinase complex activity on the endothelial cell surface by monoclonal antibodies directed against the kringle 1 domain suggested that this domain participates in the formation of the prothrombinase complex (Sugo et al., 1995). In addition, a recent study has demonstrated that kringle 1 interacts directly with factor Va during the assembly of the prothrombinase complex (Deguchi et al., 1997). In this study, both purified kringles, in the presence of factor Va, inhibited the FXa-catalyzed prothrombin activation in the absence of phospholipid. However, in the absence of both factor Va and phospholipids, kringle 2 fragment, but not kringle 1 fragment, inhibited prothrombin activation. The interaction between both kringles and factor Va were confirmed by fluorescence polarization, and the binding constants are similar to that of prothrombin binding to factor Va.

Recently, the kringle domains of prothrombin have been implicated in determining the intracellular degradation of under-γ-carboxylated prothrombin using a rat/human chimeric prothrombin (Wu et al., 1997). It has been known for some time that vitamin K antagonists such as warfarin inhibit the vitamin K-dependent γ-glutamyl carboxylation during protein processing. Secretion of under γ-carboxylated prothrombin is possible in human or bovine but not in rat. In this study, under γ-carboxylated prothrombin was secreted from warfarin-treated human HepG2 cell cultures, but not in warfarin-treated rat H-35 cell cultures. A chimeric human prothrombin with kringle 1 replaced with the rat equivalent kringle showed drastically reduced level of secretion in the transformed human kidney HEK293 cell lines treated with warfarin. Another chimera with kringle 2 from rat did not alter the secretion rate under the same conditions. Thus the kringle 1 of the rat species led to

more degradation of the under γ-carboxylated protein. Although rat and human kringle 1 share about 66 % sequence identity, the structural features of the kringle seem to be important in determining the stability of under γ-carboxylated prothrombin precursors in the endoplasmic reticulum, or at least in this case helps the quality-control mechanism between the prothrombins of the two species.

3.3b. The second kringle

As mentioned earlier, there are other blood proteins that contain multiple kringle domains and there are high degrees of sequence identity between the kringles both intra- and interspecies. The kringle 1 in prothrombin F1 had been shown to have a possible calcium binding property and may play a role in the Ca^{2+}-dependent secondary structure involved in phospholipid membrane binding, as well as limited interaction with factor Va in the prothrombinase complex. In contrast, there is little known about the function of the second kringle domain (fragment 2) of prothrombin.

Factor Va, a membrane bound protein, functions to (a) bind factor Xa to induce conformational change to improve factor Xa catalytic efficiency; and (b) bind to prothrombin to enhance the affinity for phospholipid membrane surfaces and or to alter the conformation of the substrate (prothrombin) for activation by FXa. By sedimentation equilibrium analysis, the second kringle has been shown to interact with factor Va heavy chain (Vh) (Luckow et al., 1989). The study showed that these two proteins associated to form a 1:1 complex with a moderate affinity (Kd = 10 μM). Although the activation of prothrombin and the stability of Va both required calcium ions, the interaction of factor Vh with prothrombin was completely independent of calcium ions. This suggested that the γ-carboxyglutamic acid residues play no role in the Va-prothrombin interaction. The idea was further supported by the observation that the interactions of prethrombin 1 (a prothrombin species lacking the Gla residues) and prothrombin with factor Vh were indistinguishable and both were calcium independent. The binding interaction was similar for bovine and human prethrombin 1. From these observations, it was postulated that fragment 1, absolutely essential for calcium binding, might not play any role while the second kringle region of prothrombin fragment 2 might be directly involved in the binding with factor Va (Vh). However, these experiments had the disadvantage that the factor Va dimerized during the sedimentation analysis; the

prothrombin aggregated at high concentrations of calcium ions, and the system lacked a major component of the prothrombinase complex formation, namely phospholipid membrane surfaces. Also factor Va tends to dissociate into Vh and Vl (light chain); because sedimentation is weight sensitive, Vh was used for the binding assays. Whether factor Va interacts in the same manner is unclear.

The contribution of the fragment 2 peptide to factor Va association requires further characterization. At the time, little was understood about the topographical sites on prothrombin involved in recognition and binding to factors Xa and Va in the prothrombinase complex. Because antibodies recognize and bind the same topographical regions recognized by other proteins they could be useful probes of protein structure. To probe the role of a specific domain of prothrombin during activation, five murine monoclonal antibodies to prothrombin fragment 2 (F2) have been prepared and characterized (Church et al., 1991). Each antibody was tested for its ability to block prothrombin activation. Two of these, αHII-3 and αHII-4, inhibited prothrombin activation to about 90 and 50% levels respectively, but by different mechanisms. Antibody αHII-3 inhibited the factor Va-dependent activation of prothrombin but not by factor Xa alone where as αHII-4 appeared to inhibit factor Xa cleavage directly. The peptide sites recognized by antibodies αHII-3 and αHII-4 represented possible peptide determinants on F2 that were involved in the prothrombinase recognition. However, the data were not conclusive that the sites defined by these two antibodies contained protease (FXa) or cofactor (FVa) binding sites, since the observed inhibitions could be due to steric blocking or an altered conformation resulting from antibody binding to a site removed from the actual enzyme or cofactor site. Further definition of the exact amino acids in the various epitopes needs to be further examined.

Fragment 2 has been reported to associate strongly with α-thrombin (Myrmel et al., 1976). This interaction was observed to slow the rate of inhibition of thrombin by antithrombin about 3-fold (Walker et al., 1979) suggesting a role of fragment 2 of prothrombin as a pseudo-auto inhibitor. Recently, the interaction between fragment 2 and thrombin has been localized using a recombinant protein system (Liaw et al., 1998). Plasma-derived, recombinant-derived, and synthetic peptides of various region of fragment 2 were subjected to binding studies using fluorescently labeled active site-blocked thrombin indicating that a contiguous peptide containing the inner kringle loop and connecting peptide

binds strongly to thrombin and influences its catalytic activity. This N-terminal region of fragment 2 reduces the rate of heparin-catalyzed inhibition of thrombin by antithrombin, alters the rate of thrombin-mediated hydrolysis of chromogenic substrates, and prolongs the thrombin clotting time of fibrinogen in a concentration dependent manner. These observations suggest that the binding of fragment 2 to thrombin result in an allosteric change in the conformation of thrombin, influencing its active site, exosite II (heparin binding) and exosite I. Thus the interaction of fragment 2 to thrombin seems to have significant consequences on thrombin activity, although what this means in a system where the fragment 2 is attached to thrombin remains unclear. However, localized increase of released fragment 2 after prothrombin activation may have an anti-coagulant effect.

Kringle 2 of prothrombin has been suggested to play a role in the prothrombinase complex (Esmon et al., 1974a). Kinetic studies of prothrombin, kringle 1, and kringle 2 domains have been performed to elucidate their roles in the prothrombinase complex activity. Fragment 2 has been shown to inhibit prothrombin activation by the prothrombinase complex or by factor Xa alone, in a dose dependent manner (Taneda et al., 1994). Recombinant prothrombin variants lacking either kringle 1 or kringle 2 domain were subjected to kinetic analysis using a purified prothrombinase complex system (Kotkow et al., 1995; Kotkow et al., 1993). Results indicate that the kringle 2 region is not responsible for prothrombin-phospholipid binding, but influences the catalytic rate of the prothrombinase complex. However, there is still some controversy about the exact role of fragment 2 in the prothrombinase complex. Some evidence suggests fragment 2 binds to factor Va (Kotkow et al., 1995); others have shown fragment 2 binds to thrombin (Arni et al., 1994; Liaw et al., 1998). While a kringle 2-deleted prothrombin has lower catalytic activity as shown by one author (Kotkow et al., 1995), a recent study has shown that the catalytic effect of factor Va in the prothrombinase complex is totally independent of the presence of fragment 2 domain in the substrate (Krishnaswamy et al., 1997). Thus the exact role of the kringle 2 domain, as well as that of the kringle 1 domain, remains to be examined.

3.4. Thrombin

The final protease activated by the coagulation cascade and necessary for the formation of insoluble fibrin clot is α-thrombin (EC 3.4.21.5), a glycosylated trypsin like

serine protease (Magnusson, 1971). Upon activation by the prothrombinase complex, prothrombin is converted into α-thrombin, consisting of A and B chains linked by a disulfide bridge at Cys^{293}-Cys^{339}. In human, the A chain consists of 36 amino acid residues whose function is unclear. A lysine deletion at position 312 in the A chain leads to bleeding complication (Hewitt et al., 1999) suggesting a role for A chain in thrombin function or stability. The B chain comprises the trypsin like protease of 259 residues, and is present in vertebrates as low in the evolutionary ladder as hagfish (Banfield et al., 1992). The crystal structure of human thrombin has been solved in a complex with PPACK, a small active site inhibitor synthetic peptide (D-PheProArg chloromethylketone) (Bode et al., 1989; Bode et al., 1992), with hirudin, a natural thrombin inhibitor from leech (Grutter et al., 1990; Rydel et al., 1990), with a peptide analog of fibrinogen (Martin et al., 1992), with thrombin receptor peptides (Mathews et al., 1994), and with fragment 2 of prothrombin itself (Arni et al., 1993; Arni et al., 1994; Martin et al., 1997; Van de Locht et al., 1996). The thrombin structure resembles that of a "Pacman" with the active site, similar to other serine protease, consisting of a "catalytic-triad" Ser^{195}-His^{57}-Asp^{102} (thrombin numbering), and prefering an Arg-X bond for cleavage. Besides the active site, the B chain has also been characterized to have two other anion exosites essential for its multifunctional properties.

As discussed earlier, thrombin proteolytic activity plays a major role in the coagulation process, namely the activation of platelets and the formation of fibrin. In addition, thrombin induces proliferation and chemotaxis of inflammatory and mesenchymal cells as well as activating endothelial cells. These activities are mediated either through two types of thrombin receptors, PAR-1 (protease activated receptor-1) and PAR-3 or through its non-enzymatic activity (Goldsack et al., 1998).

3.4a. Enzymatic function and thrombin receptor

Thrombin is crucial for the accelerated formation and regulation of a fibrin plug through its enzymatic action on platelets and various clotting proteins. Thrombin activates the procoagulants factors V, VIII, XI and XIII, the anticoagulant protein C. Thrombin activates and aggregates platelets through the proteolytic action on a platelet-derived receptor, a seven-transmenbrane glycosylated protein. The thrombin-receptor has been cloned (Vu et al., 1991), and has been characterized to have a thrombin cleavage site

LDPR/S in the extra cellular amino terminal domain. Upon cleavage, the new receptor amino terminus functions as a tethered ligand and activates the receptor, resulting in platelet activation and aggregation through the receptor-mediated signal transduction pathway. The mechanism was further confirmed when agonist peptides based on this tethered ligand (called TRAPs for thrombin receptor-activating peptides) also proved to be potent activators of platelets. Recently, another thrombin receptor, the protease-activated receptor 3, has been described (Ishihara et al., 1997).

Thrombin also regulates the differentiation and proliferation of neuronal cells (Cunningham et al., 1989; Grand et al., 1989). Thrombin activity on neuronal outgrowth is also linked to a receptor-mediated mechanism although the exact receptor on the neurons remains to be elucidated (Grand et al., 1989).

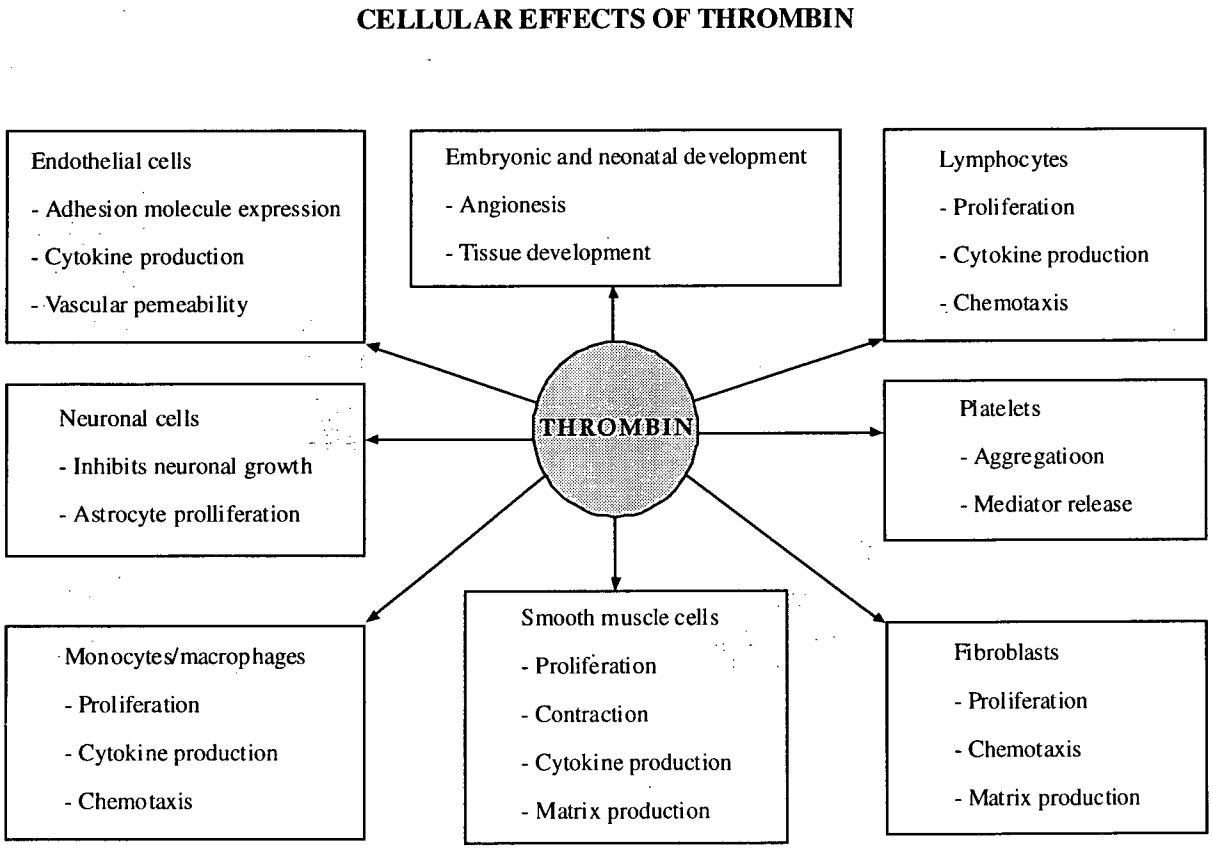
3.4b. Non-enzymatic functions

Thrombin is instrumental in the normal recovery and wound healing phases after coagulation. Thrombin possesses chemotactic properties for human peripheral blood monocytes and macrophages (Bar-Shavit et al., 1983), thus playing a role in the inflammation process. Thrombin exhibits potent mitogenic activity to a number of cells, such as fibroblasts and macrophages (Bar-Shavit et al., 1986; Chen et al., 1975), smooth muscle cells (McNamara et al., 1996; McNamara et al., 1993) and vascular endothelial cells (Harlan et al., 1986). Taken together with the observation that thrombin is also a potent mediator and activator for endothelial cell adhesion (Bar-Shavit et al., 1991) and binding of subendothelial extra cellular matrix (Bar-Shavit et al., 1989), thrombin plays a significant role in wound healing and angiogenesis, the formation of vascular vessels (Figure 10). In addition, from the transgenic mice studies, thrombin is absolutely essential for the development of vascular genesis during embryogenesis as well as post-natal tissue development (Sun et al., 1998; Xue et al., 1998).

3.4c. Antithrombotic therapy

Thrombin has been shown to participate in thrombotic disease states, such as venous thrombosis, stroke and pulmonary emboli (Fenton et al., 1998). Thrombin has been

Figure 10. The cellular effects of thrombin.



implicated in the growth and metastasis of certain cancers, and in the etiology of Alzheimer disease (Wagner et al., 1989). In addition, because of the ability of thrombin to bind to the extra cellular matrix and endothelial cells, and its mitogenic activity on these cells, thrombin may be a risk factor involved in the formation of atherosclerotic plaque. Tradditionally, coumarins and heparin are the only effective clinical antithrombotics. Orally taken coumarin and its derivatives are inhibitors of the γ-carboxylation process, resulting in production of Gla-less non-functional clotting proteases. Heparin therapy is usually used in surgical and acute thrombotic situations. Heparin and its fragments (small molecular weight heparins) enhance the inactivation of thrombin by the serpin antithrombin III. Recently recombinant hirudin and synthetic analogs, such as Bivalirudin (Hiulog©), have been advocated as potential antithrombotic agents (Fenton et al., 1998). In other areas, the field of small synthetic peptides as reversible, specific inhibitors of thrombin active site has been actively developed. Numerous small synthetic peptides and peptide analogs have been developed for the crystallographic structure of thrombin (Pavone et al., 1998), serving as important models for drug development in clinical therapy. Effective antithrombotic therapy must be vigorously regulated and monitored, however, as inhibition of thrombin and other proteases may lead to excessive bleeding complications.

4. Activation of prothrombin

The serine protease thrombin is crucial in the maintenance and regulation of the coagulation process as well as other haemostatic events through its cellular effects. Thrombin may also play physiologically significant roles during embryogenesis and tissue development. Thus the formation of the serine protease α-thrombin is an extremely important event in the body. The precursor zymogen, prothrombin, is activated to thrombin on negatively charged phospholipids of platelet and endothelial cell membranes by the proteolytic activity of an enzymatic complex termed the prothrombinase complex. The prothrombinase complex is comprised of a protease, factor Xa, a protein cofactor, factor Va, phospholipids and calcium ions. The substrate of the prothrombinase complex is the plasma protein prothrombin. There are four similar complexes in the coagulation systems: the FVIIa:TF complex, the tenase complex, the prothrombinase complex, and the protein Case or thrombin:TM (thrombomodulin) complex. The first three complexes are pro-coagulant and

are instrumental in the initiation and amplification of the clotting process, while the last complex is anti-coagulant and plays an important role in the regulation of clotting. All of the complexes are made up of similar components: a protease, a substrate, a protein cofactor, phospholipids and calcium ions (Figure 3).

4.1. The prothrombinase complex

The protease of the prothrombinase complex is the pro-coagulant factor Xa that is activated from its zymogen precursor, factor X. Factor X is a circulating plasma protein of 59,000 Daltons. The factor X structure begins with a Gla domain, followed by two epidermal growth factor (EGF) like domains and a catalytic serine protease N-terminal domain. Upon activation, factor X is converted into a two-chain peptide linked by a disulfide bridge, a light chain (Gla-EGFs region) and a heavy chain (protease region). The factor VIIa:TF (tissue factor) complex activates a small amount of factor X at the onset of coagulation. The sustained and amplified production of factor Xa is continued mainly through the activity of the tenase complex. The tenase complex consists of the plasma protease, factor IXa, a cofactor, factor VIIIa, as well as phospholipids and calcium ions.

The cofactor of the prothrombinase complex is factor Va that is activated from its precursor, factor V (330,000 Daltons). Factor V is a circulating plasma glycoprotein with a structure consisting of two homologous A-type domains, a connecting B-type domain, followed by another A-type domain and two C-type domains (Rosing et al., 1998). Factor V is activated into the pro-coagulant factor Va via the proteolytic activity of its physiologic protease thrombin. Upon activation, the B domain of factor V is cleaved off the full length protein, and factor Va is formed as a two-chain polypeptide consisting of a heavy chain (the amino terminal A1-A2 domains) and the light chain (the carboxy terminal A3-C1-C2 region). Unlike factor Xa, the light and heavy chain of factor Va are associated by a single calcium ion and not with disulfide linkages. Besides thrombin, factor V is also cleaved by factor Xa, meizothrombin, an activated intermediate of thrombin, and the anti-coagulant activated protein C.

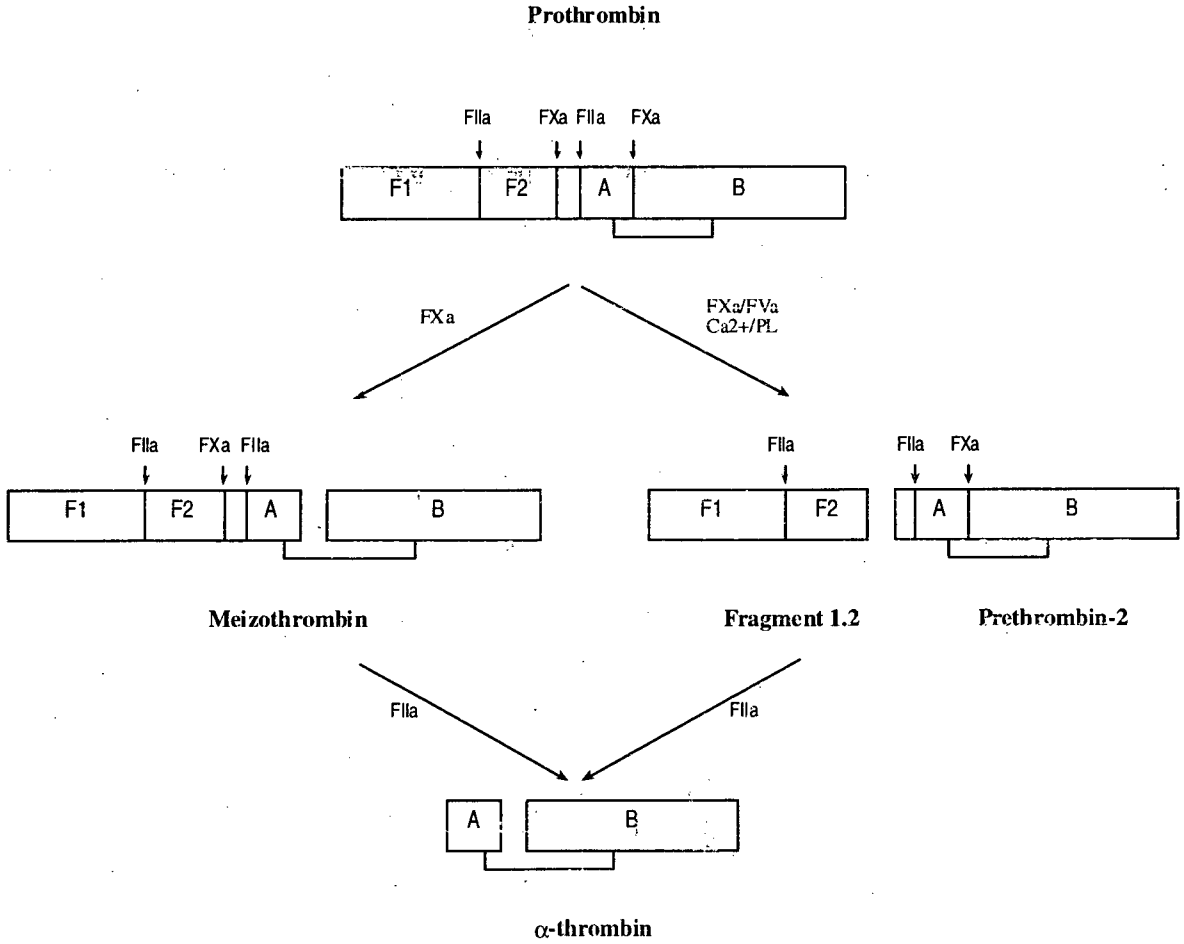
The other important cofactors for the prothrombinase complex besides factor Va are negatively charged phospholipid surfaces and calcium ions. Calcium ions present at physiological concentration in plasma play important roles in many haemostatic events.

Vitamin K-dependent proteins have γ-carboxyglutamic acid rich regions that bind calcium in a co-operative manner necessary for the optimal activity of their functions. This metal binding property is directly linked to the phospholipid binding property of these proteins. Platelet and endothelial cell membranes contain phosphotidylserines that contribute to the overall negative charge on these surfaces. When vitamin K-dependent proteins bind calcium, they undergo conformational changes in their structure that facilitate optimal binding to the membranes. In addition, calcium ions may act as bridging agents for this particular interaction between proteins and membrane surfaces.

4.2. Mechanism of activation of the prothrombinase complex

The activation of prothrombin into thrombin has been noted as a proteolytic event (Owen et al., 1974) and has been characterized extensively for decades. The exact mechanism of the activation of prothrombin by the prothrombinase complex is still not clearly understood because of the complexity inherent in a multi-component system that is distributed between a fluid phase (plasma) and semi-solid phase (phospholipid membrane). Factor Xa-catalyzed activation of prothrombin into thrombin occurs via two peptide bond cleavages at positions Arg^{271} and Arg^{320}. In the presence of the prothrombinase complex, the first cleavage at Arg^{320} is preferred giving rise to an activation intermediate termed meizothrombin (Krishnaswamy et al., 1986). Cleavage of the second site at Arg^{271} by the prothrombinase complex released the N-terminal region termed fragment 1.2, the Gla-kringle 1-kringle 2 region, and thrombin, the N-terminal catalytic protease (Figure 11). In the presence of only the protease factor Xa and calcium ions, the Arg^{271} cleavage is preferred resulting in fragment1.2 (Gla-kringle1), and prethrombin-2 (Krishnaswamy et al., 1987). In addition to the proteolytic cleavages in the prothrombin molecule catalyzed by factor Xa, thrombin formed during the activation process is capable of catalyzing two cleavages on prothrombin itself. The first thrombin-catalyzed feedback cleavage is at position Arg^{155}/Ser^{156} of fragment 1.2 yielding fragment 1 (Gla-kringle 1) plus fragment 2 (kringle 2) (Nesheim et al., 1983). The second cleavage is at position Arg^{284}/Thr^{285} on the A chain of α-thrombin resulting in a form of α-thrombin moiety with 13 residues deleted from the its amino terminus (Figure 11).

Figure 11. Activation of prothrombin. Prothrombin is activated by the proteolytic action of either the protease factor Xa (FXa) alone, or the prothrombinase complex that includes factor Xa, the cofactor Va (FVa), and phospholipid membrane (PL) and calcium ions (Ca^{2+}). The end product is the formation of thrombin (FIIa). F1 represents fragment 1 of prothrombin (the Gla domain and the first kringle). F2 is the second kringle domain. A and B are the two polypeptide chains of thrombin.


Prothrombin
FIIa
FXa
FIIa
FXa
F1
F2
A
B
FXa
FXa/FVa
Ca2+/PL
FIIa
FXa
FIIa
F1
F2
A
B
FIIa
F1
F2
FIIa
FXa
A
B
Meizothrombin
Fragment 1.2
Prethrombin-2
FIIa
FIIa
A
B
α-thrombin

The presence of the cofactor Va and phospholipids in the prothrombinase complex increase the catalytic efficiency of prothrombin activation by factor Xa between 6,000-14,000 thousand fold (Rosing et al., 1998). Under a Michaelis-Menten kinetic analysis approximation for the activity of the prothrombinase complex, phospholipids increase the K_m of prothrombin (binding constant of prothrombin to the complex) while factor Va increases the V_{max} (optimal reaction rate), as well as the K_m for prothrombin. The lowering of the K_m by cofactor Va is observed when membranes with a low phosphotidylserine content are used. Thus the cofactor factor Va and phospholipid membranes function to increase the catalytic activity of the prothrombinase complex by promoting and localizing interactions between the protease factor Xa and its substrate prothrombin.

5. Congenital prothrombin disorders

Prothrombin plays crucial roles in the coagulation, anti-coagulation, fibrinolysis, inflammation and repair systems. Thus a deficiency or an abnormality in the protein will lead to catastrophic consequences in the function and maintenance of haemostasis. The first prothrombin deficiency was reported in 1947 (Quick, 1947), and the first prothrombin abnormality was found in 1969 (Shapiro et al., 1969). Since then, many others have been characterized. Congenital prothrombin disorder can be classified into three categories: first, a "true" deficiency or hypoprothrombinemia; second, prothrombin abnormalities or dysprothrombinemia; and third, a combined disorder involving other factors (Girolami et al., 1998).

Prothrombin true deficiency is a very rare disorder, and could be either homozygous or compound heterozygous. The level of the protein antigen is markedly reduced with protein activity. Homozygous hypothrombinemia would probably be lethal at birth, as there are only about 20 cases of hypoprothrombinemia reported worldwide. Recent studies in transgenic mice deficient in prothrombin gene have shown that the inactivation of the gene led to partial embryonic death and complete lethality of surviving neonatal mice. The embryonic death (between 9.5 and 11.5 days of embryogenesis) resulted from bleeding in the egg yolk sac and tissue necrosis, while the surviving mice neonates developed fatal hemorrhagic events and ultimately died within a few days of birth (Sun et al., 1998; Xue et al., 1998).

The second type of prothrombin disorder is prothrombin abnormality, also divided into homozygous, heterozygous and double or compound herterozygous categories. These conditions are mostly characterized to have a higher level of antigen compared to the clotting activity. The antigen level can sometimes be normal; thus the disorders result from a dysfunctional prothrombin molecule, hence the term dysprothrombinemia. About twenty-four families of dysprothrombinemia have been characterized thus far (Figure 12), not all have been characterized at the molecular level. The abnormalities occur in all recognized domains of prothrombin with the most in the catalytic domain, and often as a result of a single amino acid substitution. Two main types of prothrombin defects have been characterized: defects in the activation mechanism (prothrombins Barcelona, Madrid, Clamart), and defects in the thrombin catalytic activity (prothrombins Quick, Metz, Salakta, Tokushima, Himi). Other mutations include the thrombin-fibrinogen binding site (prothrombins Molise), and recently prothrombin Greenville (Arg^{517}-Gln) (Henriksen et al., 1998), a mutation at the Na^+ binding site of thrombin severely affecting the interaction of thrombin with fibrinogen. A lysine deletion at position 312 (in the α-chain of thrombin) has recently been found in a Vancouver family (Hewitt and MacGillivray, unpublished result). A recombinant prothrombin carrying this deletion mutation has been produced in BHK cells and studied. The exact cause for the clinical phenotype of this particular mutation remains to be determined, but probably involves protein stability.

The third type of prothrombin disorder is a combined defect involving multiple mutations and or polymorphisms in prothrombin and other clotting plasma proteins. The main combined defects of prothrombin are those usually associated with clotting factors VII, IX, X, Va and protein C or S (Brenner et al., 1990), although very few documented cases of this type of disorder have been described. The defect is probably associated with the carboxylation process that is common to each of these proteins (except factor Va). Recently, a G $\rightarrow$ A transition polymorphism at nucleotide position 20,210 in the 3'-untranslated region of prothrombin gene has been reported to be associated with elevated plasma prothrombin levels and an increase risk of deep vein thrombosis (Poort et al., 1996), and cerebral venous thrombosis (Reuner et al., 1998). In addition, the G20,210A transition in prothrombin has been found to be associated with another prevalent thrombotic risk, factor V-Leiden (Ehrenforth et al., 1998; Leroyer et al., 1998; Lewandowski et al., 1998). Factor V-Leiden is

Figure 12. Congenital prothrombin deficiencies[a]

Abnormality	Activity (% of normal)	Antigen (% of normal)	Nature of defect
Barcelona (Mardrid, Obihiro)	12 (3,18)	100 (100,105)	Factor Xa cleavage (Arg^{271}-Cys)
Padua (Drahran)	50 (6)	100 (100)	Factor X cleavage (Arg^{271}-His)
Clamart	50	100	Factor Xa cleavage (Arg^{321}-Ile)
Houston	5-9	51	Factor X cleavage
Gainesville	23	70	Factor Xa cleavage
Perija	2	70	Factor Xa cleavage
Molise (Tokushima)	11 (11)	45 (42)	Thrombin catalytic region (Arg^{418}-Trp)
Habana	10	50	Thrombin catalytic region
Metz	10	50	Thrombin catalytic region
Corpus Christi	2	25	Thrombin region (Arg^{382}-Cys)
Quick I and II	<2	34	Thrombin-fibrinogen region (Arg^{382}-Cys, Gly^{558}-Val)
Himi I and II	10	88	Thrombin-fibrinogen region (Met^{337}-Thr, Arg^{388}-His)
Salakta (Frankfurt)	16 (13)	100 (91)	Thrombin-fibrinogen region (Glu^{466}-Ala)
Greenville	51	102	Thrombin-fibrinogen region (Arg^{517}-Gln)
Vancouver[b]	5	5	Thrombin region (ΔLys^{302})
Camberra	?	?	Pro-region (Glu^{157}-Lys)
San Juan	20	93	Calcium-binding site
Segovia	7-20	100	Fragment 2 defect ?
Cardeza	50	100	Prethrombin 2 region
Brussels	46	88	?
Birmingham I and II	8-10, <3	24-30, 8-14	?
Poissy	2	47	?
Magdeburg	44	90	?

[a] data were taken from Girolami, 1998

[b] unpublished result

an Arg^{506} to Gln mutation at a predominant cleavage site that leads to a factor V resistant to the proteolytic cleavage by the anticoagulant protease activated protein C (for review, see Bertina, 1997 and Rosing, 1998). The relationship at the molecular level between the combined defects and the heterogeneity of the prothrombin gene, and their clinical manifestation remain to be studied further.

True deficiency of the homozygous disorder is always accompanied by severe and several bleedings, often life-threatening. The heterozygous defect will have excessive bleeding often after a surgical procedure, such as tooth extract or tonsillectomy, but for the most part appears normal. Since heterozygosity is often asymptomatic, the homozygotes need prompt treatment in case of bleeding or adequate prophylaxis before surgical procedures. Similar to other clotting factors, therapy for prothrombin deficiency involves administration of plasma, (activated) prothrombin complex concentrates (aPCC, PCC), or both. There have been recent ventures into recombinant protein substitution for plasma concentrate, for example recombinant factor IX and recombinant factor VIIa (NovoSeven, NovoNordisk A/S), but none has been developed for prothrombin.

III. PRESENT STUDY

Structural-functional relatioships of the protease domain of prothrombin have been characterized extensively. In contrast, except for the Gla domain, the functions of the other non-enzymatic domains are poorly understood, particularly the kringle domains. The aim of this study was to establish the structural-functional relationships of prothrombin's non-enzymatic domains. The approach was to construct prothrombin variants having these domains different from those of the wild type and to produce the corresponding recombinant proteins in tissue culture. The variants were constructed using recombinant DNA techniques and the proteins were expressed in BHK cells. The secreted recombinant proteins were purified and subjected to assays such as calcium and phospholipid binding, and coagulation assays. The Gla content of the recombinant proteins were determined using a novel method and were correlated with the biological activities of the proteins. The recombinant proteins were tested for their ability to be activated by factor Xa and by a purified prothrombinase complex. The information obtained gives insight into the roles of the various domains of the protein and a correlation between the structure and its function can be established.

CHAPTER 2

MATERIALS AND METHODS

I. MATERIALS

Most laboratory chemicals were purchased from Fisher Scientific Ltd. (Nepean, ON), GibcoBRL (Burlington, Canada) or the Sigma Chemical Co. (St. Louis, MO). Restriction enzymes and DNA modifying enzymes were from Gibco BRL, New England Biolab (Mississauga, ON) and Pharmacia (Baie d' Urfé, Quebec). Sequenase 2.0 was purchased from United States Biochemicals (Cleveland, Ohio). Oligonucleotides were synthesized on an Applied Biosystems 391A DNA Synthesizer. The Bio-Rad Reagent Concentrate for protein assay was from Bio-Rad Laboratories (Richmond, CA). For tissue culture, the media DMEM-F12, new born serum (NBS), trypsin-EDTA, and AlbuMAX were from GibcoBRL (Burlington, Canada), insulin and apo-transferin and sodium selenite were from Sigma Chemical Co. (St. Louis, MO). Vitamin K1 was purchased from Abbott Laboratories Ltd, (Saint-Laurent, Quebec), and Faulding (Canada) Inc. (Vaudreuil, Quebec) provided the Methotrexate Sodium. The polyclonal sheep anti-human prothrombin antibody, SAFII-IG, was from Affinity Biologicals Inc. (Halmilton, ON), and the anti-sheep IgG Alkaline Phosphatase Conjugate was from Sigma Chemicals Co. For gamma-carboxyglutamic analysis, the amino acid standard L-aspartic acid, L-glutamic acid, L-γ-carboxyglutamic acid, electrophoresis reagent grade Tris, and FITC isomer I (98 %) were purchased from Sigma-Aldrich Canada Ltd., Mississauga, Ont., Canada. HPLC grade acetone and perchloric acid (69-72 %), and capillary tubes (open ends, 1.5-1.8-100 mm, KIMAX-51) were from Fisher Scientific Canada, Nepean, Ont., Canada. For the clotting assay, FII-deficient plasma and Thromboplastin with Calcium were purchased from Sigma Diagnostics, St. Louis, MO, USA. The PCPS and DOPE:DODAC were generously donated from Dr. Pieter Cullis's lab, Department of Biochemistry and Molecular Biology, UBC, Vancouver BC, Canada. For the enzyme kinetic experiments, plasma human thrombin, human factor Va and Xa were purchased from Haematologic Technologies Inc., Essex Junction,Vermont, USA. The chromogenic substrate S-2238 was from Chromogenix (Mölndal, Sweden). The synthetic peptides were made by the Nucleic Acid and Protein Science Unit, UBC, British Columbia.

1. Strains

Escherichia coli (E. coli) strain DH5αF' was the host strain for the transformation of circularized DNA plasmids. The genotype of this cell line is: F', endA1, hsdR17(r^-_k,m^+_k), supE44, thi-1, λ-, recA1, gyrA96, relA1, Δ(argF-laczya)U169, ϕ80dlacZM15. The ϕ80dlacZM15 marker provides α-complementation of the β-galactosidase gene from BlueScript vectors and, therefore, can be used in blue/white screening of colonies on bacterial plates containing X-gal and IPTG. The *E. coli* strain JM110 (genotype: dam dcm supE44 hsdR17 thi leu rpsL lacY galK galT ara tonA thr tsx (lac-proAB) F'[traD36 $proAB^+$ lacIq lacΔZM15]) was the host for plasmids that were subjected to restriction digest by the methylation sensitive enzyme BsmI.

For protein expression, a thymidine kinase deficient line of Baby Hamster Kidney (BHK) cells was kindly provided by Dr. R. Palmiter at Howard Hughes Medical Institute, University of Washington, Seattle, USA.

2. Vectors

The phagemids BlueScript KS and SK were obtained from Stratagene (La Jolla, San Diego) to subclone DNA fragments for propagation and amplification in bacterial hosts. The expression vector pNUT was used to produce recombinant proteins in BHK cells (Palmiter, 1987).

3. Media

Luria broth, (LB), supplemented with 100 μg/ml of ampicillin was used for bacterial growth. For selection of plasmid-containing bacteria, cells were spread on LB-agar (12.5 %) plates supplemented with 100 μg/ml of ampicillin, 25 μg/ml of IPTG and 50 μg/ml X-Gal. For the isolation of single-stranded DNA from the phagemid BlueScript, cells were cultured in TYP medium.

For tissue culture, DMEM-F12 media supplemented with 5 % NBS was used as the base medium for the mammalian BHK cells. During transfection, 0.44 mM methotrexate was added to select for sucessfully integrated plasmid into the cellular genome (Fann et al., 1999). For expression of recombinant proteins, DMEM-F12 medium was supplemented with

AlbuMax (0.2 mg/ml), insulin (1 g/L), apo-Transferrin (0.50 g/L), sodium selenite (0.67 mg/L), ethanolamine (0.20 g/L) and vitamin K1 (10 μg/L).

II. RECOMBINANT DNA TECHNIQUES

1. Agarose gel electrophoresis

Electrophoresis was the standard method for the seperation, identification and purification of DNA fragments. Agarose was a commercially available seaweed polymer of D-galactose and 3,6-anhydro-L-galactose which when melted can be cast in any size, shape and porosity necessary for a particular usage. The running buffer for the agarose gel was 1X TAE buffer (40 mM Tris, 16 mM NaOAc, 1 mM EDTA, pH 8.0). DNA fragments were visualized under ultraviolet light due to the presence of ethidium bromide (1 μg/ml) inside the agarose gel. The loading buffer was 60 % glycerol, 0.2 % xylene cyanol and 0.2 % bromophenol blue. The dyes were used to mark the migration of the DNA fragments when the gel was subjected to an electrical field at 5-10 volts/cm.

2. Denaturing polyacrylamide gel electrophoresis

Acrylamide, a monomer, was preferred over agarose in denaturing gel electrophoresis to separate small fragments of DNA because of its superior resolving power. In the presence of the free radical, ammonium persulfate, and TEMED, a cross linker, a denaturing (8.3 M urea) polymerized acrylamide gel was used mainly for DNA sequencing where DNA fragments (as small as 20 bp) of one base difference can be resolved. The buffer for electrophoretic separation was 1X TBE buffer (89 mM Tris- borate, 2 mM EDTA, pH 8.3). The loading buffer was 95% formamide, 10 mM EDTA, 0.2% xylene cyanol and 0.2 % bromophenol blue. The gel was run at constant power (35-40 W for a 20-cm x 40 cm gel; ~ 1700 volts) until the optimal resolution was obtained.

3. Polymerase chain reaction

The polymerase chain reaction (PCR) was the single most powerful technique in the laboratory today since the discovery of the synthesis of oligonucleotides. Used in biotechnology and medical diagnostics, PCR revolutionizes the analysis and amplification of very small amounts of DNA templates. In the laboratory, PCR was used often to amplify cDNAs from a variety of genomic sources, and also to make specific changes in these cDNA sequences, a process called PCR mutagenesis. Typically in a PCR reaction, a pair of oligonucleotides or primers flanking the region of the nucleic sequence of interest was allowed to anneal to a specific, denatured DNA template; the primers were then extended by the use of a thermal stable polymerase such as Taq polymerase. After extension, the DNA was denatured again, and the same process of annealing, elongation and denaturation was repeated for many cycles. A PCR reaction of 100 μl in volume would include the primers (100 ng each), DNA template (1 μg), polymerase buffer (67 mM Tris pH 8.8, 6.7 mM $MgSO_4$, 16.6 mM $(NH_4)_2SO_4$ and 10 mM β-mercaptoethanol), and Taq Polymerase (1-2 U). Beginning with a "hot start" step, a denaturing step at 95°C for 5 min, the condition for the PCR reaction was 30 cycles of denaturation at 95°C, followed by annealing at 50-60°C, and followed by elongation at 72°C. The temperature of the annealing step depends on the sequence and length of the designed primers. The amplified fragments of DNA were visualized by agarose gel electrophoresis.

4. Restriction enzyme digest

A typical reaction contained a DNA template, the appropriate buffer, and the restriction enzyme(s) in a small volume of 20-30 μl. The reaction was carried out at the appropriate temperature for the restriction enzyme, usually at 37°C for about 1-2 hr. The digested DNA fragments could be recovered and purified by electroelution and or centrifugal-elution from an agarose gel (see next section).

5. Blunt-ending of DNA fragments

Occasionally, for sub-cloning into a blunt-end site such as SmaI or HincII, the DNA obtained from a restriction enzyme digest had to be made blunt-ended. This was achieved by the use of large fragment of DNA Polymerase I (Klenow fragment) which degraded ds or ss-

DNA from a free 3'-hydroxyl end, and synthesized DNA from a free 5'-phosphate end. The reaction was identical to a restriction enzyme digest reaction, except free deoxynucleotides (dNTPs) were added for the filling at the free 5' end of the DNA fragment.

6. Purification of DNA fragments from agarose gels

The digested DNA was subjected to electrophoresis on an agarose gel and visualized under UV illumination. The DNA fragments were excised from the gel and eluted from the agarose by two different methods. The first method was electro-elution in a dialysis tubing. The DNA was submerged in a small volume of running buffer (200 µl) inside a small piece of dialysis tubing (30K MW cut-off), and was subjected to electrophoresis. The DNA fragment under the electrical field migrated out of the agarose into the buffer inside the dialysis tubing and was subsequently recovered. The second method of elution was by centrifugation: the DNA was placed in a chamber (a cutoff eppendorff tube with a needle small hole in the bottom) containing a small plug of glass wool; the chamber was placed on top of another eppendorff tube and was centrifuged at 5000 rpm for 7-10 min; the soluble DNA in the liquid phase could then be removed from the solid agarose which remained in the glass wool chamber. The recovered DNA in buffer solution was usually extracted once with phenol:chloroform and the DNA was precipitated with 3x volume 95% EtOH and 0.5 volume 7.5 M $(NH_4)_2SO_4$. The precipitated DNA was collected by centrifugation at 13000 rpm for 10 min, and washed extensively with 70% EtOH. The air-dried DNA pellet was resuspended in sterile dH_2O or TE buffer for subsequent reaction.

7. Ligation and transformation

The digested DNA fragment was ligated into a plasmid vector digested with the appropriate restriction endonuclease(s). The reaction was similar to the digestion reaction, typically containing the DNA insert, the plasmid vector, ligation buffer (50 mM Tris pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, and 0.1 mg/ml BSA). The ratio of DNA insert to vector was 3 to 1 for sticky end ligation, and ususally 5-10:1 for blunt-end ligation. The reaction was carried out at 16°C overnight for blunt-ended ligation, and at room temperature for 2-4 hr for sticky end ligation.

A small aliquot of the ligated product (5 µl) was added to 50 µl of thawed competent DH5αF' cells on ice. Mixing was done by gentle pipetting and the mixture was left on ice for 30 min. After 2 min of heat shocking at 37°C, the mixture was added to 200 µl of LB media, and was shaken in a 37°C incubator for 45 min. The mixture was spread evenly onto several X-gal (50 mg/ml)/ IPTG (25 mg/ml)/ LB-Amp (100 mg/ml) plates. For the plasmid BlueScript, ampicillin was required for the selection of successfully transformed cells, while the X-gal/IPTG were required for the selection of successful integration of the insert into the plasmid. The pNUT vector did not contain the lacZ gene in the polycloning site and therefore did not require X-gal/IPTG for the white/blue color selection. The plates were then incubated at 37°C for 12-16 hr until visible colonies were observed.

8. Isolation of plasmid DNA

8.1. Alkaline lysis method

A single colony from a transformed plate was picked and grown in 5 ml of LB-Amp (100 mg/ml) media at 37°C for 16 hr with vigorous shaking. The bacteria were collected by centrifugation on a bench top centrifuge at 3000 rpm for 15 min. The cell pellet was resuspended in 200 µl of ice cold Glucose buffer (50 mM Glucose, 25 mM Tris pH 8.0, 10 mM EDTA), and 400 µl of freshly made lysis solution (0.2 M NaOH, 1% SDS) was added to the cell suspension. The solution was mixed by inversion and placed on ice for 5-10 min. To the lysed, viscous solution was added 300 µl of 3M NaOAc pH 4.8 and a precipitate consisting of cell debris and protein appeared. The precipitate was centrifuged at 13000 rpm for 10 min, and to the supernatant was added 450 µl of isopropanol and left at RT for 5 min after vortexing. The precipitated plasmid DNA was centrifuged at 13000 rpm for 10 min, the DNA pellet was resuspended in 200 µl of TE buffer, and incubated at 68°C for 5 min to denature and precipitate contaminating proteins. To the TE solution was added 100 µl of 7.5 M $(NH_4)_2SO_4$ pH 7.5 and 1 ml of 95% EtOH. The DNA was collected by centrifugation as before and the subsequent DNA pellet was washed extensively with 70% EtOH, followed by centrifugation. The final DNA pellet was air-dried and resuspended in 100 µl of sterile dH_20

and or TE buffer. A small aliquot of 5 µl was used for restriction digestion, while 40 µl was usually needed for DNA sequence analysis.

8.2. Quick boiling method

Individual colonies of bacteria were grown in 2 ml of LB-Amp media for 16 hr at 37°C with shaking. The bacterial cells were collected in an Eppendorf tube at 13000 rpm for 1 min. To the bacterial pellet was added 200 µl of lysis buffer TELT (50 mM Tris pH 7.5, 62.5 mM EDTA, 2.5 M LiCl, 0.4% Triton X-100). The solution was heated at 95°C for 2 min and centrifuged at 13000 rpm for 10 min at 4°C. The cellular debris/protein pellet was removed using a toothpick, and 100 µl of isopropanol was added to precipitate the DNA at –20°C for 10 min. The DNA was collected by centrifugation at 13000 rpm for 10 min and was washed in a manner similar to the alkaline lysis method. The final pellet was resuspended in 30 µl of sterile dH_2O or TE buffer. The plasmid DNA (5-10 µl) was further digested with the appropriate restriction enzyme to check for the integrity of the ligation reaction. The boiling prep was usually preferred for restriction enzyme analysis of ligated plasmid DNA because of its speed, while the alkaline lysis prep (which yielded purer DNA) was better for DNA sequence analysis.

9. Isolation of phagemid single-stranded DNA

The plasmid BlueScript is a phagemid such that both dsDNA and ssDNA can be prepared by using appropriate bacterial host strains and helper phage. Usually 30 µl of helper phage M13K07 (10^8 pfu/ml) was added 4 hr after the 2 ml bacterial culture has been seeded in TYP broth (16 g/L yeast extract, 16 g/L bacto tryptone, 10 g/L NaCl, 2.5 g/L K_2HPO_4) containing Ampicillin (100 mg/ml). The culture was shaken for 60 min at 37°C for expression of Kanamycin resistance from the helper phage. Kanamycin was then added at a final concentration of 50 mg/ml to inhibit the growth of uninfected bacteria and the culture was shaken at 37°C for another 16 hr. The cells were collected the next day by centrifugation at 3000 rpm for 15 min on a bench top centrifuge. To the supernatant was added ¼ volume of PEG6000:$(NH_4)_2SO_4$ (50:50 volume of 50% PEG and 7.5 M salt) solution. The phage were precipitated by incubation at 4°C for more than 30 min followed by centrifugation at

10000 rpm for 20 min. The phage pellet was resuspended in 200 µl of TE and the phage protein was removed by extracting the solution with equal volume of phenol:chloroform (50:50) several times. Extraction was accomplished by vortexing the solution vigorously for 1 min followed by 1 min of brief centrifugation in Eppendorf tubes. To the final aqueous solution was added 100 µl of 7.5 M $(NH_4)_2SO_4$ and 1 ml of ice cold 95% EtOH and the solution was incubated at –20°C for 15 min. The ssDNA was collected by centrifugation as described previously, followed by the usual washing and resuspension. The production of ss DNA was necessary since some DNA fragments were difficult to sequence in their ds form. DNA sequence analysis with ssDNA often yielded clearer and longer sequences.

10. DNA sequence analysis

DNA sequence analysis was done by the chain termination method as described by Sanger. The dsDNA was denatured chemically by alkali and a specific primer was allowed to anneal to the denatured template. Elongation of the primer was achieved using the modified DNA polymerase Sequenase2.0 and a mixture of all four deoxyribonucleotides (including radioactive ^{35}S-dATP) and a single dideoxyribonucleotide. For every template, a set of four reactions was required for the four dideoxynucleotides. The reactions were subjected to denaturing polyacrylamide gel electrophoresis, and the gel was blotted onto Whatman paper and dried under vacuum. The dried gel-paper was exposed to Kodak film overnight at RT. Following the film development, the DNA sequences were read manually, and the sequences were entered into a personal computer and processed using the PCGene computer program.

III. PROTEIN CHEMISTRY TECHNIQUES

1. SDS-Polyacrylamide gel electrophoresis

Electrophoresis in SDS-polyacrylamide gels was used for the separation and visualization of proteins. The gel consisted of a stacking phase (0.125 M Tris pH 6.8, 0.1% SDS, 3.9% acrylamide/bis, 0.05% ammonium persulfate, 0.1% TEMED) sitting on top of a

separating phase that was similar in composition to the stacking gel except that it has 0.375 M Tris pH 8.8, and between 6-15% acrylamide/bis. The acrylamide/bis solution was made out of 29.2% acrylamide (w/v) and 0.8% N'N'-bis-methylene-acrylamide (w/v). A sample of protein was mixed with ¼ volume of 4x loading buffer (0.0625 M Tris pH 6.8, 2% SDS, 0.715 M 2-β-mercaptoethanol, 0.00125% (w/v) bromophenol blue, and 10% glycerol). The mixture was heated at 95°C for 3 min, centrifuged briefly and loaded onto the gel. The 5X running buffer consisted of Tris base (15 g/L), glycine (72 g/L), and SDS (5 g/L). The gel was subjected to electrophoresis at a constant voltage of 200 V for 30-45 min. After electrophoresis, the gel was stained with 0.1% Coomassie Blue R-250 stain in fixative (40% MeOH, 10% HOAc) for 1 hr, and was destained with 40% MeOH/10% HOAc to remove the background (usually 1-2 hr with changing). The proteins were visible as blue bands against a clear background.

2. Western blot analysis

Western blot anlysis is a very sensitive technique to identify proteins using specific antibodies as probes. The protein sample was subjected to electrophoresis via SDS-PAGE and was transferred onto a solid support such as nitrocellulose membrane. A primary antibody was absorbed specifically to the antigenic epitopes displayed by the targeted protein. A secondary antibody recognized the primary antibody, and containing a chemical conjugate (such as alkaline phosphatase (AP) or horseradish peroxidase (HRP)) attached to the secondary Ab, such that visualization of the targeted protein could be achieved. After electrophoresis, the nitrocellulose blot was rinsed well with TBST buffer (8 g/L NaCl, 0.2 g/L KCl, 3 g/L Tris pH 7.4, 0.005 % Tween-20 (v/v)), and was blocked with 2% dry powder skim milk in TBST buffer for 1 hr. After several rinses with TBST, the blot was incubated with a primary Ab in TBST for 1 hr, washed extensively, and followed by incubation with the secondary Ab/AP conjugate incubation. After a final wash, the blot was immersed in 10 ml of AP buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris pH 9.5). The substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) was converted into a blue compound by the alkaline phosphatase conjugate. To the solution was added 66 µl of NBT and 33 µl of BCIP. The color conversion takes about 1-5 min, after which the blot was photographed for a permanent record of the experiment.

3. Bio-Rad protein assay

The Bio-Rad assay (Bio-Rad Inc.) was a dye-binding assay based on the differential color change of a dye in response to various concentrations of protein. The dye binds to the peptide backbone and tryptophan residues of proteins. It was used widely for the determination of the concentration of proteins using a known standard protein. The assay was based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when bound to protein. For the microassay procedure, several serial dilutions of protein standard in 0.8 ml volume were prepared from 1 to 25 mg/ml in Eppendorf tubes. To each sample was added 0.2 ml of Dye Reagent Concentrate. The samples were mixed by inversion several times, and were incubated at RT. After a period from 5 min to 1 hr, the absorbance at 595 nm was measured against a reagent blank. A standard curve of OD_{595} versus protein concentration (μg/ml) was plotted. Unknown samples were prepared similarly and the concentrations were determined from the standard plot.

CHAPTER 3

EXPERIMENTAL APPROACH

I. CONSTRUCTION OF RECOMBINANT PROTHROMBIN VARIANTS

The plasmid pBIII containing the full-length human prothrombin cDNA (MacGillivray et al., 1986) was modified using PCR to eliminate the G/C tail. The PCR fragment was subcloned into a BlueScript vector for propagation in DH5αF' cells. The nucleotide sequence of the subcloned cDNA was determined to verify the absence of PCR-induced errors. A *NotI* site was engineered into both ends of the cDNA for subcloning into the expression vector pNut that also has a NotI site. The bovine kringle sequence was amplified from the plasmid pBIII by using specific primers (MacGillivray et al., 1984).

1. Prothrombin variants

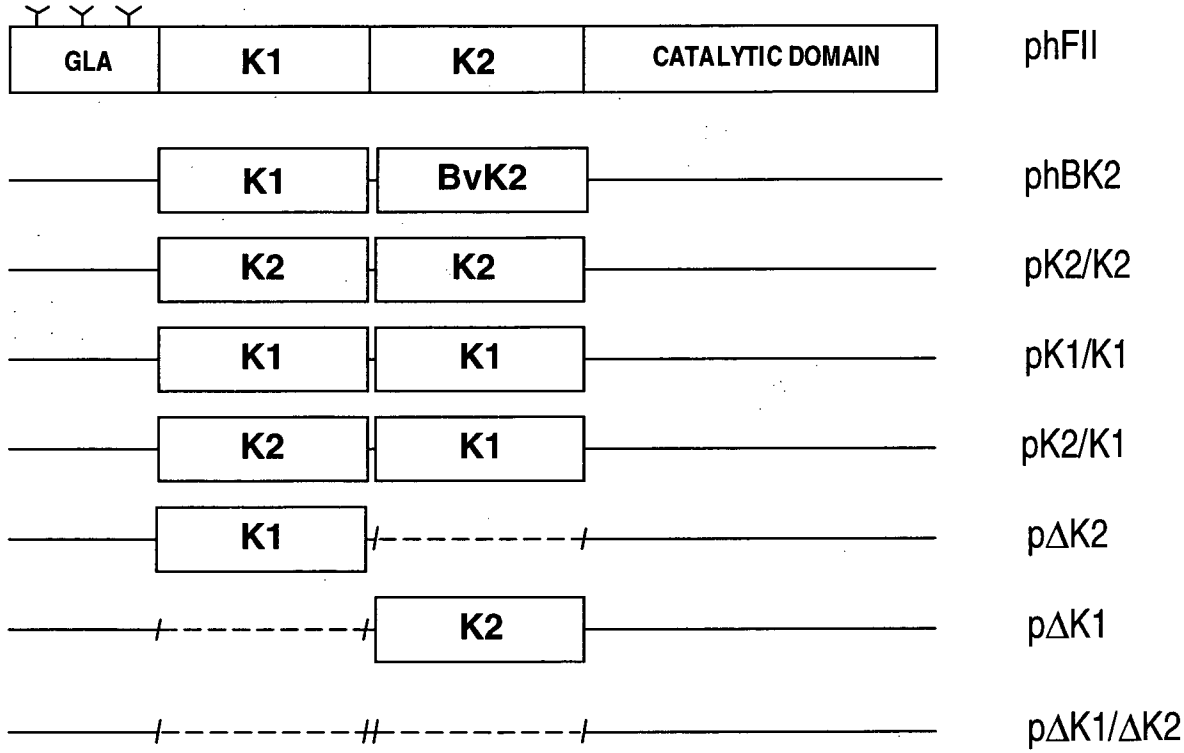
Recombinant prothrombin variants were constructed by site-directed mutagenesis so that they had the kringle domains modified either by deletion or by substitution with an equivalent domain (Figure 13). The deletion and substitution of all kringle domains started at the first cysteine and ended at the sixth cysteine of the signature tri-sulfide bridges of a kringle polypeptide. The conserved cysteines lie at the junctions of the exons coding for the kringle domains and served as useful sites for seamless joining of DNA fragments (using a technique described below). The prothrombin variants included a deletion of the first kringle (pΔK1), the deletion of the second kringle (pΔK2), and a deletion of both kringles (pΔK1/ΔK2). For the substituted species, both the first and second kringle were swapped with each other (pK1/K1; pK2/K2; pK2/K1); and another variant had the second kringle substituted with its bovine counterpart (phBK2).

2. Construction of prothrombin variants

The prothrombin variants were constructed using a site-directed PCR mutagenesis utilizing a method called enzymatic-inverse PCR (EIPCR) (Stemmer et al., 1992). This method employs class II restriction enzymes that have recognition sites that are located at the 5' end of the cut site. In this method, taking advantage of the conserved cysteines common in the DNA sequence flanking all kringle regions, an EarI restriction enzyme site was engineered in each primer for the amplification of various kringle domains, as well as the

Figure 13. Structure of prothrombin and variants. Recombinant prothrombin and variants were constructed using enzymatic-inverse polymerase chain reaction (EIPCR). The prothrombin variants included a deletion of the first kringle (pΔK1), the deletion of the second kringle (pΔK2), and a deletion of both kringles (pΔK1/ΔK2). For the substituted species, both the first and second kringle were swapped with each other (pK1/K1, pK2/K2, pK2/K1). Another variant had the second kringle substituted with its bovine counterpart (phBK2)

PROTHROMBIN and VARIANTS



regions flanking the kringle domains. EarI has a recognition site of CTCTTCN/**NNN**, and the ubiquitous (3') **NNN** overhang after the cut site was adapted to the cysteine sequence TGT/C. Thus all kringle primer sequences began with TGT (cysteine) incorporated into the EarI sites (Table 1). After amplification by PCR, the engineered EarI sites facilitate the deletion and substitution of the kringle regions without introducing unwanted sequences. After an EarI digest, all PCR fragments would have a TGT overhang that could be joined together in a ligation reaction (Figure 14). EIPCR has proven to be useful for site-directed mutagenesis independent of the presence of convenient restriction enzyme sites in the DNA sequences. Instead it helps to create restriction site(s) and makes possible the joining of DNA fragments as long as there was a conserved amino acid present. The prothrombin variants were constructed using EIPCR, subcloned into the BlueScript plasmid, and propagated in *E.coli* DH5α-F' cells. The nucleotide sequences of all constructs were verified by DNA sequence analysis and restriction enzyme digests.

II. PROTEIN EXPRESSION

1. Expression vector

The cDNAs of all prothrombin constructs in BlueScript were released from their respective vectors with NotI digestion. The cDNA fragments were separated by agarose gel electrophoresis and recovered by either electroelution or by centrifugation. The purified fragments were subcloned into a NotI-digested pNUT vector that had been dephosphorylated at the ends with alkaline phophatase. The ligated products were transformed into DH5αF'12 cells and ampicillin resistant colonies were picked and cultured. Restriction enzyme digests were carried out to confirm the successful insertion of the cDNA into the pNut vector as well as its proper orientation. Selected clones were grown to isolate dsDNA for DNA sequence analysis to confirm the restriction digest results. Frozen cultures of all clones were prepared for storage, and large scale DNA preparations were made to prepare the DNA for transfection into BHK cells for recombinant protein production. The transcription of the inserted cDNA of interest in the vector pNUT is driven by the metallothionein promotor

Figure 14. Construction of a prothrombin human-bovine kringle chimera variant. Using enzymatic inverse polymerase chain reaction (EIPCR), the restriction enzyme recognition sites for EarI were introduced into PCR fragments obtained from two plasmids containing the cDNA sequences of human prothrombin (hFII) and bovine prothrombin (pBIII) respectively. The EarI sites were introduced outside the second kringle region (K2) of prothrombin for both species. A ligated final product contained the bovine second kringle replacing the counterpart of the human prothrombin species.

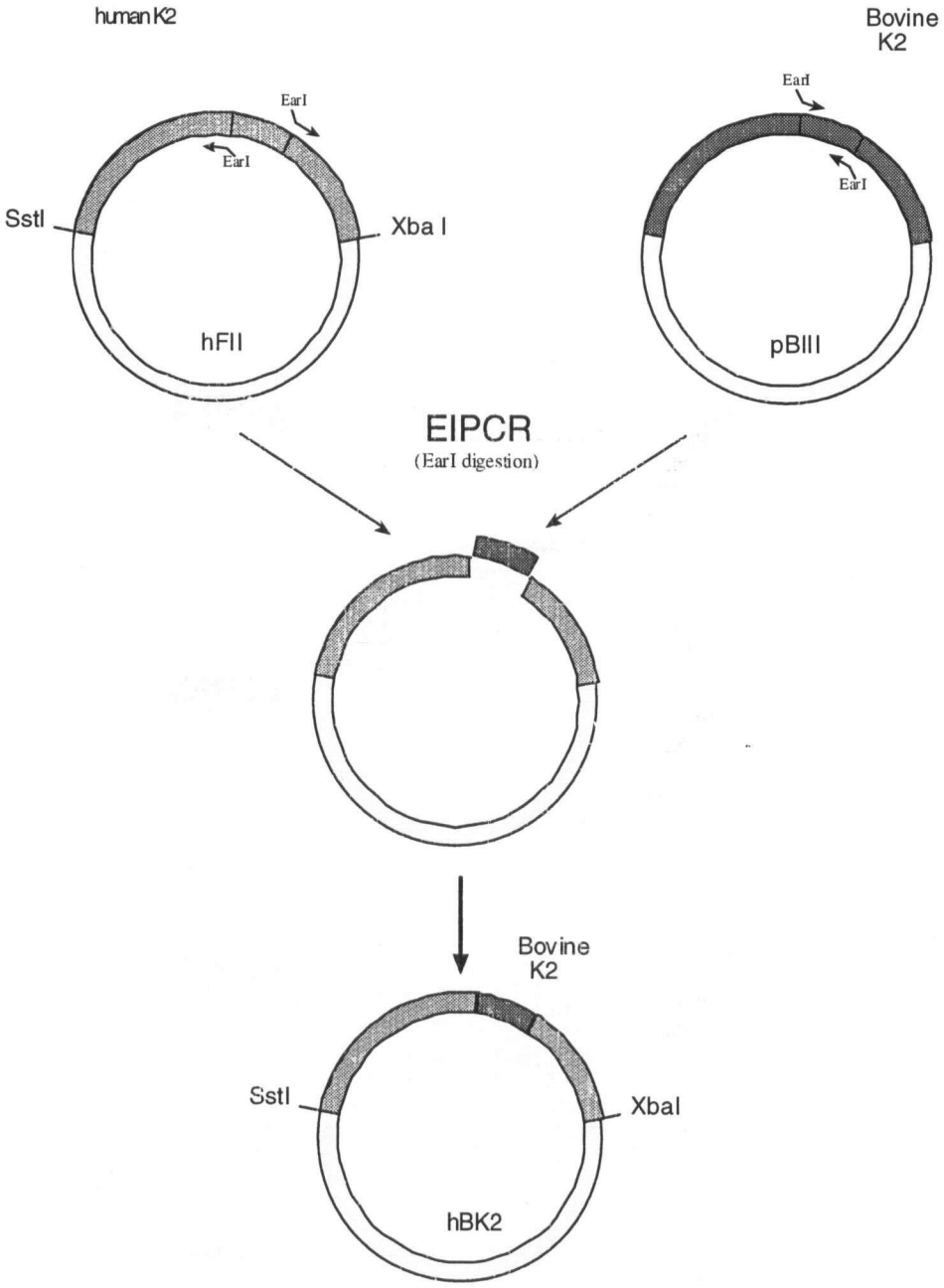

human K2
Bovine K2
EarI
EarI
SstI
Xba I
hFII
EarI
EarI
pBIII
EIPCR
(EarI digestion)
Bovine K2
SstI
XbaI
hBK2

Table 1. Oligonucleotides used for the construction of the prothrombin variants

Primer	Sequence 5' to 3'	Position	Strand	Comment
K1A	ATC*GAATTC*TCTTC **TGT**GCTGAGGGTCTGGGTAC	334	sense	5' kringle 1
K1B	ATC*GAATTC*TCTTC **CCA**CAGACAGGGATGCTGCA	572	antisense	3' kringle 1
K1C	ATC*GAATTC*TCTTC **GCA**CAGTTACCTTCCAGACA	388	sense	5' outside K1
K1D	ATC*GAATTC*TCTTC **TGT**GGCCAGGATCAAGTCAC	568	antisense	3' outside K1
K2A	ATC*GAATTC*TCTTC **TGT**GTCCCTGATCGGGGGCA	649	sense	5' of kringle 2
K2B	ATC*GAATTC*TCTTC **TCA**CAATAGTTGAGGTCGCA	887	antisense	3' of kringle 2
K2C	ATC*GAATTC*TCTTC **ACA**CACTGCTCCAATGGAGG	653	sense	5' outside K2
K2D	ATC*GAATTC*TCTTC **TGT**GAGGAGGCCGTGGAGGA	883	antisense	3' outside K2
BK2A	ATC*GAATTC*TCTTC **TGT**GTCCCGGACCGCGGCCGG	637	sense	5' bovine K2
BK2B	ATC*GAATTC*TCTTC **TCA**CAGTAGTTCAGGTCACAA	873	antisense	3' bovine K2

(The underlined letters in the oligonucleotide sequences represent the recognition sequence of the restriction enzyme EarI. The cleavage site of EarI is the gap within the sequence.

Bold letters represent the cystein sequence of the beginning and the end of a kringle region (both in the sense and antisense sequence).

The italicized letters represent the recognition site of the restriction enzyme EcoRI, that is utilized for subcloning of amplified DNA sequences into the vector BlueScript.)

(MT-1), and transcription is terminated by the human growth hormone (hGH) poly A signal. The vector contains the cDNA of the dihydrofolate reductase gene (DHFR) driven by the SV40 early promoter to allow selection in cell culture with methotrexate (MTX), as well as the ampicillin gene (amp) for the selection and propagation of the plasmid in bacterial culture (Figure 15).

2. Mammalian cell culture

Baby hamster kidney (BHK) cells were maintained in Dulbecco's modified Eagle's Medium: nutrient mixture F-12 (DMEM-F12) pH 7.4 medium containing 5% New Born Serum (NBS). Cells were grown to confluence in tissue culture flasks at 37°C under 5% CO_2 humidified conditions. Cells were removed from flasks using trypsin-EDTA (0.25% trypsin, 1 mM EDTA). Frozen cultures of cells were prepared in DMEM-F12/NBS medium containing 10% dimethylsulfoxide (DMSO) and stored in liquid N_2.

3. Transfection in BHK cells

The pNUT constructs containing the cDNAs of prothrombin and its mutants were transfected into BHK cells by either the calcium phosphate co-precipitation method (Chen et al., 1988) or by a DOPE:DODAC lipid vesicles delivery method. Healthy, vigorous cultures were set up for transfection in 90 mm sterile tissue culture plates at 40-70% confluence, this density was chosen because higher or lower cell numbers limited the number of surviving cells. The medium (9 ml/plate) was changed two hours before a transfection procedure. The transfecting DNA was purified either by CsCl gradient or by Quiagen column prep.

For the calcium phosphate co-precipitation method, approximately 10-20 μg (per plate) of highly pure plasmid dsDNA was precipitated using 0.1 volumes of 3 M NaOAc and 3 volumes of 95% EtOH. After washing in 70% EtOH, the DNA pellet was allowed to air-dry in a fume hood, and was resuspended in 450 μl of sterile dH_20 by vortexing. An aliquot of 50 μl of 2.5 M $CaCl_2$ was added to the dissolved DNA, followed by 500 μl of 2x HBS buffer (16 g/L NaCl, 0.74 g/L KCl, 0.2 g/L Na_2HPO_4, 2 g/L dextrose, 10 g/L HEPES, pH 6.95 precisely). The solution was mixed thoroughly and incubated at RT for 20 min. The solution (1 ml) was added directly to the plate containing 9 ml of media that had been

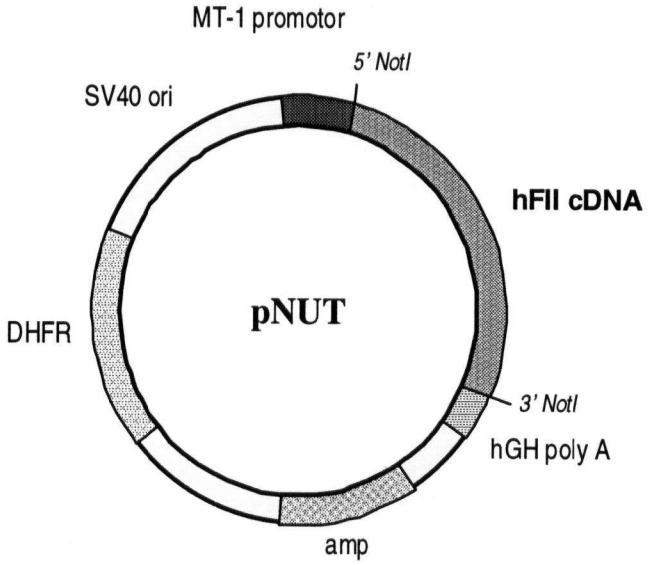


Figure 15. Schematic diagram of the expression vector pNUT. The prothrombin cDNA (hFII) was subcloned into the vector pNUT at an engineered NotI site. Prothrombin expression was driven by the metallomethionine promotor (MT-1) and controlled by the human growth hormone (hGH) poly A signal. The dihydrofolate reductase gene (DHFR) allowed seletion with methotrexate (MTX) for sucessful integration of the plasmid into baby hamster kidney cellular genome. The ampicillin gene (amp) is required for the propagation of the plasmid in bacterial culture.

changed 2 hr before hand. Mixing was done by swirling and the plates were incubated at 37°C for 4-6 hr. During this time, a fine black precipitate could be observed using the microscope. The lower pH of the buffer (6.95) allowed a slow precipitation and decreased the average particle size, resulting in better cellular uptake and thus better transfection efficiency. During this incubation period, the cells began to lose their normal healthy morphology and appeared to be dying. After 6 hr, the transfection medium was removed, the plate was washed extensively with DMEM-F12 medium, and the selection process was begun immediately.

The DNA was precipitated in a similar fashion for transfection using DOPE:DODAC cationic lipid vesicles. One microgram of precipitated plasmid DNA was resuspended in 100 µl of dH_2O, and 9 µl of 1 mM DOPE:DODAC were resuspended in 91 µl of dH_2O. The DNA solution was mixed with the cationic lipid solution and incubated at RT for 30 min to allow formation of DNA:lipid complexes . The cells were prepared in 9 mm plates similar to the calcium precipitation method, except immediately before the addition of the DNA:lipid complex, only 3 ml of DMEM-F12 media serum free was required. After the DNA:lipid solution was added to the plate, the cells were incubated at 37°C for 4-6 hr. The cells were inspected hourly as the lipids were highly toxic, and the transfection process had to be stopped if the cells appeared to be dying. Once the transfecting medium was removed by aspiration, the plates were rinsed extensively with DMEM-F12 media, and the selection process was started immediately as described below.

4. Selection

The pNUT vector contained a cDNA for dihydrofolate reductase (DHFR) that is required in the biosynthesis of both purines and thymine; thus rapidly growing cells have a high level for this enzyme. Two important anticancer drugs, aminopterin and amethopterin (methotrexate or MTX) inhibit DHFR at concentrations as low as 10^{-8} to 10^{-9} M (Metzler, 1977). Under MTX selection at high concentration, cells that had successful integration of the plasmid, leading to overexpression of the DHFR gene, would have been able to survive. As a result, most likely the inserted cDNA inside pNUT would have been co-expressed along with DHFR in the surviving cells.

The selection medium was DMEM-F12/5% NBS containing 500 μM MTX. Under these conditions, the cells took 2-3 days to be affected and eventually died off in 10-14 days. The selection medium was changed daily once the cells started to die. After 10 days, most cells had died and only successfully transfected cells survived. By then, surviving colonies were visible under the microscope, and eventually with the naked eye. Single colonies were picked and cloned by plating to limiting dilution. The cloned cell lines were expanded and analyzed for recombinant protein expression, selected and stored frozen in liquid N_2.

5. Large scale expression

Selected cell lines were grown in large flasks in serum-free expression medium DMEM-F12/ITS-K. The ITS-K media supplement consisted of insulin from bovine pancreas at a final concentration of 1 g/L, human apo-transferin at 0.50 g/L, sodium selenite at 0.67 mg/L, ethanolamine at 0.20 g/L, albuMAX at 0.20 g/L, and vitamin K1 at 10 mg/L. The media were harvested and tested for the presence of the recombinant protein using SDS-PAGE and Western Blot analysis (Figure 16). Clones with the highest expression levels were picked and grown up for large-scale expression in roller bottles. Approximately three large T-125 cm^2 flasks of confluent cells grown in normal media were needed to seed one roller bottle (1700 cm^2). The cells in the roller bottles were grown in normal DMEM-F12/NBS media until confluence. The serum medium was then removed, the cells were washed with DMEM extensively and serum-free DMEM-F12/ITS-K was introduced. The expression medium was changed every 48 hr and collected. The medium was filtered through Whatman papers to remove cellular debris, and to the media was added the protease inhibitor benzamidine (final concentration 10 mM) and 0.1% sodium azide to prevent bacterial growth. Collected media was stored either at 4°C for immediate purification or frozen at –20°C for long term storage.

6. Protein purification

The purification of the recombinant proteins was carried out as reported previously (Côté et al., 1994). Typically, the protein in the collected media was precipitated with barium citrate, washed and resuspended in buffer for anion exchange chromatography using

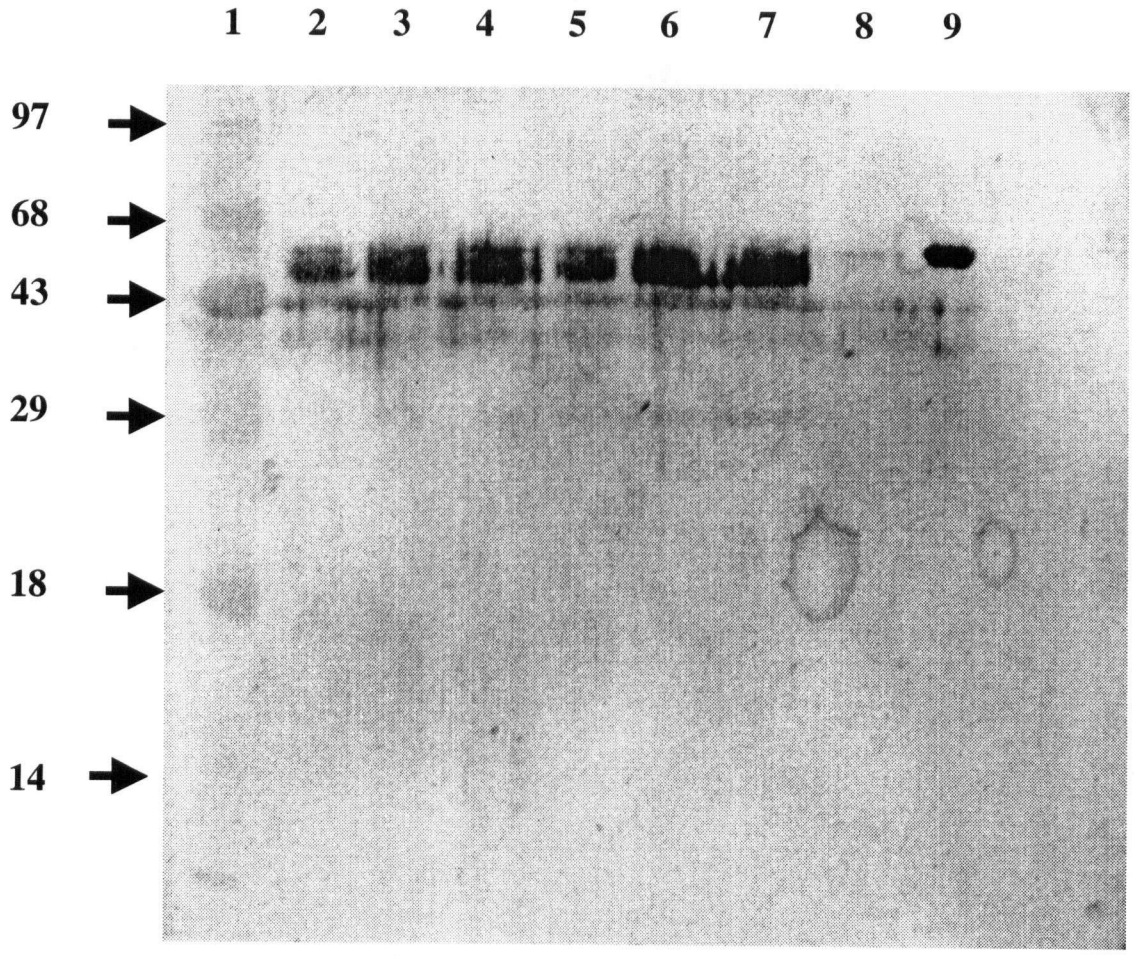


Figure 16. Western Blot analysis of the media of the recombinant variant rΔK2 clones. Samples of the media (400 μl) of transfected clones of the recombinant prothrombin variant rΔK2 were concentrated to 40 μl, and subjected to SDS-PAGE and Western Blot analysis. From left to right, lane 1 was the molecular weight standards (in kDa), lane 2 to 7 were clones 1 to 6 of rΔK2, lane 8 was the BHK/pNUT control, and lane 9 was the plasma-derived prothrombin.

FPLC. Pseudo-affinity chromatography was carried out using a calcium gradient to resolve different Gla containing protein species.

6.1. Barium citrate adsorption and precipitation

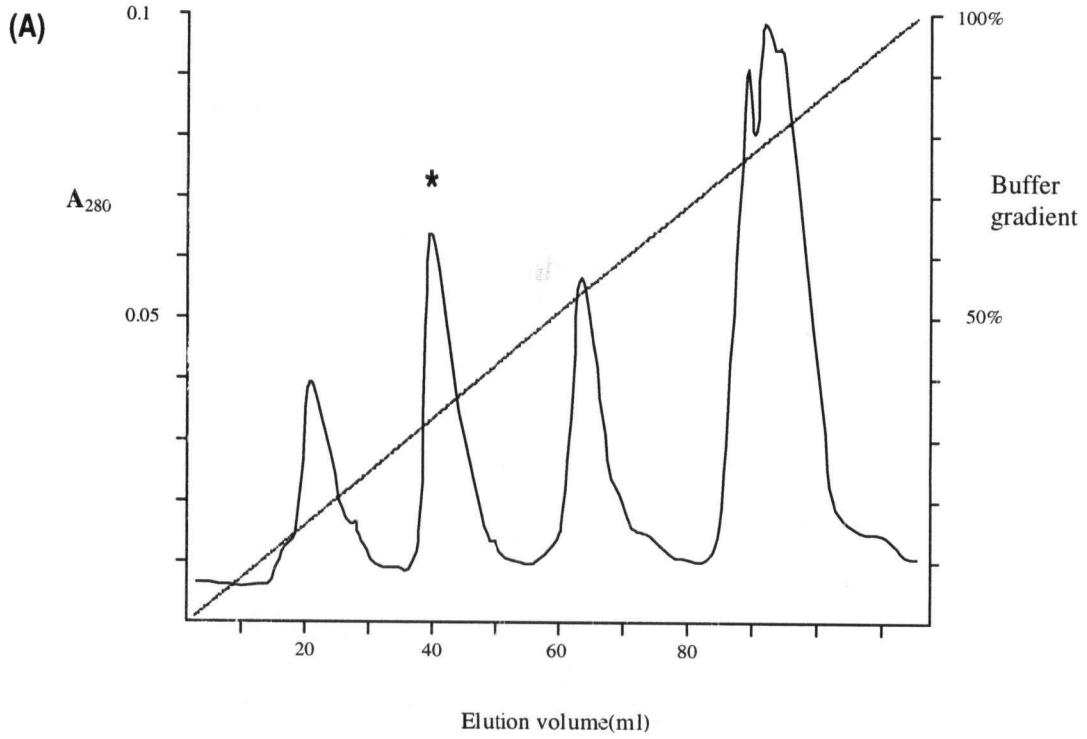
The collected media containing the recombinant prothrombin protein and its mutants were concentrated using the Amicon RS2000 concentrator with a spiral-wound membrane cartridge of 30 K MW cutoff. To the concentrated media was added slowly 1/25 volume of 0.4 M sodium citrate and 1/10 volume of 1 M $BaCl_2$. The suspension was stirred slowly at 4°C overnight. Because of the presence of the Gla residues, prothrombin would co-precipitate with the barium citrate particulate in the process. The precipitate was collected by centrifugation using a bench top centrifuge at 3000 rpm for 15 min, and the pellet was washed extensively with 0.1 M $BaCl_2$. The adsorbed protein was eluted by dissolving in either 0.2 M EDTA (1/6 of the original volume) or by 35% $(NH_4)_2SO_4$ solution pH at 6.0 containing 50 mM benzamidine. The solution was clarified by centrifugation and dialyzed in appropriate buffer for chromatography.

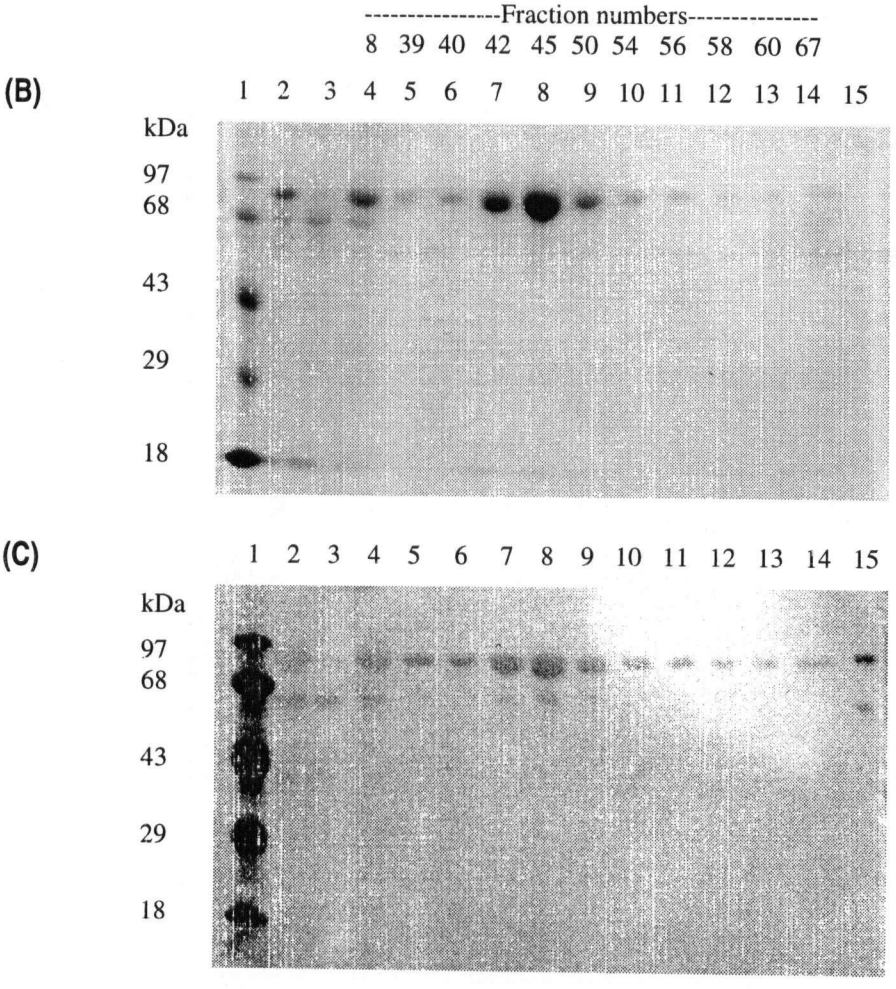
6.2. Fast Performance Liquid Chromatography (FPLC)

The eluted protein from a barium-citrate precipitation step was dialyzed extensively at 4°C in 20 mM sodium citrate pH 6.0, 100 mM NaCl and 1mM benzamidine. The solution was filtered through a 0.22 μm membrane before any column and was subjected to anion exchange chromatography using a Bio-Rad Econo-Pac FPLC High Q resin column. The eluting gradient was 0.1 to 1 M NaCl in 20 mM citrate buffer pH 6.0 (flow rate 1 ml/min) (Figure 17). The peak fractions were characterized using SDS-PAGE and Western Blot analysis (Figure 17). Alternatively the barium-citrate precipitate was dialyzed against 20 mM Tris pH 7.4, 100 mM NaCl and 1 mM benzamidine overnight at 4°C. The sample was chromatographed using the same column with a gradient of 0.1-1M NaCl in the same buffer. Fractions were characterized as described.

Pooled prothrombin fractions from either column were dialyzed in 20 mM Tris pH 7.4, 100 mM NaCl, and 1mM benzamidine. The sample was the loaded onto a Pharmacia FPLC Mono Q HR 5/5 column, and the protein was eluted using a pseudo-affinity calcium

Figure 17. Anion-exchange fast performance chromatography (FPLC) of the recombinant prothrombin variant rhBK2. The recombinant protein from a barium-citrate precipitation step was dialyzed extensively at 4°C in 20 mM Tris.Cl oH 7.4 containing 100 mM NaCl and 1mM benzamidine. The solution was filtered through a 0.22 μm membrane and was subjected to anion exchange FPLC using a Bio-Rad Econo-Pac High Q resin column. The eluting gradient (dashed line) was 0.1 to 1 M NaCl (0-100%) in 20 mM Tris.Cl pH 7.4 (flow rate 1 ml/min) (panel A). Samples were collected in 1.5 ml fractions using UV absorbance at 280 nm. The majority of functional recombinant prothrombin variant rhBK2 eluted at around the 300 mM NaCl concentration (peak *). Denatured rhBK2 eluted earlier in the first peak at around 100 mM NaCl concentration. The peak fractions (20 μl each) were characterized using SDS-PAGE (panel B) and Western Blot (panel C). For the gels in panel B, the order from left to right are (lane 1) protein standards (in kilo daltons); (lane 2) protein sample before FPLC; (lane 3) flow-through after loading; (lane 4-14) fractions 8, 39, 40, 42, 45, 50, 54, 56, 58, 60, 67; and (lane 15) plasma prothrombin (1μg). The polyacrylamide gel was stained with Coomassie dye. A duplicate of the gel was subjected to Western Blot analysis using a sheep primary anti-prothrombin antibody, followed with an alkaline-phosphatase conjugated anti-sheep antibody.


(A)
0.1
0.05
A_{280}
100%
50%
Buffer gradient
*
20
40
60
80
Elution volume(ml)


----------Fraction numbers----------
8 39 40 42 45 50 54 56 58 60 67
(B)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
kDa
97
68
43
29
18
(C)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
kDa
97
68
43
29
18

gradient (0-50 mM $CaCl_2$) (Figure 18). Fractions were analyzed as described (Figure 18). This pseudo-affinity chromatography step was developed to resolve differentially carboxylated species of Protein C (Yan et al., 1990), but can be used for other vitamin K-dependent proteins. The fully carboxylated population was eluted first from theMono Q column under these conditions, followed by one or two more Gla populations of lesser carboxylation. This observation was confirmed by Gla analysis by capillary electrophoresis in a later section of this thesis. Using this method, protein samples were purified to at least 95% purity. The purified sample was dialyzed in 20 mM Tris pH7.4, 150 mM NaCl and stored in the same buffer with 50% glycerol at –20°C. Protein concentration of wild type prothrombin was determined by absorbance readings at 280 nm ($E_{1\%}280$=13.8) (Mann et al., 1981). The recombinant mutant protein concentrations were determined by the Bio-Rad assay using wild type prothrombin as standard.

Occasionally some protein samples were also subjected to a Bio-Rad Econo-Pac hydroxyapatite column for purification. For this particular separation, the protein solution was dialyzed in 10 mM phosphate pH7.2 buffer, loaded onto the column and the protein was eluted using a gradient of 10-400 mM sodium phosphate pH 7.2. Hydroxyapatite $(Ca_5(PO_4)_3OH)_2$ is as form of calcium phosphate that can be used for the separation and purification of proteins, nucleic acids, and other macromolecules. Gla residues present in prothrombin and other blood clotting enzymes had a strong affinity for the hydroxyapatite matrix, making this column useful for the purification of these proteins.

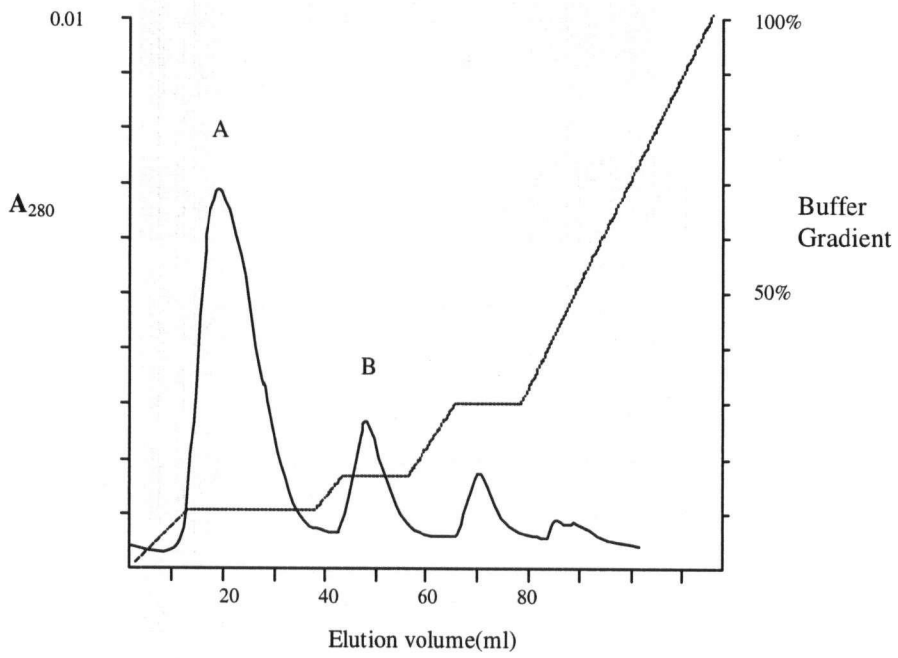
III. CHARACTERIZATION OF RECOMBINANT PROTEIN

1. Phospholipid vesicles preparation and characterization

Phosphatidylcholine:phosphatidylserine (PCPS, 75:25) vesicles were prepared as described by Hope and co-workers (Hope et al., 1985). To make 10 mM PCPS, 2.5 mM of PS was mixed with 7.5 mM of PC in chloroform. The solution was evaporated under N_2 gas and was lyophilized completely for 2-3 hr under vacuum. The dried down lipids were dispersed in 1 ml of sterile dH_2O and vortexed vigorously. The dispersed lipids were

Figure 18. Pseudo-affinity fast performance chromatography (FPLC) of the recombinant prothrombin variant rhBK2. The pooled fractions from the anion-exchange FPLC column was dialyzed extensively at 4°C in 20 mM Tris.Cl oH 7.4 containing 150 mM NaCl and 2 mM EDTA. The solution was filtered through a 0.22 μm membrane and was subjected to (calcium) pseudo-affinity FPLC using a Pharmacia FPLC Mono Q HR 5/5 column. The eluting calcium gradient was 0 to 50 mM $CaCl_2$ (0-100%) in 20 mM Tris.Cl pH 7.4 (flow rate 1 ml/min) (panel A). The dashed line represents the percentage of the eluting buffer gradient (horizontal sections meant the gradient were held during those periods). Samples were collected in 1.0 ml fractions using UV absorbance at 280 nm. The Gla-rich species (rhBK2A) of the recombinant prothrombin variant rhBK2 eluted at near 10 mM $CaCl_2$ (peak A), and the Gla-poor species (rhBK2B) eluted at around 17.5 mM $CaCl_2$ (peak B). The peak fractions (20 μl each) were characterized using SDS-PAGE (panel B) and Western Blot (panel C). For the gels in panel B, the order from left to right are (lane 1) protein standards (in kilo daltons); (lane 2) protein sample before FPLC; (lane 3) flow-through after loading; (lane 4-14) fraction 5, 10, 15, 20, 30, 40, 50, 55, 71, 82, 91; and (lane 15) plasma prothrombin (1 μg). The SDS-PAGE gel was stained with Coomassie dye. A duplicate of the gel was subjected to Western Blot analysis using a sheep primary anti-prothrombin antibody, followed with an alkaline-phosphatase conjugated anti-sheep antibody.

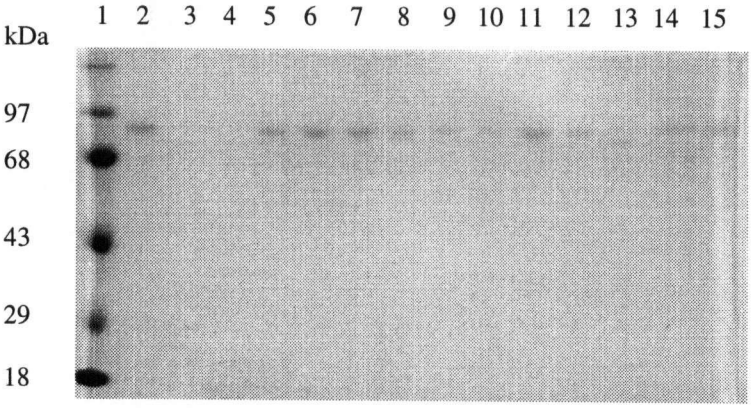
(A)



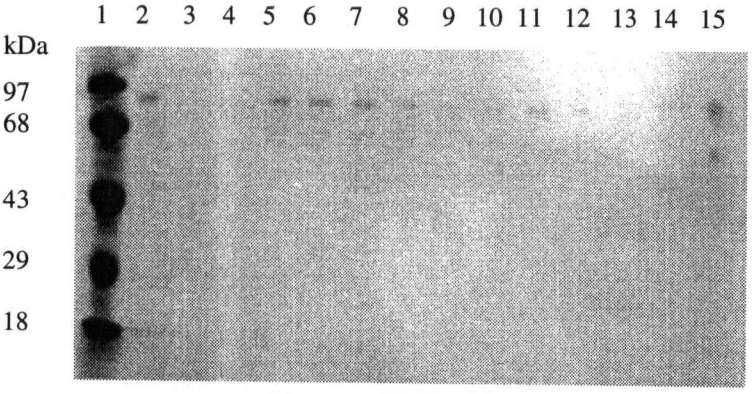
----------Fraction numbers----------

10 15 20 25 35 40 45 50 71 85 90

(B)



(C)



subjected to 5 freeze-thaw cycles using liquid N_2. After the final thawing, the solution was extruded ten times through an extruder (Lipex Biomembranes) using 2 x 100 nm pore size membranes. As a result, large unilamillar vesicles with approximate diameter of 100 nm were formed. The vesicles' concentration was determined by phosphate assays. To a series of phosphate standards, concentration from 0-200 nmol in 0.25 ml volume, was added 0.75 ml of perchloric acid each and digested at 185°C for 2 hr. To each tube was added 7 ml of ammonium molybdate and 0.8 ml Fiske reagent (150 g/L NaHSO3, 5 g/L Na2SO3 and 2.5 g/L 1-amino-2-napthol-4-sulfonic acid). The solutions were heated in boiling water bath for 15 min. After cooling, the absorbance was read at 815 nm. The concentration of the phospholipid vesicles was determined from the standard curve of the phosphate standards.

2. Ca^{2+} binding assays

The Ca^{2+} binding property of the proteins were inferred by the decrement in intrinsic tryptophan fluorescence as previously described by Nelsestuen, Prendergast and Mann (Nelsestuen, 1976; Prendergast et al., 1977). The protein sample (2 ml, 10 µg/ml in 20 mM HEPES, 150 mM NaCl pH 7.4), was stirred in a thermostated (25°C) quartz cuvette in a Perkin-Elmer Luminescence Spectrometer (LS 50). Intrinsic fluorescence was excited at 280 nm and continuously monitored at 300 to 400 nm, with excitation and emission band widths of 5 and 15 nm respectively, and with a 290 nm cut-off filter for the emission beam. An aliquot (10 µl) of 1 M $CaCl_2$ was added to give a final concentration of 5 mM. The solution was allowed to mix and the decrease in fluorescence was monitored over the emission bands (Figure 19 and Figure 20). The ratio of the magnitude of the decrement (ΔI_{340}) over the initial fluorescence (I_{o340}) of the recombinant proteins were extrapolated and compared to that of the wild type plasma prothrombin. Figure 19 and 20 show the intrinsic tryptophan fluorescence response to calcium binding of plasma-derived and recombinant prothrombin. The results indicated that the calcium binding properties were very similar for both proteins.

3. Phospholipid binding assays

The phospholipid binding property of the proteins was monitored by right angle light scattering as described previously (Bloom et al., 1979; Nelsestuen et al., 1977). A solution

Tryptophan fluorescence response to calcium binding of human wild type plasma prohrombin

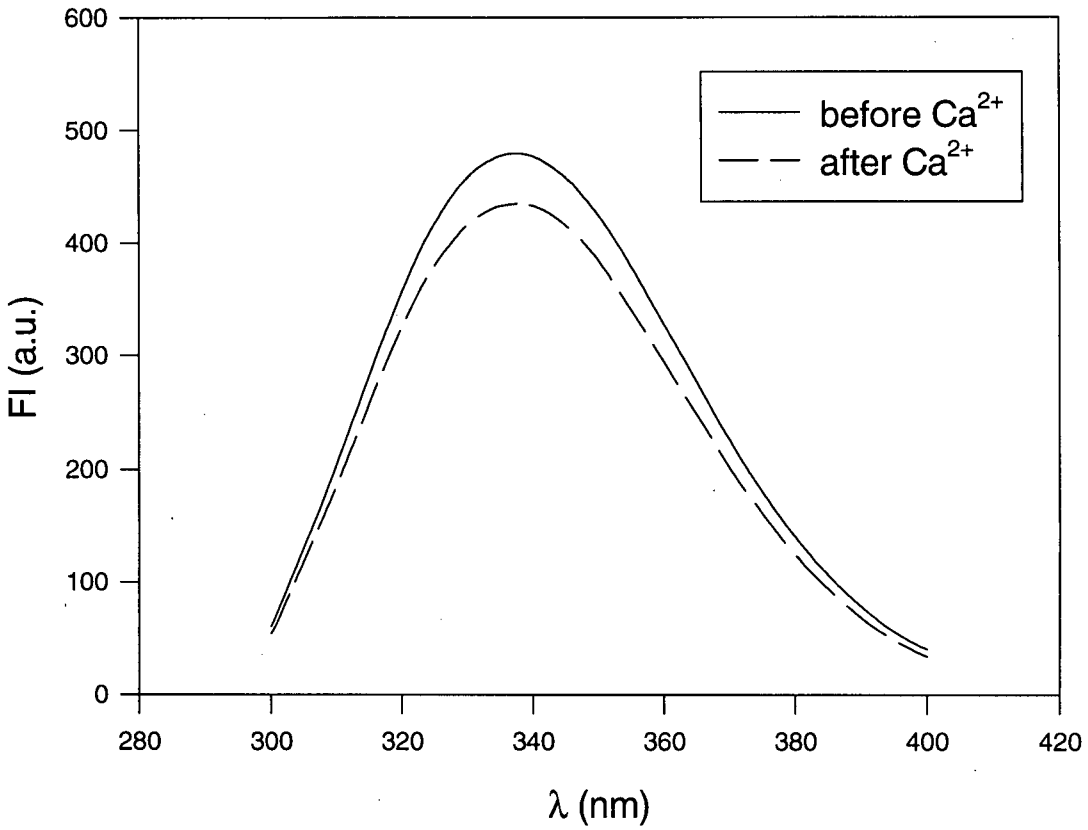


Figure 19. Tryptophan fluorescence response to calcium binding of human wild type plasma prothrombin (phFII). A sample of plasma prothrombin (phFII) in 20 mM HEPES pH 7.4 was stirred in a cuvette in a temperature-controlled luminescence spectrometer. The intrinsic tryptophan fluorescence was monitored continuously with λ_{ex} at 280 nm, λ_{em} between 300-400 nm, and respective excitation and emission band passes of 5 and 15 nm. The fluorescence was monitored before and after the addition of $CaCl_2$ (final concentration 5.0 mM) to the protein sample.

Tryptophan fluorescence response to calcium binding of human wild type recombinant prothrombin

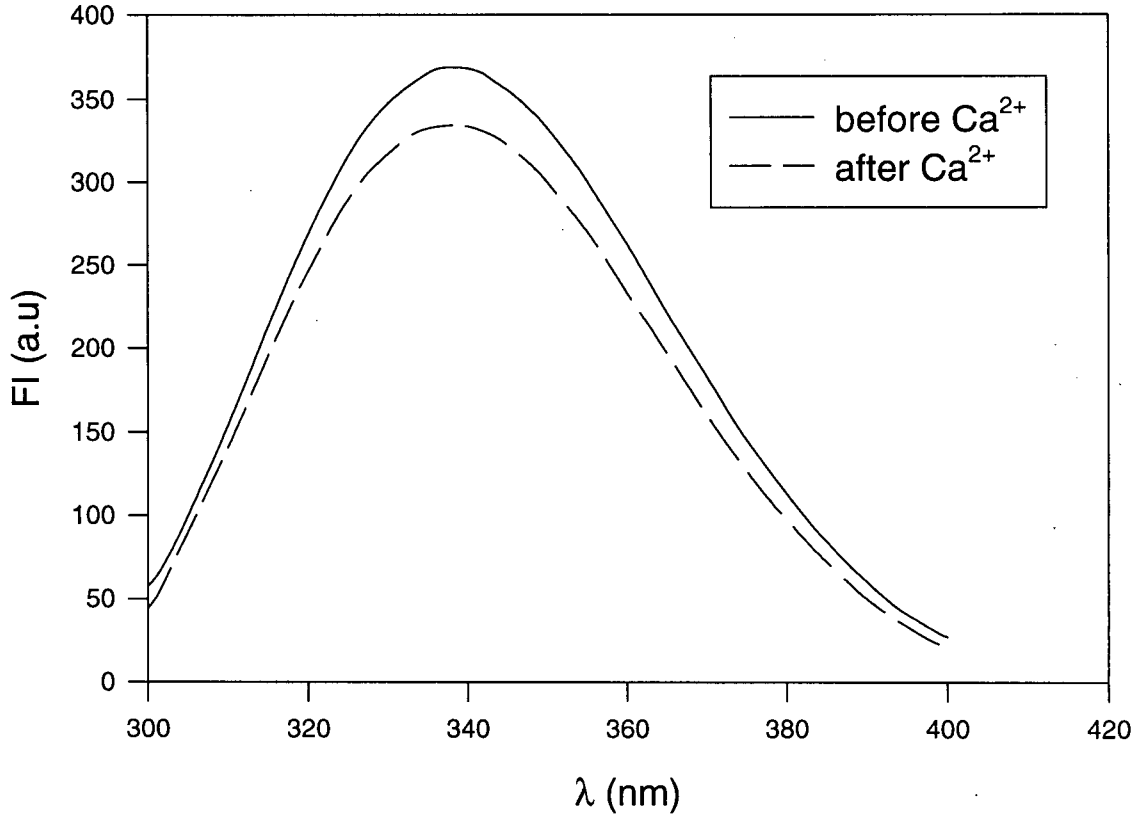


Figure 20. Tryptophan fluorescence response to calcium binding of wild type recombinant prothrombin (rhFII). A sample of recombinant prothrombin (rhFII) in 20 mM HEPES pH 7.4 was stirred in a cuvette in a temperature-controlled luminescence spectrometer. Intrinsic fluorescence of tryptophan was monitored continuously with λ_{ex} at 280 nm, λ_{em} between 300-400 nm, and respective excitation and emission band passes of 5 and 15 nm. The fluorescence was monitored before and after the addition of 5.0 mM $CaCl_2$ to the protein sample.

Right-angle light scattering response to calcium binding and phospholipid vesicles binding of plasma and recombinant prothrombin

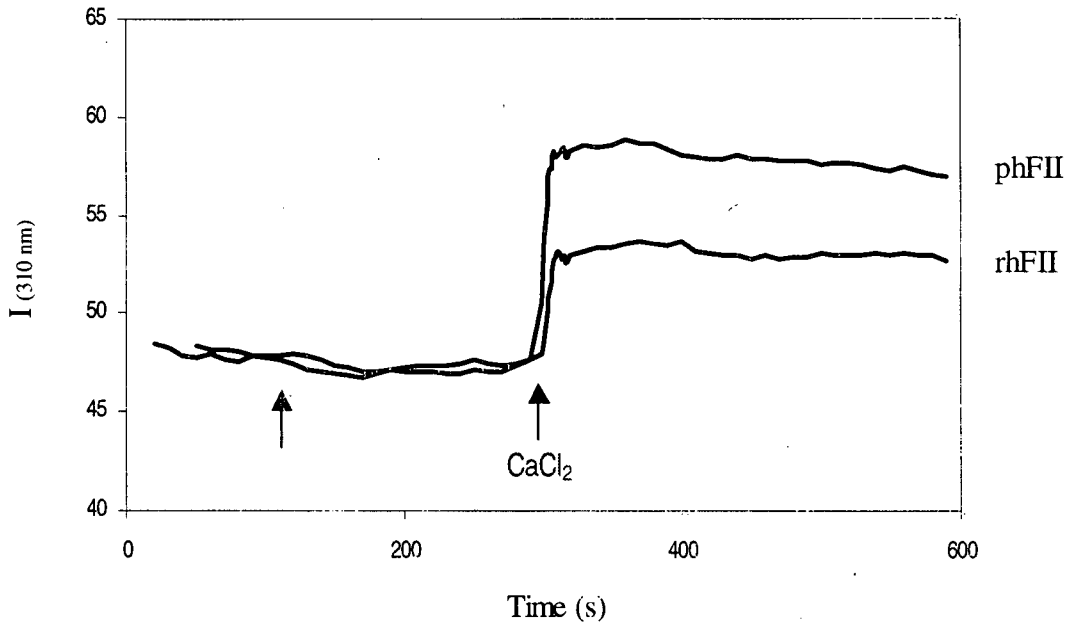


Figure 21. Right-angle light scattering response to calcium binding and phospholipid vesicles binding of plasma and recombinant prothrombin. An aliquot of phosphotidylcholine:phosphotidylserine (PCPS) vesicle (20 μg/ml) in 2 ml of 0.02 M Tris.Cl pH 7.4 containing 150 mM NaCl was stirred in a quartz cuvette in a temperature controlled luminescence spectrometer at 25°C. A sample (10 μg) of human wild type plasma prothrombin (phFII) or recombinant prothrombin (rhFII) was added and right angle light scattering intensity was monitored continuously for 3 min at 410 nm with 2.5 nm slit width. Calcium ($CaCl_2$) was added to 5 mM final concentration and the course of the scattering was monitored for another 5 min. The lines represent the light scattering of the phospholipid vesicle-protein complexes before and after the addition of the proteins and calcium ions (the arrows).

mixture (2 ml) containing PCPS (75:25) at 10 μg/ml in 20 mM Tris.Cl and 150 mM NaCl pH 7.4 was stirred in a quartz cuvette (25°C) in the spectrometer. The right angle scattering intensity was monitored continuously over 10 min with the excitation wavelength of 400 nm and emission wavelength of 410 nm, both with a 2.5 nm slit width. An aliquot of protein (10 μg) was added after 2 min interval and the scattering was continuously monitored. After 3 min, an aliquot (10 μl) of 1 M $CaCl_2$ was added to give a final concentration of 5.0 mM to induce the Ca^{2+}-dependent protein-phospholipid interaction. The time course of the increase in light scattering was monitored for 5 min until a stable reading was obtained (Figure 21). Data was collected at 1s intervals using a Perkin-Elmer Luminescence Spectrometer (LS 50). The increments in intensity of the recombinant proteins were compared with that of the wild-type plasma prothrombin. Figure 21 showed the different phospholipid-binding properties of plasma-derived and recombinant prothrombin. The addition of a protein had no effect on the right angle light scattering of the phospholipid vesicles. In contrast, however, when calcium ions were added, there was a strong and immediate response to light scattering, as the protein was able to bind onto the membrane surfaces. The results from Figure 21 indicated that the recombinant prothrombin produced from the BHK system had a lower response in right angle light scattering upon calcium ions addition than that of the plasma-derived prothrombin, indicating that the two proteins had different phospholipid binding properties.

4. Coagulation assay

Coagulation assays were performed for the recombinant prothrombin protein and variants using human-prothrombin-deficient plasma activated with rabbit brain thromboplastin and calcium ions. An aliquot (50 μl) of each protein (20 μg/ml) was mixed by pipetting with an equal volume (50 μl) of the prothrombin-deficient plasma and incubated in a water bath at 37°C for 2 min. Clotting was initiated by the addition of an aliquot (100 μl) of the thromboplastin-Ca^{2+} solution and the reaction was allowed to proceed at 37°C. The time required to detect a visible clot was recorded manually. Each protein was measured at least four times. The clotting activities of the recombinant proteins were determined from the standard curve for the clotting activity of human wild type plasma prothrombin (Figure 22). The results were shown in Table 2.

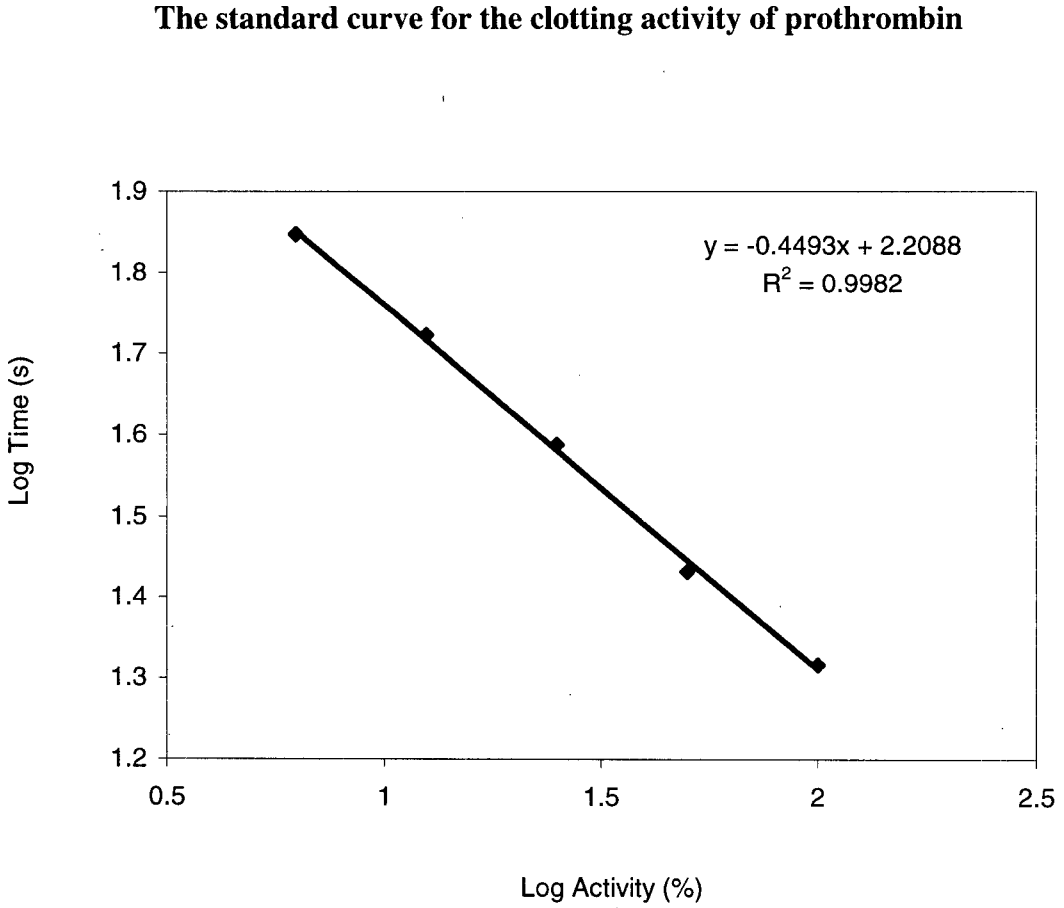


Figure 22. The standard curve for the clotting activity of prothrombin. The clotting assays were performed for human wild type plasma prothrombin using human-prothrombin-deficient plasma activated with rabbit brain thromboplastin and calcium. Aliquot (50 μl) of the protein (20 μg/ml) was mixed with an equal volume (50 μl) of the prothrombin-deficient plasma and incubated in a water bath at 37°C for 2 min. Clotting was initiated by the addition of an aliquot (100 μl) of the thromboplastin-Ca^{2+} solution and the reaction was allowed to carry out at 37°C. The assays were performed for a series of concentration of plasma prothrombin. The time required to detect a visible clot was recorded manually. The figure is a log-log graph of the activity (concentration) of plasma prothrombin and the time required for detection of a visible clot.

Table 2. Relative clotting activity of recombinant prothrombin and variants.

Protein[a]	**Clottting Time**[b] **(sec)**	**Relative Clotting**[c] **Activity (%)**
phFII	20.7 ± 0.6	100
rhFII*	25.5 ± 0.5	61.4 ± 2.9
rhBK2*	26.3 ± 1.0	57.3 ± 4.9
rΔK1	32.4 ± 1.3	36.0 ± 3.1
rΔK2	43.4 ± 0.7	18.7 ± 0.7
rK1/K1	75.8 ± 3.3	5.5 ± 0.5
Control	96.0 ± 1.4	3.2 ± 1.0

[a] the prothrombin variants studied were the human/bovine K2 chimera (rhBK2), the first kringle deleted variant (rΔK1), the second kringle deleted variant (rΔK2) and the second kringle substituted with the first kringle (rK1/K1). Water was used as a control

[b] the values represented for each protein were from at least six determinations

[c] the clotting activity of the recombinant protein was determined from the standard curve for the clotting activity of human wild type plasma prothrombin (phFII)

* the clotting time and clotting activities of the recombinant prothrombin (rhFII) and the human/bovine chimera (rhBK2) were not statistically different ($P>0.05$); all other results were statistically different ($P<0.05$); statistical analysis was determined using the T-test

IV. DISCUSSION

Using this experimental approach, human wild type prothrombin and several prothrombin variants were constructed and expressed in tissue culture cells. The recombinant proteins were expressed in large quantities and purified by anion exchange and pseudo-affinity chromatography using a Fast Performance Liquid Chromatography system. The proteins were subjected to several assays to determine the calcium and phospholipid binding properties. Coagulation assays were performed to determine the functional activity of the recombinant proteins.

1. Rationale for the construction of prothrombin variants

The prothrombin variants were constructed using the first and last cysteines of the tri-sulfide bridges of a kringle polypeptide. The conserved cysteines lie at the junctions of the exons coding for the kringle regions in prothrombin, and served as useful sites for the deletion and substitution of these regions. Since the kringle module was considered to be a true domain, thus having an autonomous folding independent from that of the parent polypeptide, deletion of the kringle regions would lead to a protein with the other domains properly folded. This approach would allow for the delineation of the role that was mediated by a specific kringle region in the context of the functional property of the whole protein. In addition, substitution of a kringle region by an equivalent domain was a better approach since this would lead to a full length, intact protein with all its domains in place and minimal perturbation in its folding structure. As kringle modules have been characterized to be very similar in their characteristic three-dimensional folding patterns, a protein containing a kringle chimera would mimic the native structure of the wild type protein much more closely than that of a protein with a deleted domain. In addition, direct domain-domain interactions essential in a protein tertiary structure may be possible that could lead to a more stable form. As such, the differences between the secondary structures and the primary sequences of kringle domains, combined together, could give useful information regarding the structure-function relationship of the domains involved.

2. Construction and expression of recombinant proteins

Out of seven prothrombin variants constructed, there were two constructs that did not yield any detectable recombinant proteins. These were the pK2/K1 and the pK2/K2 variants. Construct pΔK1/ΔK2 yielded traces of detectable protein as determined by Western blotting, but no significance amount of protein was produced. The rest of the variants expressed recombinant proteins at adequate levels, with the recombinant wild type having the highest level (Figure 23). The presence of the second kringle immediately after the Gla region seemed to lead to non-expressed proteins. The expressed prothrombin variants were purified using anion exchange FPLC and pseudo-affinity chromatography using a calcium gradient. The calcium gradient was used to resolve the different Gla-containing protein species.

2.1 The Baby Hamster Kidney cell expression system

Low level of expression of active human prothrombin has been achieved in other systems such as a Chinese hamster ovary (CHO) cell system (Jorgensen et al., 1987) and a Vaccina virus Expression system (Falkner et al., 1992). The use of the baby hamster kidney (BHK) cell line for the expression of prothrombin in this study has its advantages as well as its disadvantages. Prothrombin has been shown to be of by hepatic origin. BHK cells of kidney origin do not produce prothrombin normally, and thus any recombinant prothrombin produced in this cell line must be from the transcription and translation of the transfected cDNA stably integrated into the cellular genome. Being a mammalian cell line, BHK cells should modify the secreted protein post-translationally, for example γ-carboxylation, glycosylation, and other important events such as signal peptide processing and proper (disulfide bonding) folding and transport. Using this system, it has been shown that recombinant prothrombin could be produced in high amounts (Côté et al., 1994). However, questions remain whether the post-translational modifications in the BHK system are similar to that of a human system. Results from the purification step using (calcium) pseudo-affinity chromatography have shown that the γ-carboxylation of the secreted prothrombin was incomplete. Several species of recombinant prothrombin contained different amount of Gla residues in the Gla domain as shown in Figure 19A.

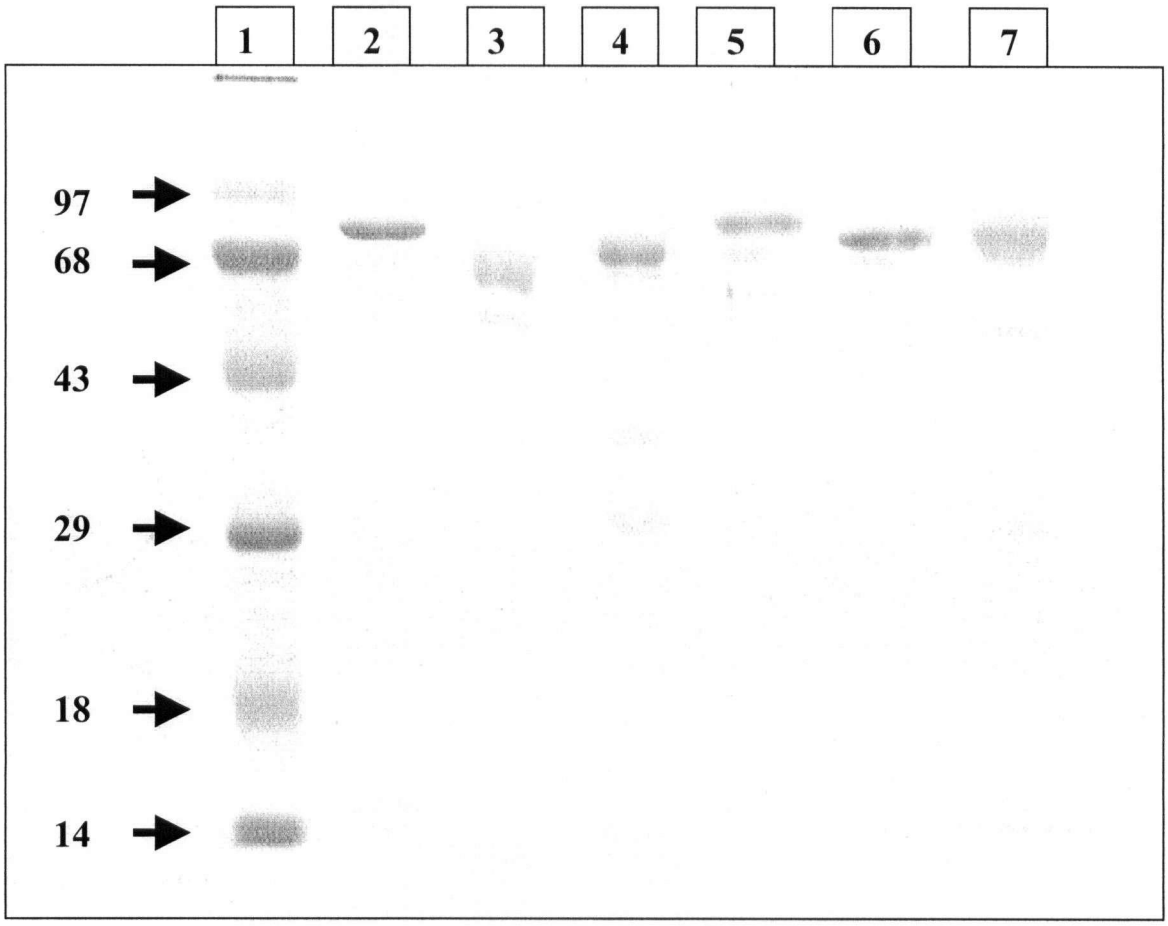


Figure 23. SDS-PAGE analysis of recombinant prothrombin and variants. From left to right (lane 1 to 7) are molecular weight standards (in kilo daltons), plasma-derived prothrombin (phFII), rΔK1, rΔK2, rhBK2, rK1/K1 and recombinant wild type prothrombin (rhFII). The proteins (5 μg each) were subjected to electrophoresis in polyacrylamide gel, and visualized by staining with the Brilliant Commassie Blue R-250 dye. Contaminating bands were degradation products of the recombinant proteins.

The Gla contents of the different species of one recombinant prothrombin variant have been characterized by a novel method (see chapter 4). The results confirmed the observation obtained from pseudo-affinity FPLC that γ-carboxylation was incomplete in the BHK system. Presumably the stress of producing high levels of recombinant protein continuously had overwhelmed the BHK cellular γ-carboxylation system leading to partial and incomplete carboxylation. Thus it seems that the advantage of a high yield expression system was offset by the under-carboxylation of the secreted protein possibly leading to a heterogenous population of different Gla-containing species.

In addition, the use of the BHK system for the expression of human prothrombin variants may have been problematic in other areas besides the incomplete carboxylation process. For example, a BHK clone stably transfected with the prothrombin cDNA did not secret any recombinant prothrombin when the cells were treated with warfarin (10 μg/ml) and in the absence of vitamin K. Thus the production of Gla-less human prothrombin was not compatible with the BHK expression system. In contrast, Gla-less prothrombin was successfully produced using a HepG2 (human hepatoma) cell line (Jamison et al., 1992). It has been noted that the inhibition of the γ-carboxylation process blocks the secretion of under-carboxylated prothrombin in the rat but not in human or bovine. Recently, it has been shown that this discrepancy was due to the structural differences in the protein (of rat and human) rather than by the origin of the cell line (Wu et al., 1997). When it was noted that under-carboxylated human prothrombin can be produced from a transformed human kidney cell line (HEK293) but not rat prothrombin, a series of human-rat chimeric prothrombin molecules were studied to determine the structural requirement for this phenomenon. When the rat kringle domain was substituted with that of the human counterpart, secretion of recombinant protein was restored. Results from this work have shown that the presence of the human kringle domain changed the stability of rat prothrombin to that of human prothrombin, and vice versa. Thus it appears that the kringle domains play important roles in the intracellular processing/degradation of carboxylated prothrombin possibly through the interactions with cellular systems involved in protein processing. In addition, when the first kringle in the human construct was replaced with that of the rat counterpart, the expression level of the recombinant protein decreased while replacement of the second kringle gave equivalent levels to wild type. These results indicated that the first kringle (of rat) was more

important than the second kringle in destabilizing the under-γ-carboxylated chimera protein precursor. It was suggested that the kringle domains interact with the cellular system(s) involved in protein processing including the Gla domain and the propeptide domain. When the γ-carboxylation fails, this interaction might be disturbed leading to selective degradation and improper processing in the ER of the cell.

It seems, therefore, the presence of the first kringle was probably important for the successful production of recombinant prothrombin in a non-human cell line. As was mentioned earlier, three constructs in this study, the pΔK1/ΔDK2 (both kringle deleted), pK2/K1 (both kringle switched places) and the pK2/K2 (second kringle substituting first kringle) did not yield detectable level of secreted protein. This observation could be explained based on the results discussed above. The absence of the first kringle and the presence of the second kringle in the first kringle domain might lead to a disruption in the protein precursor carboxylation process resulting in subsequent degradation and loss of secretion of these proteins in the BHK cells.

2.2. Other post-tranlational modifications

Other important post-translational modification events need to be examined for the BHK system such as glycosylation and signal peptide processing. Specifically, glycosylation seemed to be important for the successful expression of recombinant prothrombin using the BHK system. In work unpublished, a series of prothrombin variants was constructed in which the asparagine residues required for glycosylation were mutated (Hewitt et al., 1999). The three glycosylation sites in prothrombin are Asn-77, Asn-101 and Asn-373. At least one of these sites was needed for the expression of recombinant prothrombin in BHK cells. Combinations of any two sites yielded secreted protein, whereas a complete knockout of all three sites led to no protein expressed. Thus there seems to be an important role for the glycosylation event in protein processing, suggesting that glycosylation and secretion are linked.

Processing the signal peptide and propeptide is another important factor that needs to be considered when using the BHK system. Improper processing of the signal peptide has been implicated in BHK cells for the expression of a recombinant factor Xa variant (results unpublished). In addition, the secreted recombinant prothrombin produced using the BHK

system has a lower specific activity to that of the wild type plasma derived prothrombin (for detailed discussions, see section 3 below). Thus it seems the production of recombinant prothrombin using the BHK cell system was hampered by several processes, probably cellular events involved in the post-translational protein processing pathway and the inherent structural requirements for species-specific protein production.

3. Functional properties of recombinant prothrombin variants

The recombinant prothrombin and variants produced from BHK cells were purified and characterized using several functional assays. The calcium binding and the phospholipid binding properties of the proteins were assessed, as well as their clotting activity. These properties were determined and compared relative to those of wild type prothrombin derived from plasma.

3.1. Calcium binding property

For the calcium-binding assay, the intrinsic tryptophan fluorescence response to calcium binding was measured in the absence and presence of calcium ions at near physiological concentration. The decrease in fluorescence in response to calcium binding was determined from the ratio ($\Delta I/I_o$) of the fluorescence measured at 340 nm (Figure 24). The results showed that all recombinant proteins have a response in tryptophan fluorescence to calcium binding similar to that of the plasma prothrombin. The variant rK1/K1 had a slightly higher response probably due to the presence of the first kringle in place of the second kringle. The first kringle of human prothrombin has been shown to contain a calcium binding site (Berkowitz et al., 1992; Sugo et al., 1990; Welsch et al., 1988). Thus additional calcium binding and subsequent secondary structure folding may contribute to this slightly higher response. The addition of two more tryptophans (Trp-90 and Trp-126) in the first kringle in this variant might also lead to a higher tryptophan fluorescence response when bound to calcium. In contrast, the prothrombin variant with the deletion of either the first kringle or the second kringle domain both had similar calcium binding responses to that of the wild type protein. Thus it seemed that the kringle domains play a very minimal role in the calcium binding function of the protein; presumably, the Gla domain represents the major

calcium binding region for these prothrombin molecules. The calcium binding of prothrombin was a specific one, as another protein, the ferric binding protein (FBP) from the bacteria *Nesseria gonorrhoeae* did not elicit a response to calcium binding (Figure 24). As has been discussed, calcium binding was necessary for prothrombin undergoing a conformational change necessary for the phospholipid binding property.

3.2. Phospholipid binding property

The phospholipid binding properties of recombinant prothrombin and its variants were assessed by using a right angle light scattering assay where the ability of the protein to bind onto the surfaces of PCPS vesicles was measured. Synthetic PCPS vesicles were made containing a ratio of 75% PC to 25% PS to mimic negatively charged biological membrane. In the presence of calcium ions, prothrombin bound onto the surface of the PCPS vesicles increasing the effective radius of the vesicles and caused an increase in light scattering. The increment in light scattering measured at a right angle was determined from the ratio ($\Delta I/I_0$) of light scattering measured at 410 nm (Figure 25). The results showed that recombinant prothrombin and prothrombin variants have a calcium-dependent response different from that of the wild type plasma prothrombin. In fact, the ability to scatter light for the recombinant proteins was lesser than that of the plasma derived form. It could be inferred from this observation that the recombinant proteins did not bind to the negatively charged PCPS vesicles as efficiently as the plasma protein. Although the recombinant proteins had calcium binding responses similar to the plasma form (as measured by tryptophan fluorescence) the calcium induced conformation of the recombinant proteins may be somewhat different when bound on PCPS vesicles' surfaces. Probably, the PCPS-bound conformation of the recombinant proteins was significantly different from that of the plasma form, and thus leading to a different response in light scattering. The cause for this discrepancy is as yet unknown.

Extensive studies have been carried out by many investigators to determine the membrane contact sites for the calcium-dependent structure of prothrombin as well as other vitamin K-dependent proteins. It is understood that the Gla domains of these proteins play a significant role through the calcium dependent structure of the protein. It has been shown recently that the Ca^{2+}-specific membrane contact sites in the Gla domain of vitamin

Relative tryptophan fluorescence response to calcium binding of recombinant prothrombin and variants

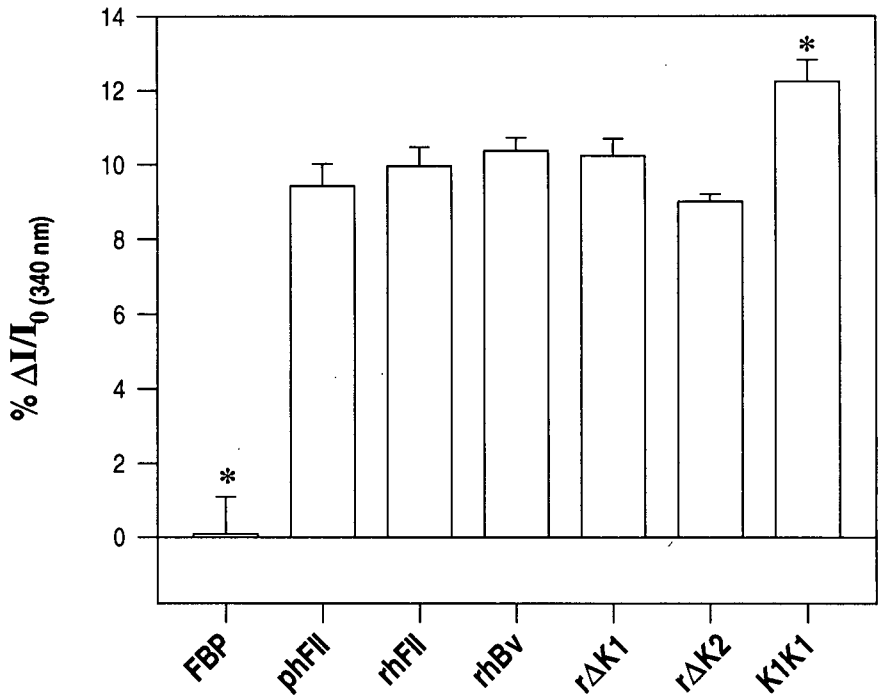


Figure 24. Relative tryptophan fluorescence response to calcium binding of prothrombin and variants. The tryptophan fluorescence response ($\Delta I/I_o$) to calcium binding of recombinant prothrombin (rhFII) and variants were measured at 340 nm and compared to that of plasma prothrombin (phFII). The prothrombin variants include rhBK2 (second kringle substituted with the bovine counterpart), rΔK1 (first kringle deleted), rΔK2 (second kringle deleted) and rK1/K1 (second kringle substituted with the first kringle). Ferric binding protein (FBP) was used as a negative control. Values represented for each protein were from three determinations. Only rK1/K1 and the control ($_*$) had values statistically different from the rest ($P<0.05$).

Relative right angle light scattering response to calcium binding and phospholipid binding of prothrombin and variants

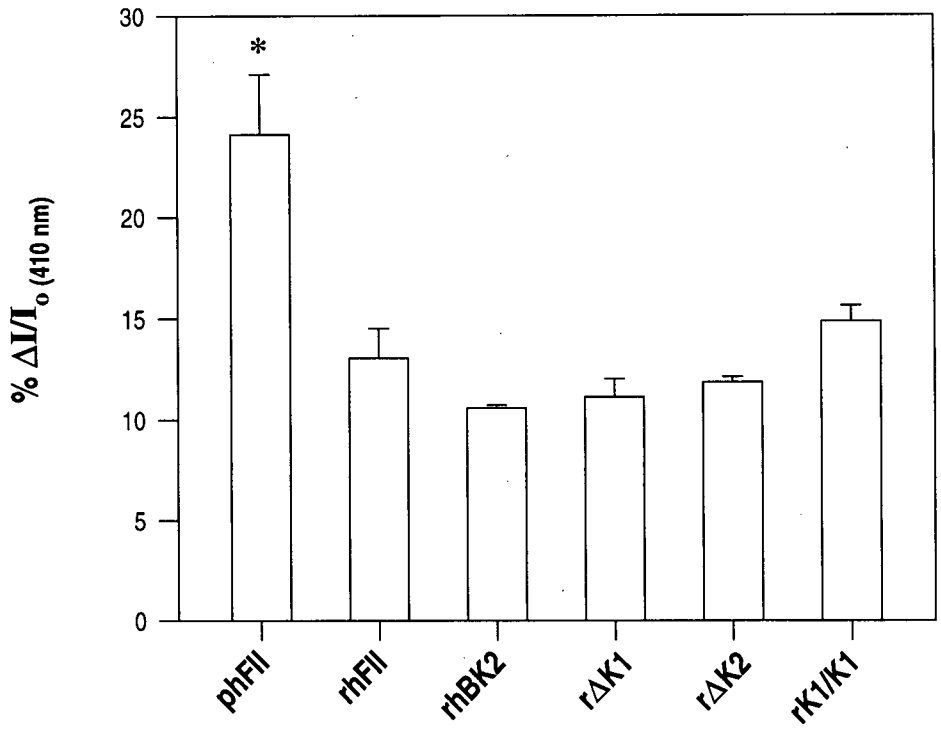


Figure 25. Relative right angle light scattering response to calcium binding and phospholipid binding of prothrombin and variants. The light scattering increment ($\Delta I/I_o$) response to calcium and PCPS binding of recombinant prothrombin (rhFII) and variants were measured at 410 nm and compared to that of plasma prothrombin (phFII). The prothrombin variants include rhBK2 (second kringle substituted with the bovine counterpart), rΔK1 (first kringle deleted), rΔK2 (second kringle deleted) and rK1/K1 (second kringle substituted with the first kringle. Values represented for each protein were from three determinations. Only phFII had a value statistically different from all the rest ($P<0.05$).

K-dependent proteins are similar despite the differences in the primary structures of these proteins (Ellison et al., 1998). Since the Gla domain of the recombinant prothrombin and variants had not been changed, the secondary structure of the Gla domain should also be very similar to that of the plasma form. The fact that they did not exhibit similar PCPS vesicle binding properties would suggest differences other than that of primary structures. Besides the Gla content, other processing events such as glycosylation in BHK cells could lead to the production of recombinant proteins that were different in structure from the plasma derived protein. In addition, intermolecular interactions between the various domains within a protein may play a role in determining the specific secondary structure of the polypeptide under certain conditions. Any gross structural changes such as the deletion and substitution of a domain may lead to a significant perturbation of this specific secondary structure.

3.3. In vitro clotting activity

Clotting assays were performed for the recombinant prothrombin protein and variants using human prothrombin-deficient plasma activated with rabbit brain thromboplastin and calcium ions. The time taken for a visible clot to appear was recorded, and the relative clotting activity was determined from a standard plot using wild type plasma derived prothrombin (Table 2). The average uncertainty error was less than 5% for the clotting activity. The clotting activity of recombinant prothrombin (rhFII) determined under this system was less than that of the plasma protein (hFII) (61% compared to 100% respectively). The prothrombin variant with the second kringle substituted with the bovine counterpart (rhBK2) had a similar relative activity of 57%. The deletion of the kringle domains led to a substantial reduction in relative clotting ability with 36% for the first kringle-deleted variant (rΔK1) and 19% for the second kringle-deleted variant (rΔK2). However, the least active variant in the ability to clot *in vitro* was the prothrombin variant with the second kringle replaced with the first kringle (rK1/K1). This particular protein had only 6% relative clotting activity compared to that of the plasma derived protein, almost at the background level. There was a correlation in reduced clotting activity and reduced PCPS binding property for recombinant human prothrombin expressed from BHK cells (Table 3). The rhBK2 variant having a slightly lower PCPS vesicle binding response also had a slightly lower clotting activity compared to those of recombinant prothrombin. For these two recombinant proteins,

Table 3. Relative calcium and phospholipid binding property and the clotting activity of recombinant prothrombin and variants

Protein	Trpyptophan response $\%\Delta I/I_o$ (340 nm)[a]	Relative Ca^{2+} binding activity (%)	Right angle light scattering $\%\Delta I/I_o$ (410 nm)[a]	Relative PCPS binding activity (%)	Clotting Time (sec)[b]	Relative clotting activity (%)
phFII	0.0943 ± 0.0059	100 ± 6	0.2413 ± 0.0296*	100 ± 12	20.7 ± 0.6	100
rhFII	0.0997 ± 0.0050	106 ± 5	0.1305 ± 0.0148	54 ± 6	25.5 ± 0.5	61.4 ± 2.9
rhBK2	0.1037 ± 0.0035	110 ± 4	0.1060 ± 0.0014	44 ± 1	26.3 ± 1.0	57.3 ± 4.9
rΔK1	0.1023 ± 0.0046	108 ± 5	0.1113 ± 0.0090	46 ± 4	32.4 ± 1.3	36.0 ± 3.1
rΔK2	0.0900 ± 0.0020	95 ± 2	0.1186 ± 0.0028	49 ± 1	43.4 ± 0.7	18.7 ± 0.7
rK1/K1	0.1223 ± 0.0058*	130 ± 6	0.1485 ± 0.0078	62 ± 3	75.8 ± 3.3	5.5 ± 0.5

[a] values represented for each protein were from at least three determinations

[b] clotting time was recorded manually; values represented for each protein were from at least six determinations; (for statistical analysis, see table 2)

* values were statistically different from all the others ($P<0.05$) within each assay; statistical analysis was determined by the T-test

the ability to bind PCPS vesicles efficiently may lead to a more active form for the subsequent coagulation process. For the other variants, other factors may be responsible for the reduced functionality other than just the PCPS vesicle binding properties.

CHAPTER 4

ANALYSIS OF THE GLA DOMAIN

I. BACKGROUND

The formation of γ-carboxyglutamic acid residues is very important for functional vitamin K-dependent blood clotting proteins including prothrombin. For studies involving recombinant prothrombin and or other recombinant Gla-containing proteins, there is a need for a robust and sensitive method for the determination of the Gla content in these proteins. Several previous studies have reported the qualitative and quantitative determination of Gla in proteins. Typically, the assays involve the hydrolysis of the protein to free amino acids followed by derivatization, separation and quantification. A variety of reagents and methodologies have been developed including chemical modification (Wright et al., 1984), ion exchange chromatography and analysis on an amino acid analyzer (Gundberg et al., 1979; James, 1979; Madar et al., 1979), detection of an N-isobutyloxycarbonyl trimethyl ester derivative by gas-liquid chromatography (Matsu-ura et al., 1981), specific tritium labeling or tritium exchange of Gla in protein or free Gla followed by HPLC or mass spectrometry (Carr et al., 1980; Hauschka, 1979; Rose et al., 1980), and derivatization with the fluorescent agent phenylisothiocyanate followed by reverse-phase HPLC (Smalley et al., 1988). Recently, capillary electrophoresis has been used to compare the differences in Gla content in small peptides (Black et al., 1997). Currently, the favoured method is a modification of a technique by Kuwasa and Katayama (Kuwada et al., 1983; Kuwada et al., 1981), in which the protein is hydrolyzed with base, followed by a pre-column derivatization with o-phthalaldehyde and anion exchange HPLC. This method yields the most consistent result for the quantification of Gla by calculating the relative ratio of the peak areas of Glu over Gla in a protein and using Asp as an internal standard. Although these methods are suitable for the quantification of Gla from purified proteins or peptides sources, they are time consuming, not very sensitive, and require substantial amount of protein. In addition, these methods are not very reliable for the analysis of biological tissues, biological fluids or impure protein preparations. The protein sample has to be highly purified, requiring extensive purification procedures that might take days or weeks.

A new method has been developed for the analysis of Gla in proteins by labeling a base hydrolysate with fluoresceinisothiocyanate (FITC) followed by separation and detection by capillary electrophoresis and laser-induced fluorescence (CE-LIF) (Britz-Mckibbin et al., 1999; Vo et al., 1999). The method has been used to determine the Gla content in several

recombinant prothrombin variants expressed in baby hamster kidney cells. The Gla content has been correlated with the physical and biological properties of these prothrombin variants.

II. PRINCIPLES OF CAPILLARY ELECTROPHORESIS

Capillary electrophoresis (CE) is a simple yet extremely powerful analytical technique capable of separating a wide range of compounds, such as small and complex ions, amino acids, chiral drugs, oligonucleotides and DNA fragments, peptides and proteins, carbohydrates and complex polymers. Other benefits of CE include small sample size requirements, simple and automated instrumentation, high efficiency, and short analysis time. There are as many modes of operation in CE just as many modes in chromatography, including capillary zone electrophoresis (CZE), micellar kinetic capillary electrophoresis (MKCE), capillary electrophoresis chromatography (CEC), capillary isoelectric focusing (CIEF), and capillary isotachophoresis (CITP) to name a few. However, they all have common principles arising from the union of two separation techniques: electrophoresis and chromatography. In CE, both chemical equilibrium and differential migration of analytes (separating molecules) in an electric field can be utilized. Equilibrium is the driving force for separation in chromatography, and electric field is the driving force for separation in electrophoresis. Capillary electrophoresis is, as the name implies, electrophoresis in a capillary where the separating molecules are resolved based on their relative size to charge ratios. Separation is achieved by exploiting the differential migration rates of solutes in an electric field. In the absence of any other forces, such as equilibrium, only charged molecules (ions) move in an electric field. The electrophoretic mobility of an ion is governed by the equation:

$$\mu_{ep} = \frac{Q_{eff}}{6\pi r \eta}$$

where, μ_{ep}: the electrophoretic mobility of an ion

Q_{eff}: the effective charge of the ion

η: the viscosity of the solution

r: the (hydrated) radius of the ion (assumed spherical shape)

The mobility of an ion in an electric field is directly proportional to its charge, and inversely proportional to its size. Thus a positively charged molecule in CE would migrate toward the cathode end of the capillary, while a negative ion to the anode (Figure 26). For biological molecules possessing weakly acidic and basic functionalities, the pH of the solution becomes an important factor determining their relative migration. The pH of the background in CE is one of the most important parameters in separation optimization. The electrophoretic mobility and the electric field then determine the migration of an ion in CE as follow:

$$U = \mu_{ep}.E$$

where, U: the migration rate of an ion in CE

μ_{ep}: the electrophoretic mobility

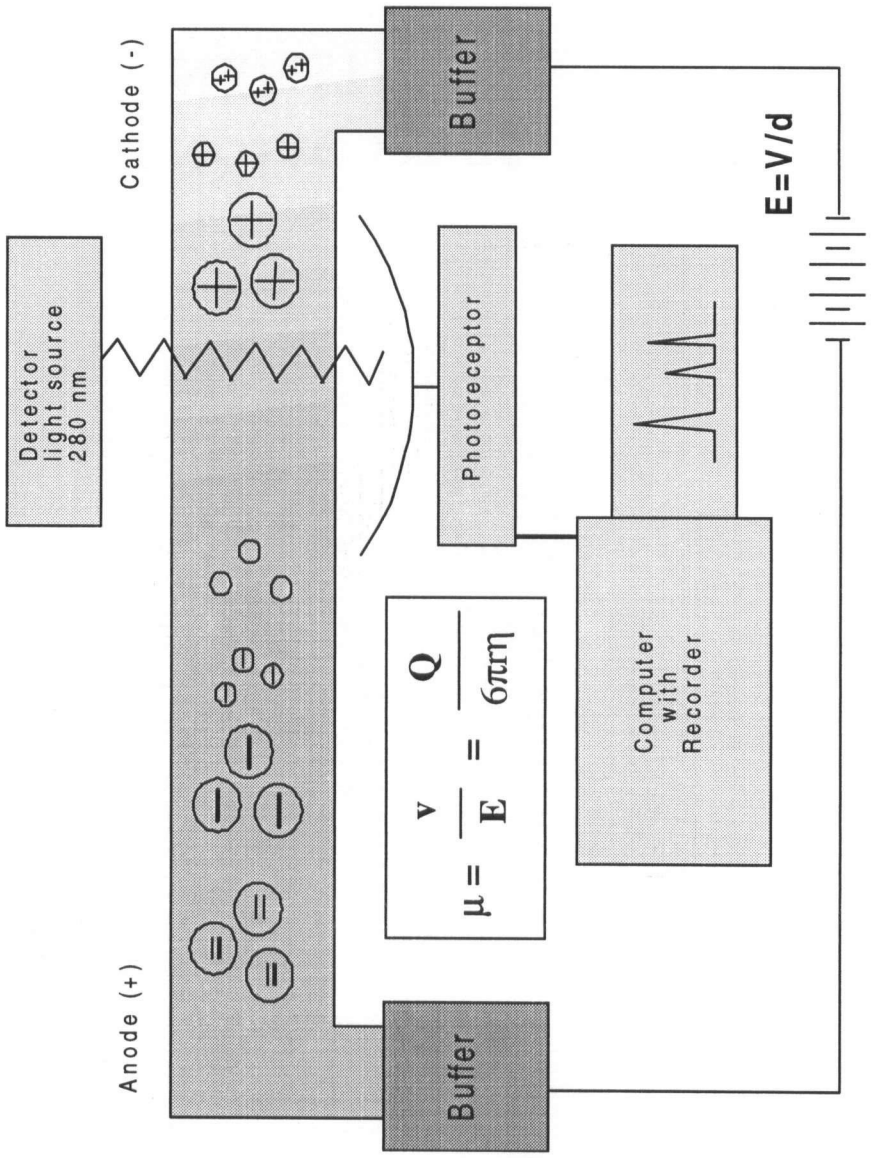
E: the applied electric field

One of the inherent advantage of CE is the ability to apply high voltages, up to 30,000 volts, because of the excellent heat transfer properties of the narrow bore capillary. The high applied voltage creates a high electric potential across the capillary resulting in extremely efficient and rapid separation.

1. Electro-osmotic flow

An important phenomenon in CE is electro-osmotic flow (EOF). Capillaries are made out of fused silica in which the inner wall hydrolyzes to form free silanol (SiOH) groups when in contact with water. Under certain pH condition, usually at high alkaline pH, the SiOH groups become deprotonated to their anionic forms (SiO^-), resulting in an accumulation of negative charges along the inner capillary wall surface. In order to neutralize this negative surface, hydrated cations collect at the inner surface of the capillary but can not fully neutralize the negatively charged wall. The electric field generated by the unneutralized negative charges attracts more cations to form a diffuse layer. A potential

Figure 26. Schematic diagram of a CE instrument and the principles of CE separation. The capillary is immersed in reservoirs containing separating buffers necessary for the production of an electric field supplied by a high voltage source. Positive ions migrate towards the (-) anode; negative ions towards the (+) cathode; while neutral ions have no electrophoretic mobility. The detection window is where a light source records the absorbance spectrum of eluting molecules into a photoreceptor, which is attached to a computerized system for automation.


Detector
light source
280 nm
Cathode (-)
Anode (+)
Buffer
Buffer
Photoreceptor
Computer
with
Recorder
E=V/d
$\mu = \frac{v}{E} = \frac{Q}{6\pi\eta}$

difference, known as the zeta potential, is present across the diffuse layer and falls off to zero towards the centre of the capillary. When a voltage is applied across the capillary, the cations forming the diffuse layer migrate towards the cathode, which is traditionally located at the buffer outlet reservoir. Due to solvation and hydrogen bonding, the solvated cations drag the bulk of the solution inside the capillary creating the electroosmotic flow or bulk solvent flow. The migration rate of an ion is then a combination of the electrophoretic mobility and the EOF. For this reason, when the EOF is high enough, negatively charged molecules and neutral molecules are pushed through toward the cathode end of the capillary. Thus the presence of the EOF makes CE a robust analytical system for the effective separation of all kind of molecules regardless of their charges.

In this system, amino acids are separated based on both their size-to-charge ratios and the strength of the EOF of the separation condition. Positively charged amino acids elute the fastest migrating with the EOF. Neutral amino acids elute next followed by the negative amino acids eluting against the EOF. Residue such as γ-carboxyglutamic acid having two negatively charges will elute even later because of the higher charge present due to the extra carboxylate group.

2. Capillary electrophoresis-laser induced fluorescence

Many types of detection systems can be employed with capillary electrophoresis. Commonly used on-line detection systems are ultraviolet (UV), photodiode array (PDA), and laser-induced fluorescence (LIF) detectors. For these optical systems, a detection window is made on the capillary where a light source can shine through and record the absorption (spectrum) of the migrating molecule. The detection limit for direct UV or PDA absorption is governed by Beer's Law, which states:

$$A = \varepsilon b c$$

where, A: absorbance

ε: molar absorptivity (extinction coefficient)

b: path length

c: concentration of the absorbing species

In CE, the optical path length for on-line detection is the very small, inner diameter of the capillary, usually in the μm range. It is apparent that the smaller the path length, the lower the resulting absorbance. That is why one of the on-going problems faced by researchers in capillary electrophoresis is the relative insensitivity of UV detection, resulting in poor concentration limits of detection (LOD). This restricts the use of UV detection in trace and ultratrace level analysis. More sensitive detection methods are therefore required to increase the LOD of small traces of materials. There are several adaptations to increase detection sensitivity including on-line focusing and sample stacking (increasing the sample concentration), increasing the path length (resulting in poor separation), and fluorescence detection.

Laser-induced fluorescence (LIF) detection has the potential to enhance sensitivity, since more monochromatic light can be focused into the small inner diameter of fused capillaries. This results in the excitation energy more effectively applied to the very small sample volume. Since most biological compounds such as proteins, peptides and amino acids do not possess native fluorescence properties at any one of the commonly available laser lines, chemical derivatization with a fluorescent label is required. However, problems with kinetics and labeling chemistry can adversely affect sensitivity resulting in a much higher concentration limit of detection than would normally have been achieved or expected. Fluorescein isothiocyanate (FITC) has been widely used as a fluorescent labeling reagent for antibody labeling, immunofluorescence procedures, and it is frequently applied in the labeling of proteins and amino acids. The chemistry of FITC labeling is depicted in Figure 27, where the isothiocyanate group reacts with the primary amine of an amino acid to form the thiocarbamylated (FTC) amino acid. At room temperature, the reaction is light sensitive and the mechanism of action requires several hours for adequate labeling (derivatization being the rate-determining step). Acidic and basic amino acids react relatively slowly in comparison to the others, probably due to charge repulsion. The use of FITC and fluorescence detection decreases the LOD in CE several thousand folds compared to direct UV detection. The use of this reagent in electrokinetic methods was reported by Cheng and Dovichi in 1988 when subattomole ($<10^{-18}$ M) analysis of some amino acids was demonstrated (Cheng et al., 1988).

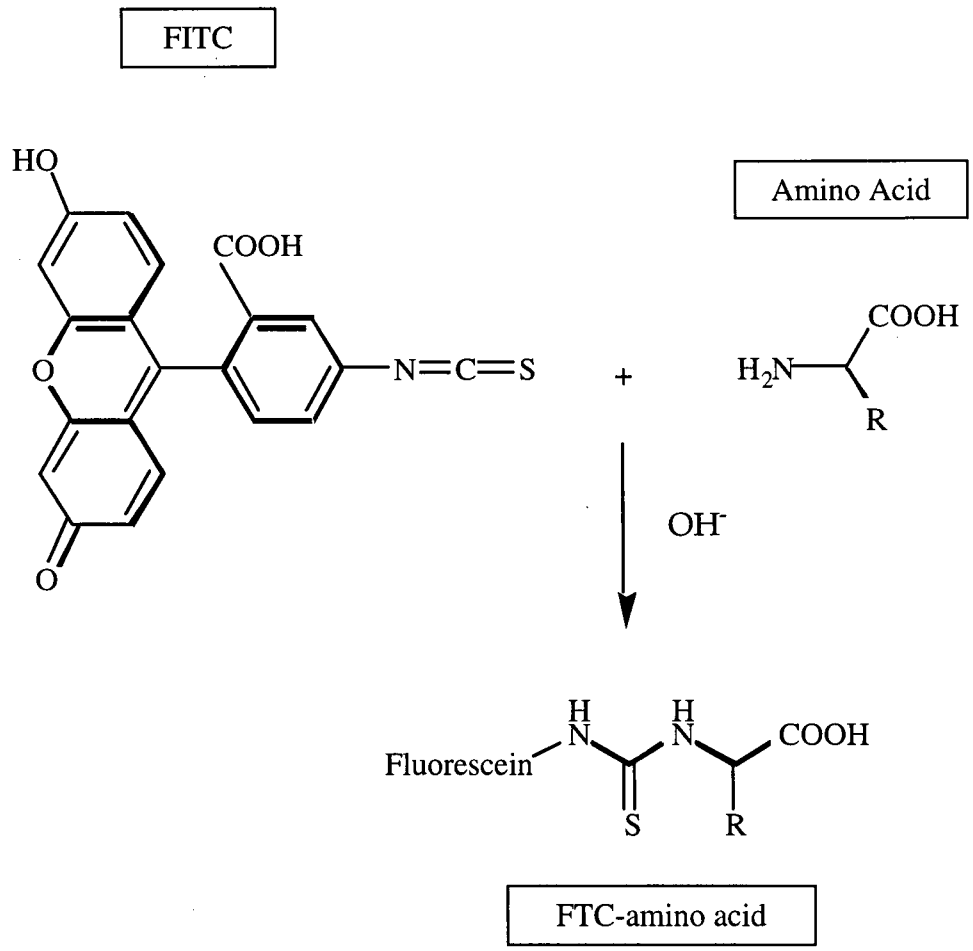


Figure 27. Derivatization of an amino acid with fluorescein isothiocyanate (FITC). The primary amine group of the amino acid reacts with the isothiocyanate group of FITC to form the fluorescein thiocarbamyl (FTC) amino acid. The reaction requires a basic condition (OH^-) and is light sensitive.

III. GLA ANALYSIS BY CAPILLARY ELECTROPHORESIS

1. Prothrombin and variants

The γ-carboxyglutamic acid (Gla) contents of several variants of human prothrombin have been measured by using capillary electrophoresis and laser-induced fluorescence (CE-LIF). The protein variants were recombinant proteins produced in tissue culture using BHK cells, as discussed in the previous chapter. The recombinant proteins were purified using a pseudo-affinity chromatography method and characterized for their calcium and phospholipid binding properties, as well as the ability to clot using a two stage clotting assay. These properties were correlated with their Gla content as measured by capillary electrophoresis. The recombinant prothrombin studied was a chimeric protein in which the second kringle domain had been replaced with the equivalent region of bovine prothrombin (rhBK2). The chimera rhBK2 has been described in chapter 3, using the EIPCR method.

The purification of the proteins consisted of precipitation from media with barium citrate, anion exchange chromatography by FPLC as described previously. A last step of purification was pseudo-affinity FPLC chromatography using a calcium gradient on a column of Mono Q HR 5/5 to resolve the different Gla-containing protein species. During the pseudo-affinity chromatography step, rhBK2 eluted as two populations (designated rhBK2A and rhBK2B) eluting at 10 mM $CaCl_2$ and 17.5 mM $CaCl_2$ respectively. Other recombinant proteins also showed sub-populations that are resolved using this pseudo-affinity method.

The expression system using BHK cells grown on a media supplemented with ITS-K was sufficient to produce adequate amount of recombinant protein. The extent to which the protein was modified is still unclear. There are several post-translational steps required before protein secretion such as γ-carboxylation, glycosylation and signal processing. However, the Gla content that is crucial for the functionality of the protein was undetermined thus far. The Gla content of the recombinant protein was measured indirectly using two methods: a calcium binding assay and a phospholipid binding assay. The results obtained from these assays were qualitative in nature and had to be compared to that of wild type protein. The technology of capillary electrophoresis was then employed to develop a quantitative analysis of the Gla content of the recombinant proteins. The Gla-content of the

variants of the human/bovine construct, rhBK2A and rhBK2B, were compared with human plasma prothrombin (rhFII) and human recombinant prothrombin (rhFII). The method was validated using wild type plasma factor X and osteocalcin protein. Typically, the assay for protein analysis involved a sequential three-step method. First, the protein was hydrolyzed with base, followed by a derivatization step, and finally the electrophoresis of the derivatized products using CE-LIF.

2. Base hydrolysis of proteins

The protein sample (1 μg of a 1 mg/ml solution in 20 mM Tris buffer, pH 7.5 containing 150 mM NaCl) was added to 30 μl of 2.5 M KOH for alkaline hydrolysis, or 30 μl of 6 N HCl for acid hydrolysis. The mixture was transferred into a 1.5 x 100 mm capillary tube and heat-sealed at both ends such that the air space was minimal. The tubes were then submerged completely in the wells of a heating block filled with industrial oil, and the hydrolysis was carried out at 110°C for 16 hours. After hydrolysis, the hydrolysate was transferred from the capillary tube to an eppendorf tube and the silicate byproduct was removed by centrifugation at 13,000 rpm for 5 min.

3. Urine and plasma preparations

Human urine samples were collected from healthy volunteers immediately after rising in the morning. Urine was stored frozen at –20°C and subsequently thawed, centrifuged at 13 000 rpm for 10 min, and used directly for fluorescent labeling. Pooled plasma was obtained from 30 normal donors and stored frozen at –80°C. Plasma was deproteinized by adding a half volume of HPLC grade acetonitrile or by filtering the sample through a Millipore Eppendorf tube with a 5000 MWCO filter at 3000g for 30 min prior to derivatization.

4. Fluorescein isothiocyante (FITC) derivatization of amino acids

Prior to derivatization, the alkaline hydrolysate was neutralized by addition of small aliquots of ice-cold 70 % and 7 % perchloric acid until the pH of the hydrolysate was approximately 4-7 (testing was done using litmus paper). After neutralization, the mixture was left on ice for 15 min and the insoluble potassium perchlorate was removed from the

solution by centrifugation at 13 000 g for 10 min, at room temperature. The supernatant was made up to 28 µl with 0.2 M sodium bicarbonate buffer pH 9.0, and 2 µl of 50 mM of fluoresceinisothiocyanate (FITC) in actone:pyridine (80:20, v:v) was added. The derivatization reaction was carried out in the dark at room temperature for 24 hr. The mixture was diluted 400 times with deionized water prior to CE. For urine and plasma, the samples were made up in 0.2 M carbonate buffer to a volume of 28 µl, to which was added 2 µl of 50 mM FITC.

For the standard calibration curve, standard amino acid solutions were made up as 0.2 M solutions in 0.2 M sodium bicarbonate buffer, pH 9.0. To ensure a total and complete reaction, the FITC concentration was ten-fold in excess for both the protein hydrolysate and the standard amino acids. In addition, using the plasma-derived prothrombin, an experiment in labeling time was performed over the course of 72 hr. The results showed that the Gla fluorescence signal was barely detectable after 30 min of labeling time but increased to noticeable level after 2 hr. The signal intensity reached a plateau after 12 hr with only very slight increase after 24 hr. In addition, the signal was stable for three days with storage at -20°C. The fluorescence intensities of FTC-Glu and FTC-Asp also behaved in similar fashion. The relative peak area ratio of Gla over Glu seemed to be constant at all time after the first initial 2 hr. Based on this observation, all analyses were carried out with a 24 hr labeling time.

5. Electrophoresis of FTC-amino acids

The fluoresceinthiocarbamyl (FTC) derivatives of the amino acids were separated using an automated Beckman P/ACE 5000 CE system. Uncoated fused-silica capillaries with inner diameter of 75 µm, outer diameter of 360 µm, and length of 57 cm were used. A new capillary was rinsed for 20 min with aqueous 1 M NaOH followed by a 20 min rinse with separation buffer. The separation buffer was 100 mM Tris.Cl buffer pH 8.25. The capillary was then allowed to equilibrate overnight in separation buffer before being used. Before each separation, the capillary was rinsed with 0.1 M NaOH for 2 min followed by a 4 min rinse with separation buffer. The sample was introduced by a 3 s low pressure injection (0.5 psi) and the separation was carried out for 20 min at 30 kV and 25°C. A 4 mW argon ion laser was used for detection; excitation was at 488 nm and the emission was monitored at

520 ± 20nm with a Beckman fluorescence detector. Data were collected and processed using System Gold software.

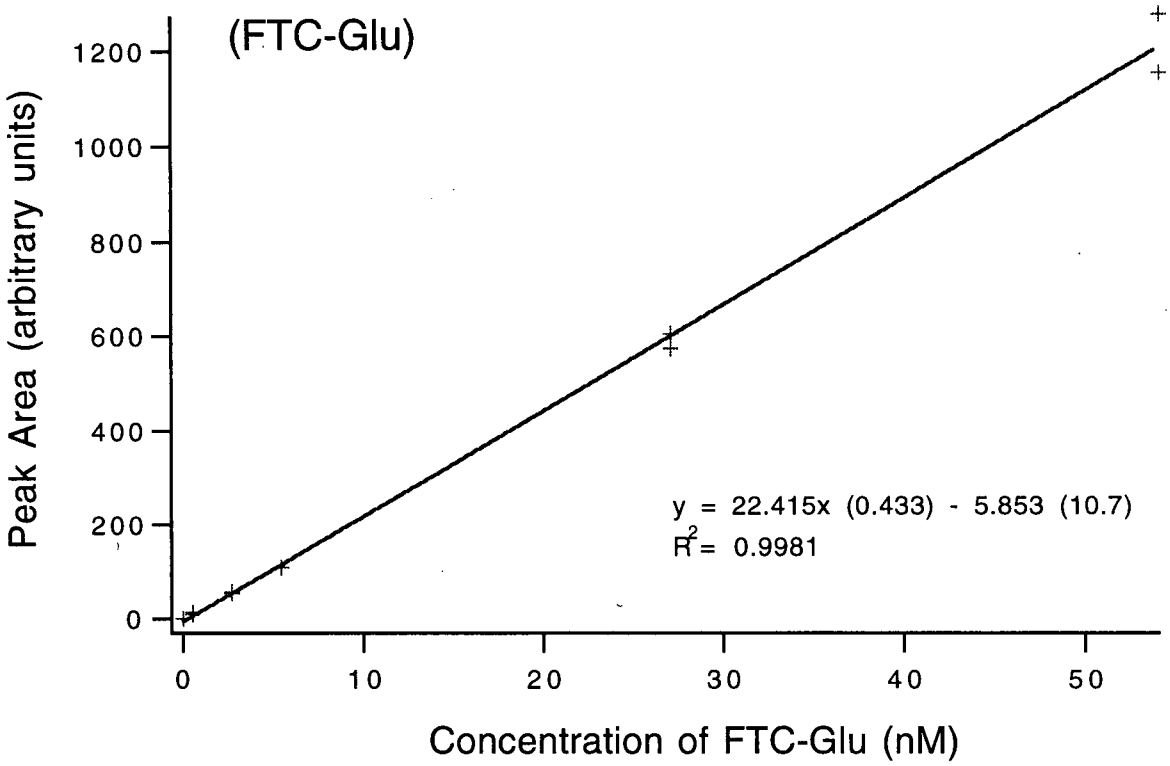
6. Calibration plots of FTC-Gla and FTC-Glu

Standard gamma-carboxyglutamic acid and glutamic acid were used for calibration curves. A mixture of FITC (1.0×10^{-5} M) and standard amino acid (1.0×10^{-3} M) were mixed together in a 1 ml volume solution. The solution was covered in thin foil and stirred at room temperature for 4 hours for the Glu/FITC solution and for 8 hours for the Gla/FITC solution. After derivatization, the solution was serially diluted at appropriate concentrations and samples were taken for CE-LIF analysis. The linear dynamic range was 10^{-7} to 10^{-10} M for both Glu and Gla with R^2 values of 0.9981 and 0.9999 for FTC-Glu and FTC-Gla respectively. The curve fit for FTC-Glu had the linear equation y = 22.415 x (± 0.433) - 5.853 (± 10.7), and for FTC-Gla y = 17.068 x (± 0.054) - 0.88514 (± 1.2); y was the fluorescence for the peak area, and the x value the concentration of the FITC labeled amino acid in nM concentration (Figure 28).

7. Gla content of recombinant prothrombin and variants

To assess the carboxylation status of prothrombin expressed using the pNUT-BHK system, samples of human plasma prothrombin (phFII) and recombinant human prothrombin (rhFII) were hydrolyzed, derivatized with FITC and the FTC-labeled acidic amino acids were analyzed by CE-LIF. As a control, the non-Gla containing ferric iron binding protein from *N. gonnhoreae* was also analyzed. Figure 29 showed the results of CE-LIF analyses of both acid and base hydrolysates of rhFII. The expected FTC-Gla peak was observed in the alkaline hydrolysate of rhFII (panel C); however, no FTC-Gla peak was observed in the acid-hydrolyzed samples of rhFII (panel B) nor in the base-hydrolyzed sample of ferric iron binding protein (panel A). In addition, the FTC-Glu to FTC-Asp ratio of the acid-hydrolyzed sample of rhFII was slightly higher than that of the base-hydrolyzed sample. These results were consistent with the decarboxylation of Gla to Glu during the acid hydrolysis step. In some electropherograms, an unidentified peak appeared that eluted between the FTC-Asp and FTC-Gla peaks; however, this peak did not interfere with the quantification of the three

Figure 28. Calibration plots for FTC-Gla and FTC-Glu peak areas. A mixture of FITC (1.0×10^{-5} M) and standard amino acid (1.0×10^{-3} M) was mixed together in a 1 ml volume solution. The solution was covered in thin foil and was stirred at room temperature for 8 hours for derivatization. After derivatization, the solution was serially diluted at appropriate concentrations and samples were taken for CE-LIF analysis. Linear regression was performed to determine the relationship between the fluorescence response and the concentration of the FTC-Gla and the FTC-Glu peaks.


(FTC-Glu)
Peak Area (arbitrary units)
1200
1000
800
600
400
200
0
0
10
20
30
40
50
Concentration of FTC-Glu (nM)
y = 22.415x (0.433) - 5.853 (10.7)
R2= 0.9981

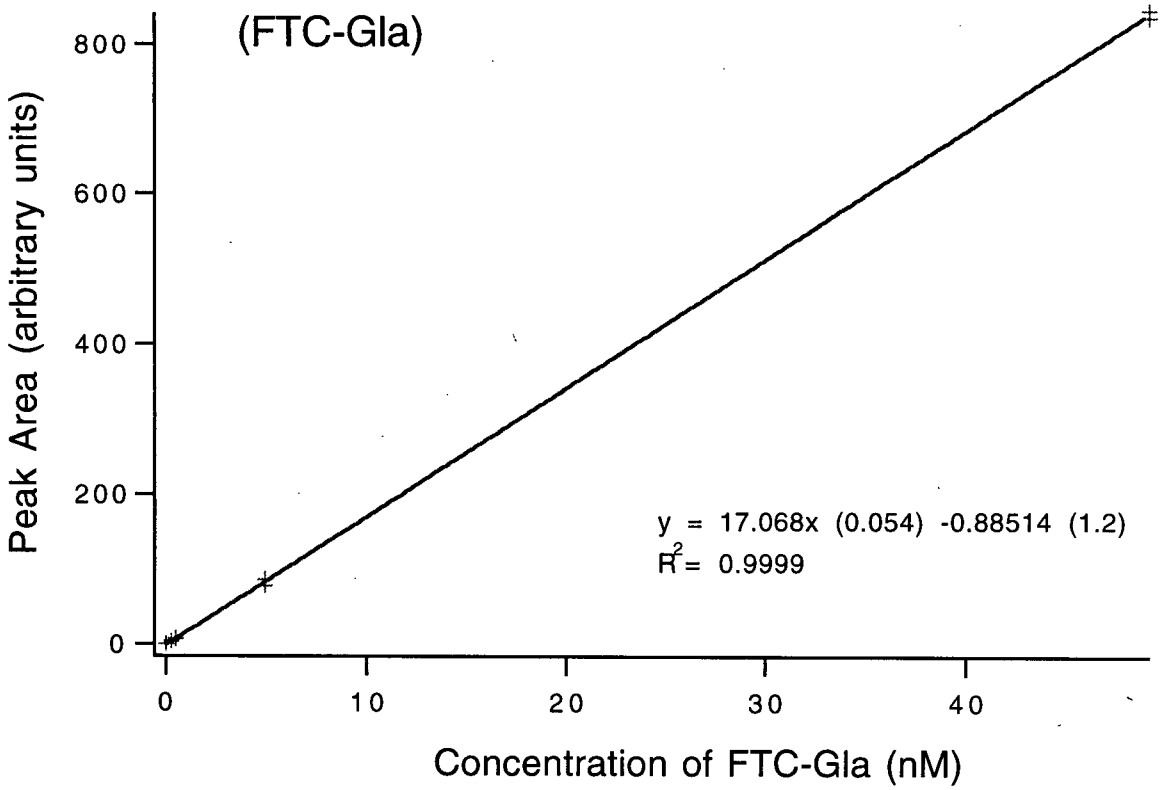

(FTC-Gla)
Peak Area (arbitrary units)
800
600
400
200
0
0
10
20
30
40
Concentration of FTC-Gla (nM)
y = 17.068x (0.054) -0.88514 (1.2)
R2= 0.9999

Figure 29. Electropherograms from the hydrolysates of recombinant human prothrombin analyzed by CE-LIF. Panel A: Alkaline hydrolysis of ferric iron-binding protein. Panel B: Acid hydrolysis of recombinant human prothrombin (rhFII). Panel C: Base hydrolysis of rhFII. The peaks corresponding to the FTC-labeled acidic amino acids were determined from their migration times; where necessary, the identity of the FTC-Gla peak was confirmed by adding an aliquot of standard FTC-Gla to the sample, and repeating the CE-LIF analysis. The peaks corresponding to FTC-Glu (1), FTC-Asp (2) and FTC-Gla (3) are labeled.

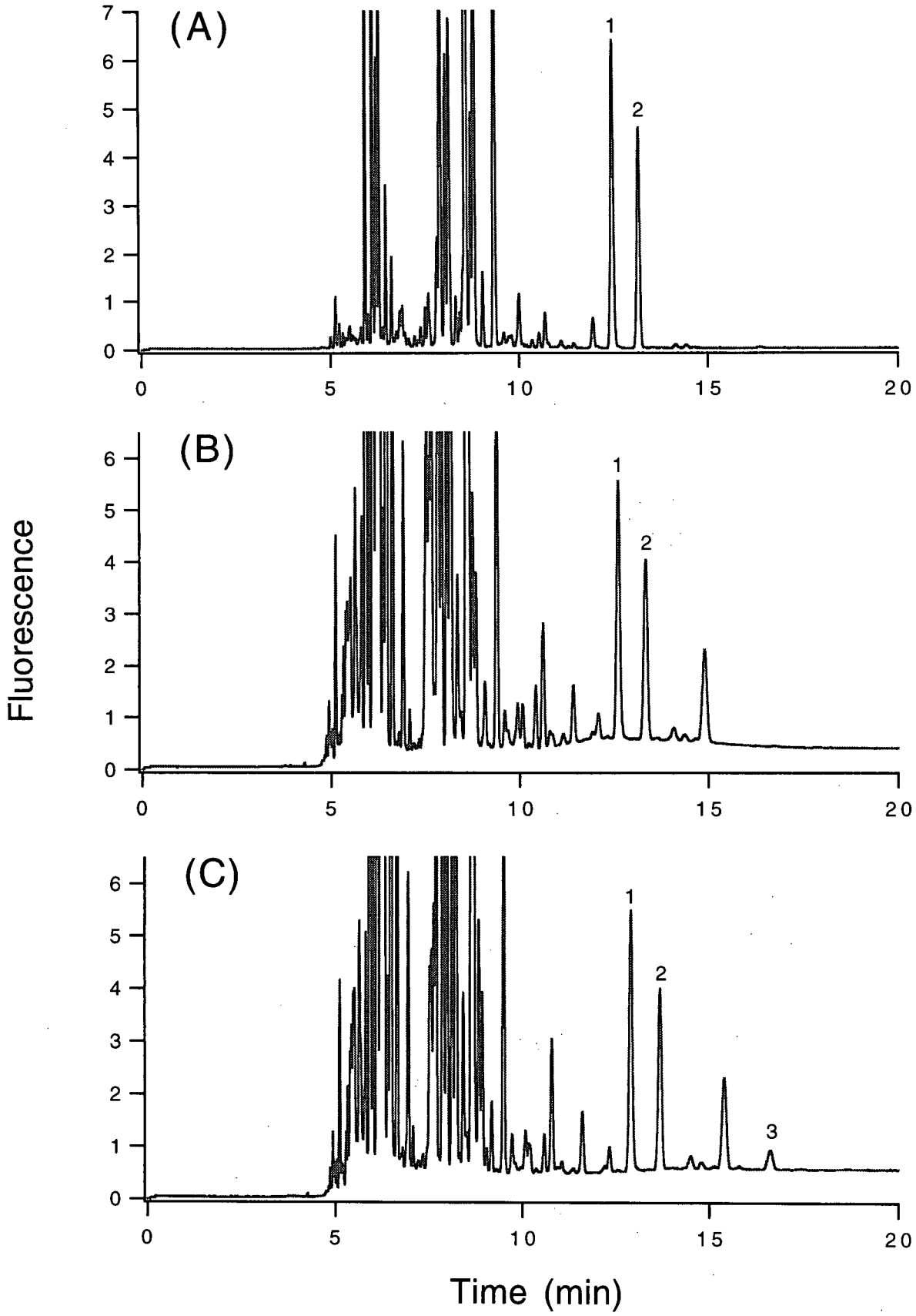
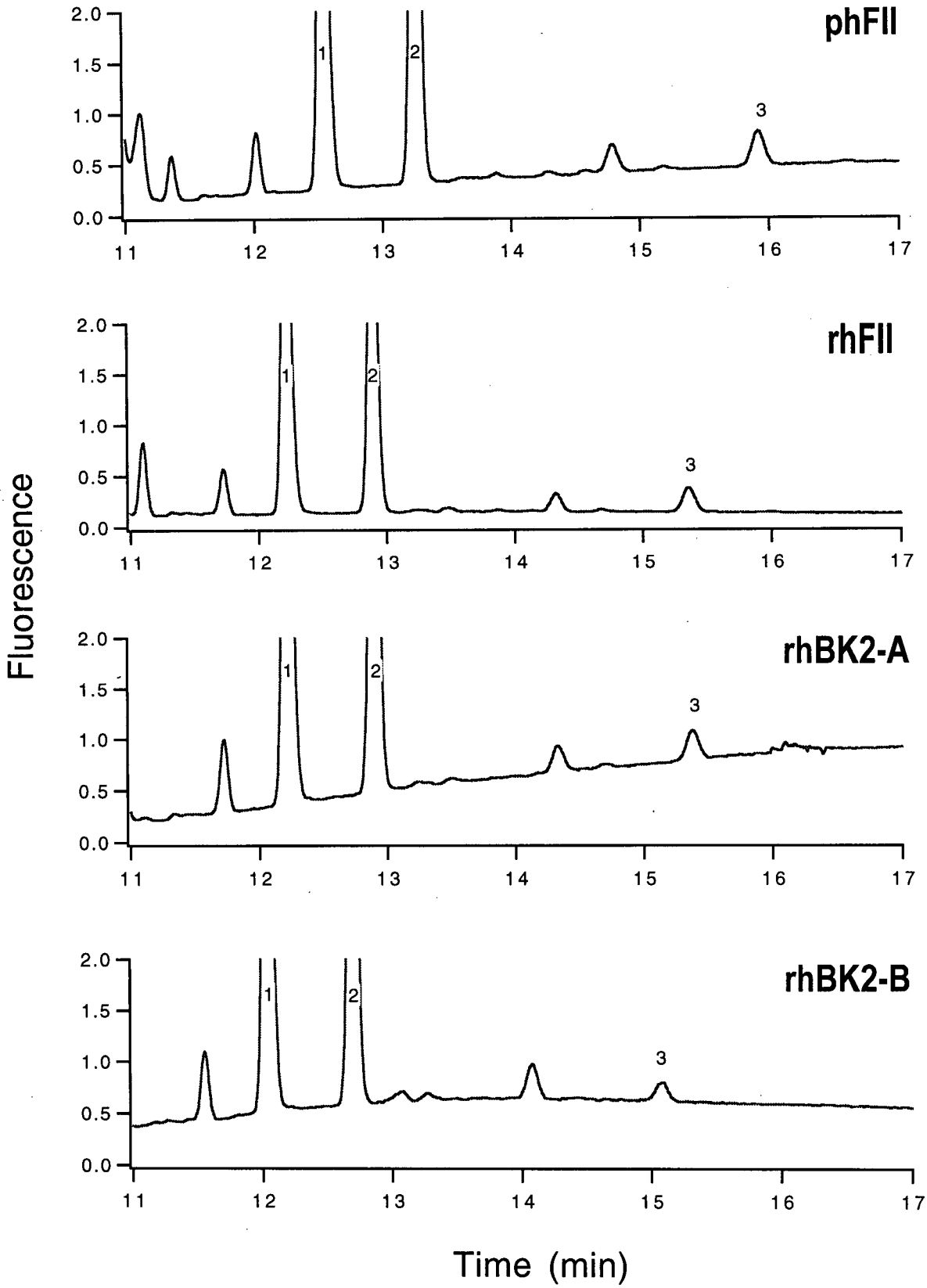

(A)
(B)
(C)
1
2
3
Fluorescence
Time (min)
0
1
2
3
4
5
6
7
0
5
10
15
20

Figure 30. Electrophoregrams from alkaline hydrolysis of prothrombin variants analyzed by CE-LIF. Panel A: phFII. Panel B: rhFII. Panel C: rhBK2A. Panel D: rhBK2B. The peaks corresponding to to FTC-Glu (1), FTC-Asp (2) and FTC-Gla (3) are labeled.


phFII
rhFII
rhBK2-A
rhBK2-B
1
2
3
2.0
1.5
1.0
0.5
0.0
11
12
13
14
15
16
17
Fluorescence
Time (min)

acidic amino acids of interest. The Gla contents of both plasma and recombinant human prothrombin were summarized in Table 4. Under CE-LIF analysis, both the phFII and rhFII samples had Gla/Glu ratios corresponding to the ten residues of Gla per molecule of protein. The high Gla content was consistent with the purification of rhFII by pseudo-affinity chromatography using calcium gradients; this method was developed to enrich for fully carboxylated species of recombinant human protein C (Yan et al., 1990).

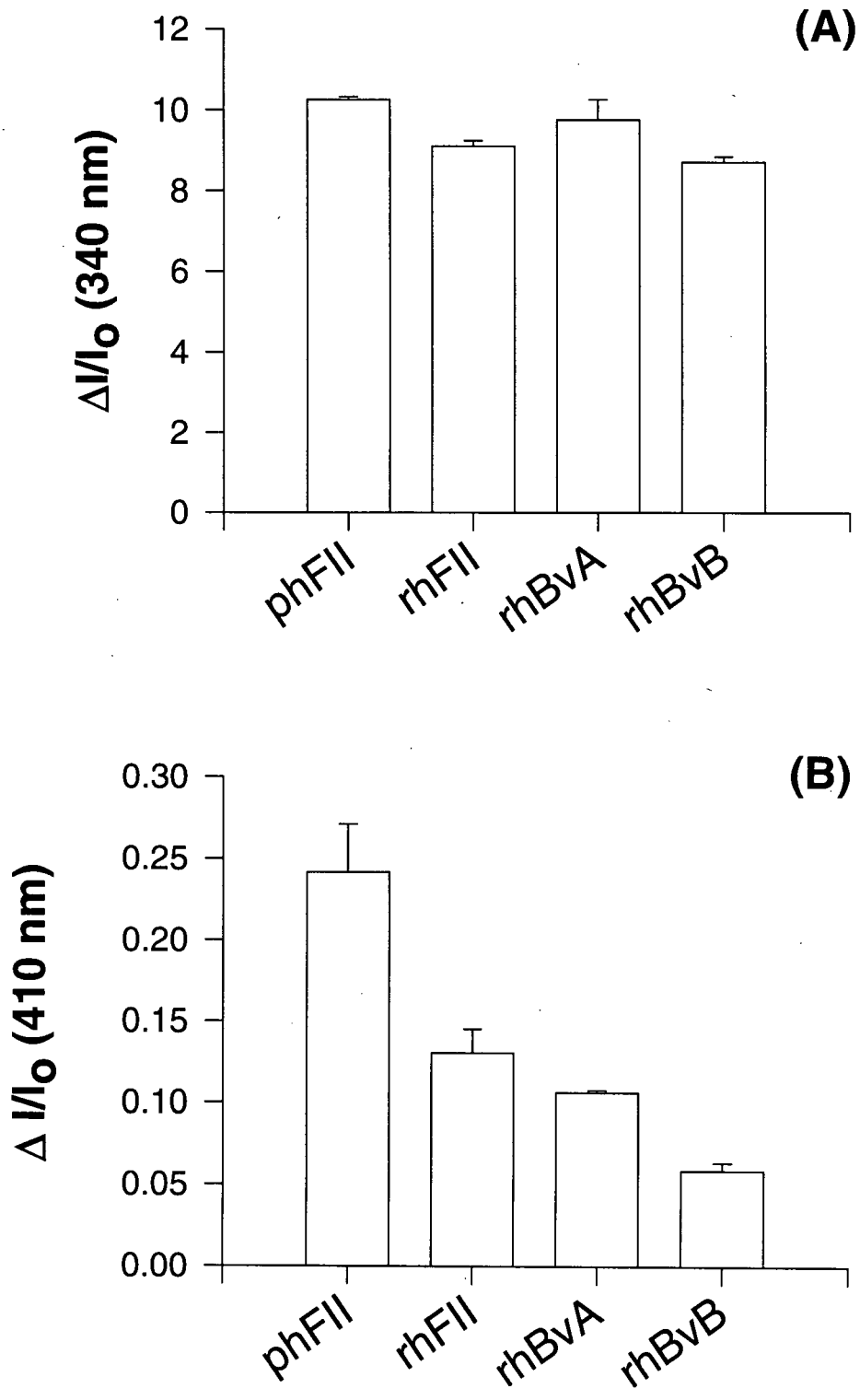
Samples of the two prothrombin variants, rhBK2A and rhBK2B, were also analyzed in a similar manner. Analysis of Gla/Glu ratios showed that rhBK2A and rhBK2B contained 8.6 ± 0.1 and 6.2 ± 0.7 residues of Gla per molecule of protein respectively (table 4). As shown in Figure 30, the Gla peak of rhBK2B (panel D) was lower than those of phFII, rhFII, and rhBK2A (panel A, B, and C respectively).

IV. PROTEIN CHARACTERIZATION

1. Calcium and phospholipid binding properties

To correlate the Gla content with the Ca^{2+}-induced conformational change, the calcium and phospholipid binding properties of rhBK2A and rhBK2B were assessed using intrinsic tryptophan fluorescence and right angle light-scattering, respectively (table 4). The results were compared with those obtained from phFII and rhFII. The results of the fluorescence quenching and light scattering on calcium binding were shown in Figure 31. The proteins rhFII and rhBK2A showed very similar tryptophan fluorescence quenching responses that were similar to those observed for phFII. The relative tryptophan fluorescence quenching responses were 89% and 95% for rhFII and rhBK2A respectively. The results suggested that the proteins underwent a similar conformational change upon calcium binding. Consistent with a lower Gla content, rhBK2B exhibited a slightly smaller response (85%) on the addition of calcium. In the right angle light scattering experiments, the addition of calcium to the suspension of protein and PCPS phospholipid vesicles promoted the formation of protein-lipid interactions resulting in increased light scattering intensity as the effective radius of the phospholipid vesicles increases. Figure 32 showed the increment in right angle

Figure 31. Calcium-binding and phospholipid-binding properties of prothrombin variants. Panel A: The relative change in intrinsic tryptophan fluorescence after calcium addition. Panel B: The relative change in light scattering on calcium-dependent binding to phospholipid vesicles. Each experiment represents the average of three independent measurements, and the bars represent the standard deviation. PhFII, plasma derived prothrombin; rhFII, recombinant prothrombin; rhBK2A, Gla-rich prothrombin variant with bovine kringle 2; rhBK2B; Gla-poor prothrombin variant with bovine kringle 2.


(A)
12
10
8
6
4
2
0
ΔI/Io (340 nm)
phFII
rhFII
rhBvA
rhBvB
(B)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Δ I/Io (410 nm)
phFII
rhFII
rhBvA
rhBvB

Right angle light scattering response to calcium binding and phospholipid vesicles binding of the two species of the recombinant prothrombin variant rhBK2

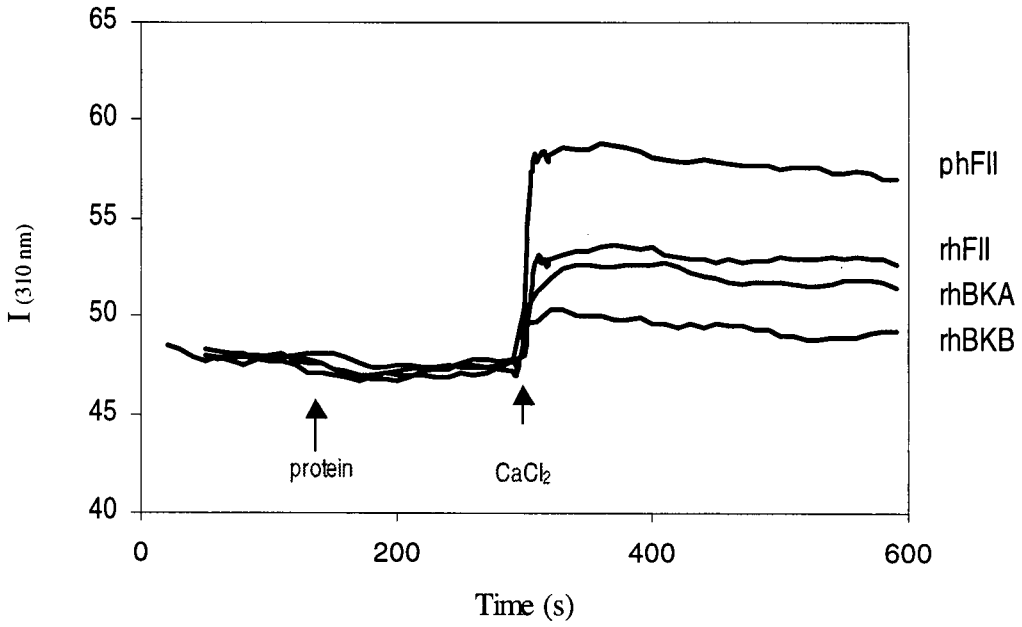


Figure 32. Right angle light scattering response to calcium binding and phospholipid vesicles binding of the recombinant prothrombin variant rhBK2. An aliquot of phosphotidylcholine:phosphotidylserine (PCPS) vesicle (20 μg/ml) in 2 ml of 0.02 M Tris.Cl pH 7.4 containing 150 mM NaCl was stirred in a quartz cuvette in a temperature controlled luminescence spectrometer at 25°C. A sample (10 μg) of human wild type plasma prothrombin (phFII) or recombinant prothrombin (rhFII) or the two prothrombin variants rhBK2A and rhBK2B was added and right angle light scattering intensity was monitored coninously for 3 min at 410 nm with 2.5 nm slit width. Calcium ($CaCl_2$) was added to 5 mM final concentration and the course of the scattering was monitored for another 5 min. The lines represent the light scattering of the phospholipid vesicle-protein complexes before and after the addition of the proteins and calcium ions (the arrows).

Table 4. Correlation of Gla content and biological activities of plasma prothrombin, recombinant prothrombin and variants.

Protein	Gla/Glu ratio	Gla residue (per mole protein)[a]	$\Delta I/I_o$ Trp[a,b] (340 nm)	Relative Ca^{2+} binding activity (%)	$\Delta I/I_o$[a,c] (410 nm)	Relative PCPS binding activity (%)	Clotting Time[d] (sec)	Relative clotting activity (%)
phFII	0.07377 ± 0.0002	10 ± 0.3*	10.25 ± 0.05	100	0.25 ± 0.03	100	20.7 ± 0.6	100
rhFII	0.07371 ± 0.0013	10 ± 0.2*	9.10 ± 0.10	89 ± 1	0.13 ± 0.02	53 ± 6	25.3 ± 0.6	62 ± 3*
rhBvA	0.06366 ± 0.0008	8.6 ± 0.1	9.75 ± 0.35	95 ± 3	0.11 ± 0.01	43 ± 1	25.4 ± 0.5	62 ± 3*
rhBvB	0.04584 ± 0.0052	6.2 ± 0.7	8.70 ± 0.10	85 ± 1	0.06 ± 0.01	24 ± 2	61 ± 1	8.8 ± 0.3

a values represented for each protein were from three determinations

b tryptophan fluorescence responses to calcium binding measured at 340 nm

c phospholipid binding properties measured by right angle light scattering at 410 nm

d one-stage clotting assay using prothrombin-deficient plasma and thromboplastin; values represented for each protein were from six determinations

* within each assay, these values were not statistically different (P>0.05); statistical analysis were determined using the T-test

light scattering of the protein samples upon the addition of calcium, monitored over a time course of 10 min. On addition of calcium, all the recombinant proteins showed diminished binding to phospholipid compared to the plasma wide type prothrombin (table 4). The relative response in right angle light scattering of rhFII was 54 % compared to phFII. The rhBK2A protein had a lesser response (44%) while the rhBK2B protein (with the least Gla residues) had only a 24% response relative to phFII. Thus, the number of Gla residues correlated with the ability of prothrombin to bind to phospholipid vesicles. These results indicated that the phospholipid binding property of rhBK2B was severely affected when the Gla content was reduced to an average of approximately six residues.

2. Clotting activity

To correlate these binding studies with biological activity, clotting assays were performed on phFII and the recombinant proteins (Table 4). As reported previously (Côté et al., 1994), rhFII had only 62% of the clotting activity of phFII. The mutant rhBK2A had a similar clotting activity to rhFII, both with 62% relative to wild type, suggesting that the point mutations introduced with the bovine kringle 2 region did not affect the assembly and activity of the prothrombinase complex under the conditions used. In contrast, the mutant rhBK2B had only 9% of the clotting activity of phFII. Therefore, the six residues of Gla in rhBK2B were insufficient for the function of prothrombin in the prothrombinase complex. These results also indicated that the number of Gla residues on prothrombin and its ability to be activated upon binding onto the surface of phospholipid membrane were closely related.

V. DISCUSSION

A simple and sensitive method was presented for the analysis of γ-carboxyglutamic acid (Gla) by capillary electrophoresis. A protein hydrolysate was derivatized with FITC and the FTC-labeled amino acids were detected by CE-LIF. The dynamic range of the method spanned several orders of magnitude in concentration for γ-carboxyglutamic acid and the limit of detection was in the attomol (10^{-18}) range. The analysis of both the plasma and recombinant wild type prothrombin yielded a value of 10 ± 0.2 mol of Gla per mole protein

that was in agreement with the protein sequence data. The Gla contents of the two sub-populations of a recombinant prothrombin variant rhBK2 (rhBK2A and rhBK2B) were found to be 8.6 ± 0.1 and 6.2 ± 0.7 per mole protein respectively. The variant rhBK2 had the second kringle domain replaced by its bovine counterpart. During the purification of rhBK2, the Gla-rich component (rhBK2A) was separated from the Gla-poor component (rhBK2B) by pseudo-affinity chromatography. While both species bound Ca^{2+}, only the Gla-rich species bound significantly to phospholipid vesicles, and was active in a clotting assay. Thus, the direct measurement of Gla content in the recombinant proteins correlated well with clotting activity.

CE-LIF is the method of choice for the analysis of small amounts of amino acids and peptides. The small size and speed of analysis are well suited to the assessment of the carboxylation status of recombinant vitamin K-dependent proteins. In addition, Gla analysis by this method can potentially be used for protein sample from a partially purified source by transferring the protein band of interest from a SDS-PAGE gel, thus eliminating the need for time consuming purification steps. The method was further validated by the analysis of the Gla content of plasma protein factor X and osteocalcin as well as urine and plasma samples.

1. Validation of Gla analysis by CE-LIF

The reproducibility of separately prepared samples from all analysis was very consistent with a relative standard deviation (RSD) of less than 5%. The limit of detection for Gla using this method was about 5.0×10^{-11} M. These figures demonstrated that the method for the analysis of Gla content from a protein hydrolysate was highly sensitive, consistent, and reproducible. For the analysis of protein samples, alkaline hydrolysis was required to prevent the decarboxylation of the γ-carboxyglutamic acid that occurs under acid hydrolysis conditions. The signals of the FTC-conjugated Glu, Asp and Gla were well resolved under the described condition with an elution time under 20 min. The fluorescence response factor of Gla was substantially lower than both Glu and Asp, as observed previously (Hauschka, 1979; Smalley et al., 1988). The lower fluorescence yield was probably due to the quenching of the fluorescent signal by the carboxylate groups present in Gla. Identification of Gla was confirmed by spiking the analyzed sample with Gla standard prior to CE analysis; the absence of the Gla peak in the acid-hydrolyzed sample was also a

confirmation of the identity of the Gla peak as well. The method of Gla analysis by CE-LIF was further validated by the analysis of the Gla content of two commercially-available proteins, clotting protein factor X (human) and osteocalcin (bovine) or bone gla protein. The free Gla content in urine and plasma samples were also measured. Factor X has been reported to contain 11 Gla residues and bovine osteocalcin has between 2 to 3 residues. The results show a value of 11.0 ± 0.8 Gla residues per molecule of protein for human factor X and 2.1 ± 0.1 Gla residues per molecule of protein for bovine osteocalcin (Figure 33). Thus the results agreed well with data from published protein sequence. Free Gla in urine and plasma was measured by CE-LIF with FITC derivatization (Figure 34). The Gla peak was readily detected and identified in the urine sample. Peak identification was confirmed by spiking with known standard and by observing the disappearance after acid hydrolysis. The Gla concentration from a urine sample of a healthy individual was measured to be $(9.2 \pm 0.2) \times 10^{-6}$ M. For Gla analysis of pooled plasma, the sample was first deproteinized by either acetonitrile precipitation or by ultrafiltration (centrifugation) prior to derivatization. No Gla peak was detected when the plasma sample was deproteinized by acetonitrile precipitation, probably because the free Gla was co-precipitated with other macromolecules such as proteins, lipids or other Gla-binding complexes. When ultrafiltration was employed, traces of Gla were detected corresponding to a concentration of $(4.9 \pm 0.3) \times 10^{-8}$ M. Thus the recovery and analysis of Gla content in plasma using this method seems to be dependent on the method of deproteinization. Free Gla level in plasma has been reported in healthy individuals to be about $(14.6 \pm 0.3) \times 10^{-8}$ M (Hanss et al., 1994) which is in the same range as the current study. The free Gla content in both urine and plasma varied greatly among individuals and may warrant a systematic study to correlate the Gla level with other biologically relevant markers.

2. Application of CE to biological systems

CE-LIF analysis of Gla from a protein hydrolysate offers many advantages because it is simple and highly efficient. The relatively high stability of FTC-labeled amino acids is advantageous compared to some other method in which analysis has to be carried out immediately because signal intensity diminishes quickly over time. In addition, minimal

Figure 33. Electropherograms from the hydrolysates of plasma human factor X and bovine osteocalcin analyzed by CE-LIF. Panel A: Alkaline hydrolysis of human plasma factor X. Panel B: Alkaline hydrolysis of plasma bovine osteocalcin. The peaks corresponding to FTC-Glu (1), FTC-Asp (2) and FTC-Gla (3) are labeled.

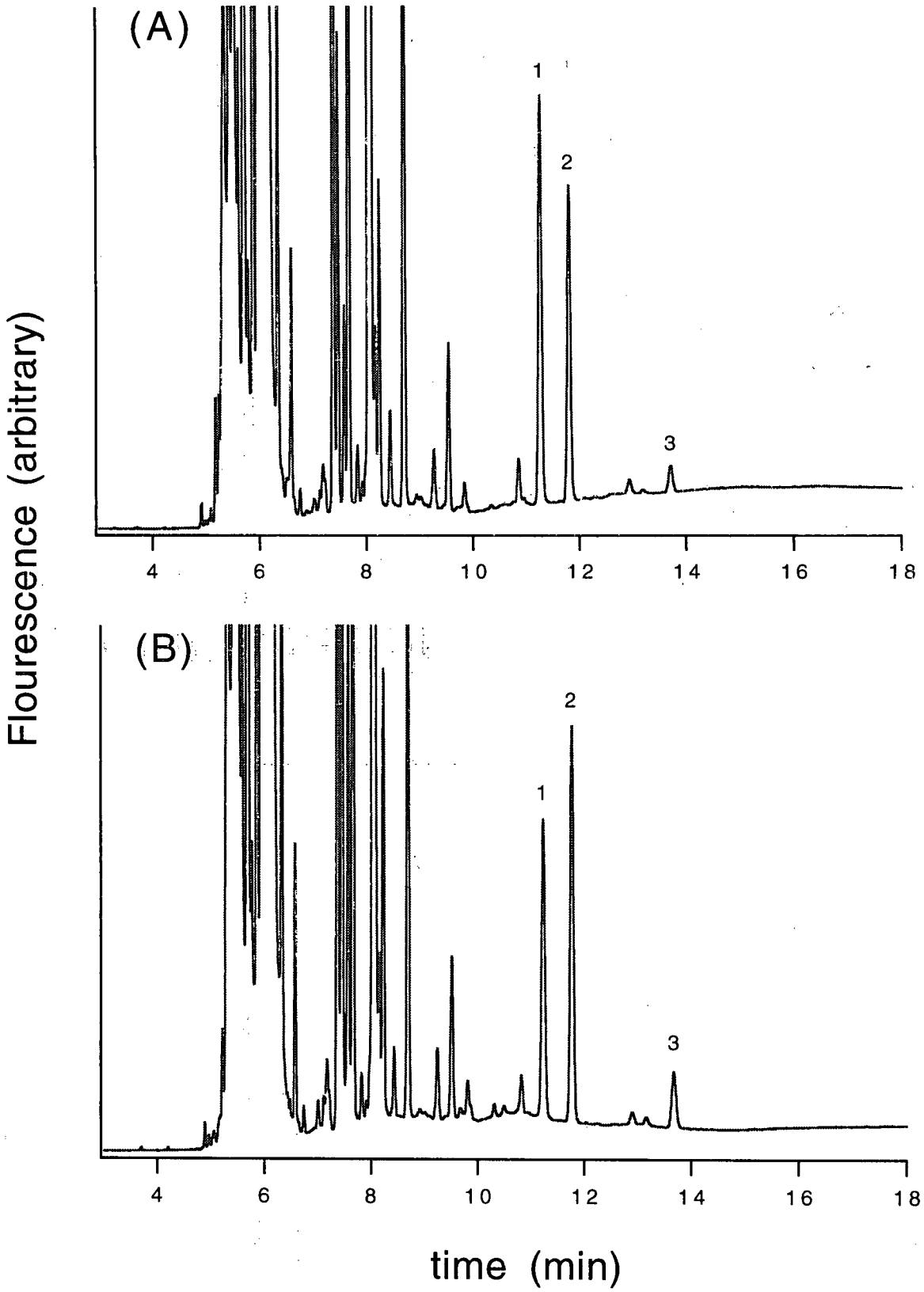
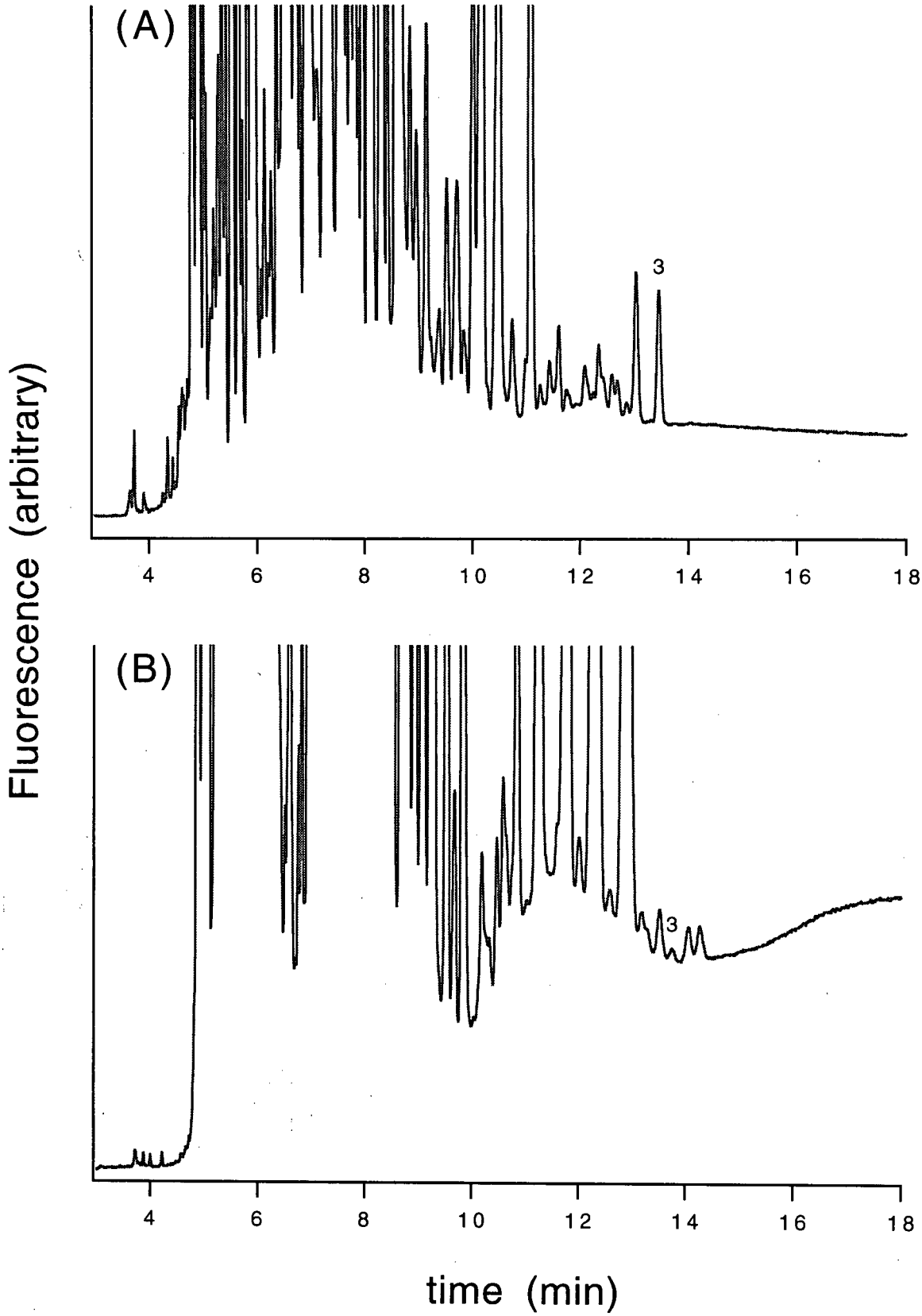

(A)
1
2
3
4
6
8
10
12
14
16
18
(B)
1
2
3
4
6
8
10
12
14
16
18
Flourescence (arbitrary)
time (min)

Figure 34. Electropherograms of free Gla content from urine and plasma samples analyzed by CE-LIF. Panel A: Urine. Panel B: pooled plasma. The peaks corresponding to FTC-Gla (3) are labeled (*).


(A)
3
4
6
8
10
12
14
16
18
(B)
3
4
6
8
10
12
14
16
18
Fluorescence (arbitrary)
time (min)

pre-column preparation is required for CE analysis and the separation time is less than 20 min. The method is highly sensitive, requiring very low amounts of starting material (sub-microgram), and offers a very high limit of detection (attomol). In addition, this is highly reproducible and consistent. The developed method has great potential for the analysis of a partially purified protein sample. A partially purified protein sample can be readily obtained from SDS-PAGE gel. The detection limit in polyacrylamide gel is in the range of ten of nanograms of protein (Coomassie and silver staining). This amount is more than required for the Gla analysis by CE/LIF. Thus long sample purification and preparation time can be avoided. This method should be particularly useful for the analysis of other vitamin K-dependent clotting factors that are recombinant proteins expressed in tissue culture cells.

The presence of free Gla in serum and urine samples has been characterized as important marker in the assessment of a wide variety of clinical situations, such as bone turnover (Epstein, 1988), osteoporosis and Paget's disease (Gundberg et al., 1983), liver disease and diabetes mellitus (Goto et al., 1994), deep vein thrombosis and intravascular disseminated coagulation (Hanss et al., 1994), primary hyperparathyroidism (Yoneda et al., 1986), and calcium oxalate crystallisation (Nishio et al., 1990). The method described in this paper should be easily modified for these analyses.

3. Carboxylation of prothrombin in BHK system

A variety of different expression systems have been used to express recombinant vitamin-K dependent clotting factors including prothrombin, factor X, factor IX, factor VII and protein C (Roddie et al., 1997). Although prothrombin has been expressed at low yields in a fully carboxylated form (Jorgensen et al., 1987) increasing the yield of recombinant protein is associated with the appearance of partially carboxylated forms (Côté et al., 1994). It has been proposed that high levels of recombinant protein overwhelm the vitamin K-dependent carboxylation capacity of the tissue culture cell lines resulting in variants of partially carboxylated forms. To enrich for fully carboxylated species, purification techniques have been developed such as affinity chromatography using conformation-dependent antibodies (Jorgensen et al., 1987; Rudolph et al., 1997), and pseudo-affinity chromatography with calcium gradients (Yan et al., 1990). In this study, the Gla contents of phFII and rhFII have been measured directly. Consistent with previous studies, the rhFII

produced in the pNUT-BHK cell system has a lower clotting activity than phFII. Using the pNUT-BHK expression system, the expressed recombinant protein could have as much as four different Gla-containing species as resolved by pseudo-affinity calcium gradient chromatography. It seems then that the advantage of a high yield expression system is offset by the under-carboxylation of the secreted protein.

When recombinant prothrombin was expressed in BHK cells in the presence of coumarin, a vitamin K agonist, and in the absence of vitamin K, no recombinant protein was secreted into the media. The disruption of the Gla formation by the vitamin K dependent carboxylase led to the production of a Gla-less protein that was observed to be non-secreted in BHK cell line. As noted previously chapters, Gla-less prothrombin has been produced in human and bovine cell lines but not in a rat cell line. Under-γ-carboxylated prothrombin was shown to be degraded in the endoplasmic reticulum in warfarin-treated rat cell cultures (Wu et al., 1997).

4. Structure of the Gla domain

The results from CE analysis showed that the number of Gla residues in prothrombin was related to the ability of the protein to bind phospholipid vesicles. The γ-carboxyglutamic acid residues of prothrombin play significant roles in calcium binding and in phospholipid binding, and are essential for the activation of the protein into the protease thrombin. These results indicated that the functional activity of the recombinant prothrombin mutant rhBK2B was severely affected when the Gla content was reduced to approximately 6 residues. As a consequence, the relative response to right angle light scattering was severely reduced (24%) in phospholipid binding studies. The calcium-dependent tryptophan fluorescence quenching response was also reduced although at a lesser extent (85%). Together, the reduction on calcium and phospholipid binding properties lead to a reduced clotting activity as measured by clotting assay (9%). In contrast, the Gla-rich prothrombin variant (rhBK2A) that had 10 Gla per mole protein has similar properties compared to that of recombinant wild type prothrombin. Thus, it seemed the point mutations introduced in the second kringle region of rhBK2 did not contribute significantly to the functionality of the protein, particularly in the ability to be activated by the prothrombinase complex.

As originally described for plasma-derived prothrombin, there was an abrupt loss of functional activity associated with the loss of as few as three of the ten Gla residues found in the plasma protein (Malhotra et al., 1985). Previous studies have shown that most if not of all of Gla residues are required for complete prothrombin function, probably through the Gla involvement in the calcium-binding dependent conformational change and phospholipid binding preceding the assembly of the activating complex. Previous studies have shown that the Gla domain in vitamin K-dependent proteins is responsible for the metal binding induced conformational change of the protein preceding phospholipid binding.

5. Function of the Gla domain

New insight has been shed on the mode of interaction of Gla-containing proteins with biological membranes in recent studies. Important factors have been identified to be the side chains of the Gla residues exposed on the surface of the protein and a cluster of three hydrophobic amino acids in the interior of the protein. Upon calcium binding, the Gla residues fold into the core of the domain where they form a rigid secondary structure and ligate calcium ions in a cooperative manner. This conformational change leads to the exposure of the hydrophobic amino acids Phe, Leu and Val that are normally hidden in the calcium-free structure (Figure 35). Mutagenic studies of bovine prothrombin and protein C have demonstrated that mutations to aspartic of Gla residues 6, 7, 16, 26, and 29, the calcium-binding channel, lead to complete loss of biological activity and phospholipid binding property of the protein (Radcliffe et al., 1976; Yan et al., 1990). In contrast, changes in Gla-14, 19, and others have little if any effect. The calcium binding property of the Gla domain is a cooperative one: co-operative binding of the first 2 to 4 calcium ions leads to a conformational change of the peptide as measured by fluorescence quenching of Trp-41. As shown in Figure 35, the calcium binding results in the formation of a linear channel by the N-terminus exposing the three hydrophobic residues, Phe-5, Leu-6, and Val-9, a prerequisite for the interaction with anionic phospholipid membrane (Sunnerhagen et al., 1995; Zhang et al., 1994). Leucine-5, (of protein C), a conserved amino acid in all vitamin K-dependent clotting factors, when mutated to Glu, resulted in normal calcium binding but a total loss of anionic phospholipid binding. Other amino acid residues in the Gla domain also play significant role in the interaction with membrane surfaces besides the hydrophobic cluster. For example,

Figure 35. Crystal structure of the Ca^{2+}-Gla domain of bovine prothrombin. The picture was created using the file "2PF2" downloaded from the protein database Entrez of the National Centre for Biotechnology Information (website: "http://www.ncbi.nlm.nih.gov"). The figure was created in Rasmol and modified using Raster3D and MolScript. The polypeptide backbone was shown as a purple line with the alpha helixes as yellow ribbon and the beta sheets as green arrows. The functional groups (red branches) of the gamma-carboxyglutamic residues are shown interacting with the calcium ions (blue circles). The side chains of the hydrophobic residues are shown in green.

cis-proline-22 may be important for membrane-binding conformation (Evans et al., 1996). Glycine12-Arg, a naturally occurring mutation in hemophilia B (factor IX), leads to less than 1 % of biological activity, and antigen level of ~ 45 %. Thus cooperativity in calcium and phospholipid binding property of the Gla domain is apparent, and the ordered secondary structure of the peptide, contributed by all individual amino acids, is probably the most important factor in determining its property.

Gla has a unique calcium binding property due to the extra carboxyl group at the gamma carbon. In the clotting factor prothrombin, ten Gla residues in the N terminal region are responsible for calcium binding that leads to a calcium-dependent conformational change of the protein. This conformational change is absolutely required for interaction with phospholipid membrane surfaces during prothrombin activation by the prothrombinase complex. Due to this calcium binding property, Gla residues in proteins play very important roles in the structure and function of many biological systems.

CHAPTER 5

ANALYSIS OF THE KRINGLE DOMAINS

I. BACKGROUND

1. The kinetics of the conversion of prothrombin to thrombin

The conversion of prothrombin to thrombin is a dynamic and complex process involving a multi-component enzymic system, the prothrombinase complex. Besides the presence of the protease, factor Xa, in the complex, there are three important cofactors required: the protein cofactor Va, phospholipid membranes and calcium ions (Esmon et al., 1974b). Calcium ions function to enhance the specific structural conformation for all three proteins necessary for the binding to negatively charged lipid membrane, which serves as a docking station for the assembly of the proteinous components. The cofactor Va amplifies the catalytic efficiency of the factor Xa proteolytic activity by serving as the anchor point for prothrombin and factor Xa on the lipid-docking surface and by providing further structural conformation for both these proteins necessary for optimal activity. This hypothesis is an extremely simplified version of a very complex process that has been studied extensively over the last couple of decades. Little is understood however about the exact molecular mechanism of prothrombinase assembly. The macromolecular interactions within the prothrombinase complex are still unknown, as investigators have tried to break down little by little the many components to a more simplified system. To date, to study the role of the prothrombinase complex, steady state kinetics of thrombin formation are often measured in a purified components system. The activity of the protease factor Xa alone is compared to those of the protease in combinations with other cofactors using model membranes. The substrate, prothrombin, and the cofactors factor Va and phospholipid membranes, could also be studied in similar fashions. With the advance of recombinant DNA techniques and site-directed mutagenesis, it has become possible to make changes in the structure of a protein and study the effect in a functional system. The function of prothrombin is best related to its ability to clot blood and its activation by factor Xa and the prothrombinase complex. Thus the recombinant prothrombin variants with the kringle domains changed can give useful information about the role of the kringles when subjected to these functional assays.

The prothrombinase complex formation is a process that requires several equilibrated steps. Initially, factor Xa and the cofactor Va assemble on the negatively charged

phospholipid membrane to form the catalytic complex (Krishnaswamy, 1990; Krishnaswamy et al., 1988). This is the rate-determining step for the activity of the prothrombinase complex. A phospholipid –dependent conformation of prothrombin is required for the interaction with the catalytic complex (Lentz et al., 1994; Walker et al., 1994a). The mechanism of the reaction has been shown to be a multi-step process involving multiple bond-cleavages (Krishnaswamy et al., 1987; Krishnaswamy et al., 1986) (see Chapter 1). Briefly, the activation of prothrombin to thrombin occurs via two factor Xa-catalyzed cleavages; one at the Arg^{273}-Thr^{274} between the second kringle and the serine protease domains, and the other at the Arg^{322}-Ile^{323} within the disulfide loop in the serine protease domain. This releases the serine protease domain of prothrombin, thrombin, which catalyzes cleavage of prothrombin further in the activation of fragment 1.2 (fragment 1 and fragment 2 domains). Thus the complete activation of prothrombin gives rise to fragment 1 (Gla and first kringle domains), fragment 2 (second kringle domain) and thrombin (the catalytic domain). Although factor Xa can catalyze prothrombin cleavage, the macromolecular interactions in the prothrombinase complex can enhance this catalytic efficiency by many thousand of fold (Mann et al., 1988). In most studies, the activity of the prothrombinase complex has always been assumed to follow Michaelis-Menten kinetics. The catalytic complex of factor Xa, cofactor Va and PCPS membrane is often considered one entity, the enzymic complex. Thus when steady state kinetics of prothrombin conversion to thrombin is measured, the equilibrium constant K_m and the catalytic efficiency k_{cat} of the prothrombinase complex can be determined. A comparison of these values with those of the activation of prothrombin by factor Xa in the absence of the cofactor Va can give useful information about the protein-protein interactions of the participating components.

2. The components of the prothrombinase complex

The components of the prothrombinase complex (factor Xa, the cofactor Va, calcium ion and phospholipid membrane) have been studied extensively *in vitro*. For the negatively charged membrane, many different surfaces have been employed including those of activated platelets (Bouchard et al., 1997; Larson et al., 1998), monocytes/macrophages (Camire et al., 1998; Tracy et al., 1996), and many different kind of phospholipid surfaces. Factor X and prothrombin have actually been characterized to share binding sites on activated human

platelets and may have significant impact on the assembly of the prothrombinase complex (Scandura et al., 1996). In fact, a recent study has shown that plasma lipoproteins also support prothrombinase and other pro-coagulant enzymatic complexes (Moyer et al., 1998). However the most utilized surface has always been those of synthetic phospholipid membranes for the study of prothrombinase complex in a purified system. Numerous characterizations of the different properties of the synthetic phospholipid membrane have been carried out including the composition of the different phospholipids (Kung et al., 1994; Larson et al., 1998; Lu et al., 1996b) and size such as small unilamellar vesicles or large unilamellar vesicles (Lu et al., 1996a). The most often used composition of a negatively charged phospholipid membrane is phosphatidyl choline (PC) and phosphatidyl serine (PS) at a composition ratio of 75:25 respectively. The presence of the negatively charged phosphotidylserine in greater than 10 % composition in synthetic membrane seems to lead to optimal activity for the prothrombinase complex and the optimal concentration has been characterized for the prothrombinase assay (Walker et al., 1994b; Ye et al., 1995).

For the rest of the prothrombinase complex, the optimal concentrations of the protease factor Xa and the cofactor Va have been characterized in many purified systems (Kotkow et al., 1995; Ye et al., 1995). Although the binding of factor Xa to the cofactor Va has been shown to be a stoichiometric interaction of 1:1 ratio (Mann et al., 1990), in practice the concentration of Va has often been in excess ranging from 2 to 10 fold higher compared to that of factor Xa. The excess cofactor Va ensures that factor Va is not the limiting component for the rate-limiting equilibrated binding of factor Xa-cofactor Va on negatively charged membrane surfaces. Thus before the activation of the substrate to such a complex, adequate time must be required for this binding that is essential for the formation of the prothrombinase complex, and has been shown to be around 2 minutes for low non-saturating concentration of phospholipid (Ye et al., 1995).

II. KINETICS OF PROTHROMBIN ACTIVATION

To study the protein-protein interactions of prothrombin with its protease factor Xa and the prothrombinase complex, steady state kinetics of recombinant prothrombin and kringle variants have been determined in a two-reaction process. The recombinant proteins were subjected to activation assays using a purified system: (1) prothrombin was activated

with factor Xa, calcium and PCPS phospholipid; and (2) prothrombin was activated with the same complex in the presence of the cofactor Va. The end product of the activating assay was the formation of the protease thrombin, whose quantity was measured subsequently by its proteolytic activity on the chromogenic substrate S-2238. In addition, three synthetic peptides based on the amino acid sequence of the second kringle were included in the activation of plasma prothrombin by factor Xa and the prothrombinase complex.

1. Thrombin activity assay

The activity of thrombin was measured from its ability to cleave the chromogenic substrate S-2238 (H-D-Phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochlroride or H-D-Phe-Pip-Arg-NH-Benz-NO_2.2HCl). Thrombin cleaved S-2238 at theArg-NH bond to release pNA (para-nitroanilide), a colored moiety that can be measured spectrometrically:

$$\text{H-D-Phe-Pip-Arg-NH-Benz-NO}_2\cdot\text{2HCl} \xrightarrow{\text{thrombin}} \text{H-D-Phe-Pip-Arg-OH + pNA}$$

The method for the determination of activity was based on the difference in optical density (absorbance) between the pNA formed and the original substrate. The rate of pNA formation, i.e. the increase in absorbance per second at 405 nm, was proportional to the enzymatic activity and was determined with a spectrometer.

To establish a standard plot for thrombin activity, 25 µl of thrombin at various dilutions was mixed with 25 µl of thrombin assay buffer (TAB) (20 mM Tris.Cl pH 7.4 containing 150 mM NaCl, 20 mM EDTA and 0.1 % albumin). The mixture was incubated in a 37°C water bath for 5 min, followed by the addition of 50 µl of 0.5 mM S-2238 substrate. The solution was transferred immediately to a quartz cuvette and the absorbance was monitored at 405 nm for up to 10 min. The rate of absorbance increase over time (in minute) was determined and plotted with the concentration of thrombin used (Figure 36). The dynamic linear range for thrombin activity as measured by this method was from 0-10 nM in concentration for thrombin (or 0-10 ng for total protein amount). At higher concentration the rate of absorbance increase at 405 nm was unreliable. The equation for the linear response was determined to be $y = 0.0088x - 0.0001$ with the value for $R^2 = 0.9996$.

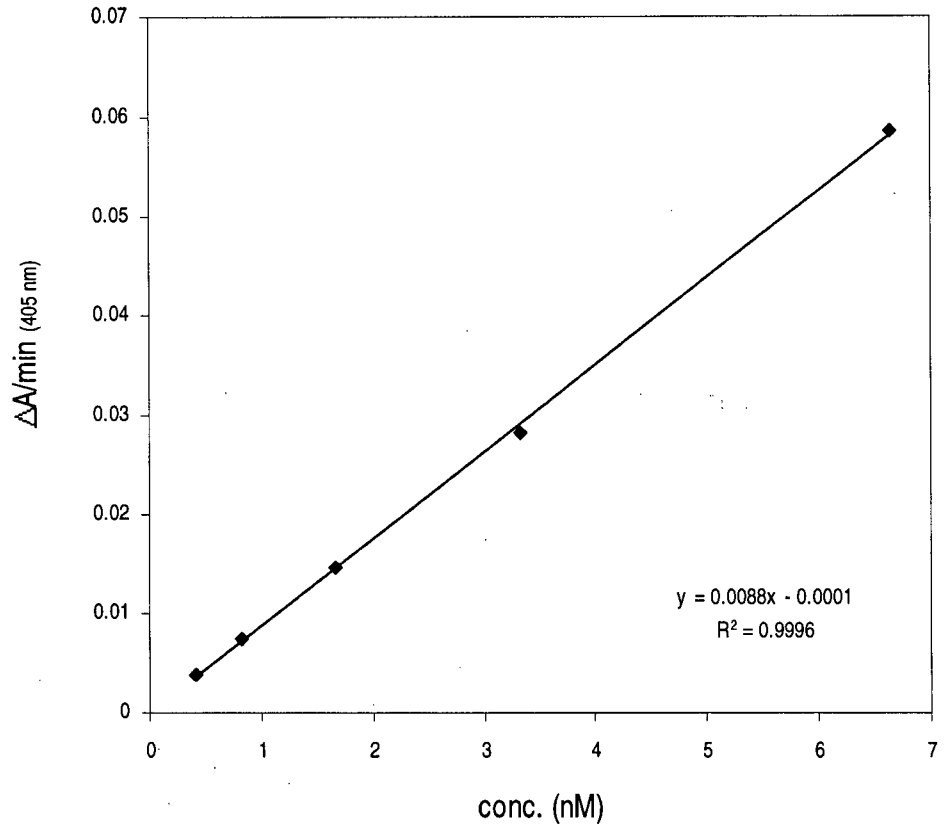


Figure 36. Thrombin activity standard curve. 25 µl of thrombin at various dilutions was mixed with 25 µl of thrombin assay buffer (20 mM Tris.Cl pH 7.4 containing 150 mM NaCl, 2 mM EDTA and 0.1 % albumin). The mixture was incubated in a 37°C water bath for 5 min, followed by the addition of 50 µl of 0.5 mM S-2238 substrate. The solution was transferred immediately to a quartz cuvette and the absorbance was monitored at 405 nm for 10 min. The rate of absorbance increase over time (in minute) was determined and plotted with the concentration of enzyme used.

Kinetics analysis of recombinant prothrombin and variants were characterized using this standard curve to determine the amount of thrombin produced.

2. Prothrombin activation by FXa, calcium and phospholipid

The substrates, recombinant prothrombin and variants, were activated by factor Xa in the presence of calcium and PCPS phospholipid complex. The reactions were carried out and the results were compared with those of plasma prothrombin. Factor Xa (5 nM) was incubated in the presence of calcium ions (5 mM) and PCPS phospholipid vesicles (35 μM) in TBS buffer (20 mM Tris.Cl pH 7.4 containing 150 mM NaCl and 0.1% BSA) at 37°C for 2 min. The substrate (prothrombin and variants) at various concentrations (0.1 to 3 μM) was added to the mixture for a final volume of 100 μl. Aliquots (25 μl) of the reaction mixture were taken out at 0, 15, 25, and 40 min intervals and subjected to thrombin activity assay as described above. Kinetic constants were determined by Michaelis-Menten equation using Sigma Plot software.

3. Prothrombin activation by the prothrombinase complex

The substrates, recombinant prothrombin and variants, were activated by the prothrombinase complex consisting of factor Xa, calcium and PCPS phospholipid complex in the presence of the cofactor Va. The reactions were carried out and the results were compared with those of plasma prothrombin. Factor Xa (0.01 nM) was incubated in the presence of cofactor Va (10 nM), calcium ions (5 mM), and PCPS phospholipid vesicles (35 μM) in TBS buffer (20 mM Tris.Cl pH 7.4 containing 150 mM NaCl and 0.1% BSA) at 37°C for 2 min. The substrate (prothrombin and variants) at various concentrations (0.1 to 3 μM) was added to the mixture for a final volume of 100 μl. Aliquots (25 μl) of the reaction mixture were taken out at two minute intervals and subjected to thrombin activity assay as described above.

4. Kinetic constants of recombinant prothrombin and variants

Prothrombin substrate was activated by factor Xa, calcium ion and phospholipid in the absence and presence of the cofactor Va. Recombinant prothrombin and variants were

subjected to these assays using a purified system. The initial rate of thrombin produced was plotted against the substrate concentration, and the kinetic constants were determined using the steady state Michaelis-Menten equation:

$$V = \frac{V_{max}\,[S]}{Km + [S]}$$

where,

V is the observed initial velocity of the reaction

V_{max} is the maximal velocity of the reaction

K_m is the constant representing the concentration of the substrate that gives half the numerical maximal velocity.

A double reciprocal plot of Lineweaver and Burk was utilized to show a linear relationship of the Michaelis-Menten equation:

$$\frac{1}{V} = \frac{K_m}{V_{max}}\left(\frac{1}{S}\right) + \frac{1}{V_{max}}$$

where, the y-intercept is $1/V_{max}$ and the x-inercept is $-1/K_m$. Thus, the relationship between the substrate, the enzyme concentration, and the velocity of the enzyme-catalyzed reaction can be developed. Using steady state kinetic, one assumption must be kept in mind, however, is that the "true" enzyme is actually an enzymic complex consisting of the real enzyme and the various cofactors, i.e. the enzymic complex for prothrombin activation is the complex of factor Xa, calcium ions and PCPS phospholipid membrane, as well as the cofactor Va in the prothrombinase complex. When prothrombin forms the substrate-enzyme complex, it is interacting not only with its protease factor Xa, but also with the cofactors present in the enzymic complex as well. Differences in the kinetics of prothrombin activation by factor Xa alone and by the prothrombinase complex give insight into the differences in prothrombin interaction with each of the components in the complexes.

4.1. Activation by factor Xa, calcium and PCPS

Recombinant prothrombin was activated by factor Xa in the presence of calcium ions and PCPS (75:25) phospholipid. The results were compared to that of plasma-derived prothrombin. The initial rate of thrombin appearance was plotted against the concentration of the substrate (prothrombin) (Figure 37), and the kinetic constants were determined from Michaelis-Menten equation using non-linear regression analysis from the Sigma Plot program. The kinetic constants were listed in Table 5. The K_m for plasma-derived prothrombin and recombinant prothrombin were 0.12 ± 0.01 μM and 0.23 ± 0.02 μM respectively, while the V_{max} were 0.48 ± 0.01 U/ml/min and 0.46 ± 0.01 U/ml/min respectively. The percentage curve (fitting) variations (CV%) for both proteins were very good ranging from 1.6% to 8.1%. Based on these results, the activation of recombinant prothrombin was similar to that of plasma-derived prothrombin with almost identical V_{max} and a very similar K_m.

The ability of recombinant prothrombin variants to be activated by factor Xa, calcium ions and PCPS were determined next and compared with that of the recombinant wild-type protein. There were three prothrombin variants characterized: with the first kringle deleted (rΔK1), with the second kringle deleted (rΔK2) and with the second kringle replaced by the bovine second kringle (rhBK2). The kinetic constants of these proteins were determined and compared to that of the recombinant wild-type protein (Table 5). The percentage curve (fitting) variations (CV%) were very good for all recombinant proteins ranging from a low of 1.13% to a high of 5.56%, indicating a fairly accurate curve fitting calculation. The variant rΔK1 showed similar V_{max} (0.40 ± 0.02 U/ml/min) with that of the wild type recombinant protein, but a K_m (0.96 ± 0.11 μM) at approximately three fold higher. The %CV for the K_m and V_{max} of rΔK1 were 1.1% and 5.6% respectively (Figure 38). The variant rΔK2 exhibited the most variation with both a K_m (1.03 ± 0.27 μM) about three fold higher and a V_{max} (0.29 $\pm$ 0.04 U/ml/min) at about 2 fold lower compared to those of the recombinant wild type. The %CV for K_m and V_{max} of rΔK2 were 2.6% and 1.3% respectively (Figure 38). The full length recombinant prothrombin variant with a bovine second kringle (rhBK2) had a K_m value (0.31 ± 0.04 μM) very similar compared to the wild type and a very slightly lower V_{max} (0.34 ± 0.01 U/ml/min); the %CV for the K_m and V_{max} were 1.1% and 3.8% respectively (Figure 39).

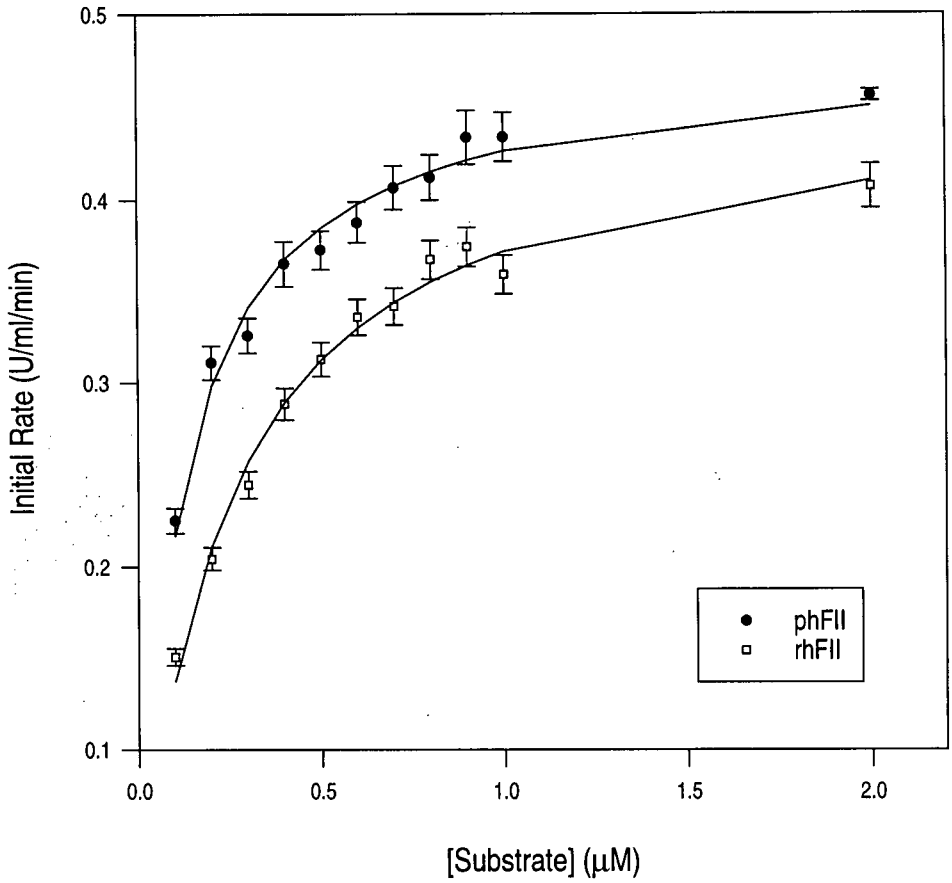


Figure 37. Activation of plasma-derived prothrombin and recombinant prothrombin by factor Xa, calcium ions and PCPS phospholipid. Plasma-derived prothrombin (phFII) and recombinant prothrombin (rhFII) were activated with factor Xa (5 nM), calcium ions (5 mM) and PCPS phosphlipid (35 μM) pre-incubated at 37°C. Aliquots of the reaction mixture was taken out at 15, 25 and 40 min intervals and were subjected to the thrombin activity assay using the chromogenic substrate S-2238. Initial rate of the reaction (units of thrombin produced per ml per min) was plotted against substrate concentrations and kinetic constants were determined using Michaelis-Menten equation and Sigma Plot non-linear regression analysis. The values and the error bars represented for each protein were from the average of two determinations.

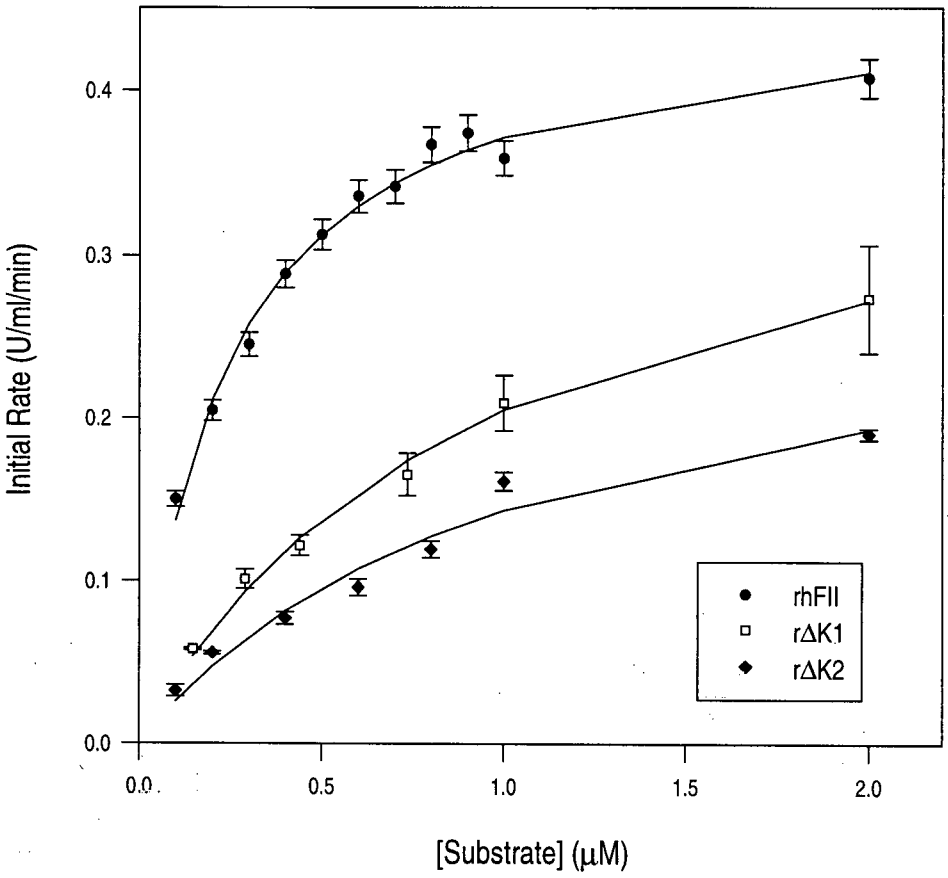


Figure 38. Activation of recombinant prothrombin and kringle variants by factor Xa, calcium ions and PCPS phospholipid. Recombinant prothrombin (rhFII) and variants with the first kringle domain (rΔK1) and the second kringle domain (rΔK2) deleted were activated with factor Xa (5 nM), calcium ions (5 mM) and PCPS phosphlipid (35 μM) pre-incubated at 37°C. Aliquots of the reaction mixture was taken out at 15, 25 and 40 min intervals and subjected to the thrombin activity assay using the chromogenic substrate S-2238. Initial rate of the reaction (units of thrombin produced per ml per min) was plotted against substrate concentrations and kinetic constants were determined using Michaelis-Menten equation and Sigma Plot non-linear regression analysis. The values and the error bars represented for each protein were from the average of two determinations.

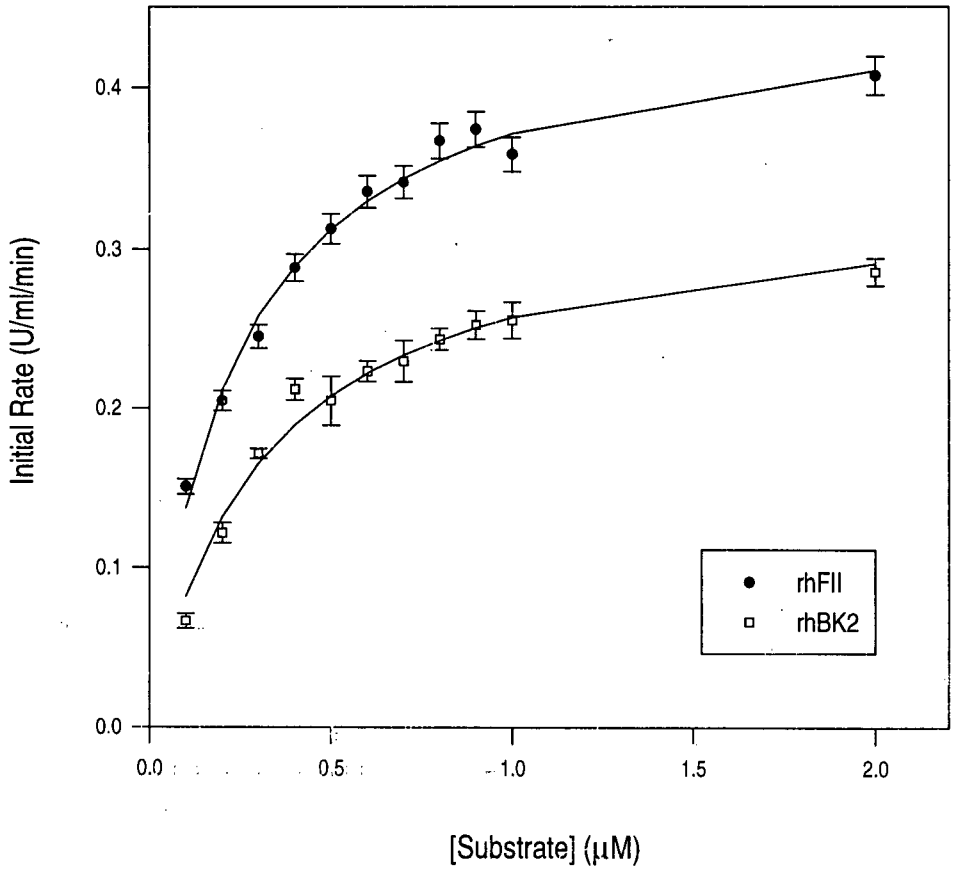


Figure 39. Activation of recombinant human-bovine prothrombin chimera by factor Xa, calcium and PCPS phospholipid. Recombinant wild type prothrombin (rhFII) and the prothrombin variant with the second kringle domain substituted by the bovine counterpart (rhBK2) were activated with factor Xa (5 nM), calcium ions (5 mM) and PCPS phosphlipid (35 μM) pre-incubated at 37°C. Aliquots of the reaction mixture were taken out at 15, 25 and 40 min intervals and were subjected to the thrombin activity assay using the chromogenic substrate S-2238. Initial rate of the reaction (units of thrombin produced per ml per min) was plotted against substrate concentrations and kinetic constants were determined using Michaelis-Menten equation and Sigma Plot non-linear regression analysis. The values and the error bars represented for each protein were from the average of two determinations.

Table 5. Kinetic constants of the activation of recombinant prothrombin and variants by two enzymic complexes

	Activation by factor Xa, calcium and PCPS[a,c]				Activation by the prothrombinase complex[b,d]			
	k_m (μM)	V_{max} (U/ml/min)	k_{cat}[e] (10^{-3}. s^{-1})	k_{cat}/k_m $(s.\mu M)^{-1}$	k_m (μM)	V_{max} (U/ml/min)	k_{cat} (s^{-1})	k_{cat}/k_m $(s.\mu M)^{-1}$
phFII	0.12 ± 0.01	0.48 ± 0.01	2.09 ± 0.06	0.01740	0.22 ± 0.05	0.91 ± 0.08	1.98 ± 0.25	9.01
rhFII	0.23 ± 0.02	0.46 ± 0.01	2.00 ± 0.06	0.00871	0.24 ± 0.01	0.36 ± 0.01	0.78 ± 0.03	3.27
rhBK2	0.31 ± 0.04	0.34 ± 0.01	1.48 ± 0.06	0.00478	0.91 ± 0.15	0.66 ± 0.06	1.44 ± 0.18	1.58
rΔK1	0.96 ± 0.11	0.40 ± 0.02	1.74 ± 0.12	0.00182	0.16 ± 0.05	0.20 ± 0.02	0.44 ± 0.06	2.72
rΔK2	1.03 ± 0.27	0.29 ± 0.04	1.26 ± 0.25	0.00123	7.05 ± 1.87	0.57 ± 0.12	1.24 ± 0.37	0.18

[a] substrates were activated with 5 nM factor Xa, 5 mM $CaCl_2$ and 35 μM of PCPS (75:25)

[b] substrates were activated with 0.01 nM factor Xa, 10 nM cofactor Va, 5 mM $CaCl_2$ and 35 μM PCPS (75:25)

[c] deviations were from two value determinations

[d] deviations were from a single value determination

[e] $k_{cat}=Vmax/([E_t]*0.765*60)$ where 0.765 is the conversion unit from nM to U/ml and 60 is the conversion from min to second

4.2. Activation by the prothrombinase complex

Recombinant prothrombin was activated by factor Xa, calcium ions and PCPS (75:25) phospholipid in the presence of the cofactor Va. The results were compared to those of plasma-derived prothrombin. The initial rate of thrombin appearance was plotted against the concentration of the substrate (prothrombin), and the kinetic constants were determined from Michaelis-Menten equation using non-linear regression analysis from the Sigma Plot program. The K_m for plasma-derived prothrombin and recombinant prothrombin were 0.22 ± 0.05 μM and 0.24 ± 0.01 μM respectively, while the V_{max} were 0.91 ± 0.08 U/ml/min and 0.36 ± 0.01 U/ml/min respectively (Figure 40). The percentage curve (fitting) variation (CV%) for the values of K_m and V_{max} for both proteins were good being 2.0% and 8.4% for plasma derived prothrombin and 4.1% and 1.3% for recombinant prothrombin respectively. Based on these results, the activation of recombinant prothrombin was relatively similar to that of plasma-derived prothrombin with an almost identical K_m and a V_{max} about 2.5 fold lesser than that of the plasma derived protein.

The ability of the recombinant variants to be activated the prothrombinase complex were then determined and compared to that of the recombinant wild-type protein. There were three prothrombin variants characterized: with the first kringle deleted (rΔK1), with the second kringle deleted (rΔK2) and with the second kringle replaced by the bovine second kringle (rhBK2). The kinetic constants of these proteins were determined and compared to that of the recombinant wild-type protein (Table 5). The percentage curve (fitting) variations (CV%) were very good for all recombinant proteins ranging from a low of 1.0% to a high of 9.7%, indicating a fairly accurate curve fitting calculation. The variant rΔK1 showed similar K_m (0.20 ± 0.02 μM) with that of the wild type recombinant protein, but a V_{max} (0.16 ± 0.05 U/ml/min) at approximately 4.5 fold lower (Figure 41). The %CV for the K_m and V_{max} of rΔK1 were 3.1% and 1.0% respectively. The variant rΔK2 exhibited the most variation with a K_m (7.05 ± 1.87 μM) about thirty fold higher and a V_{max} (0.57 ± 0.12 U/ml/min) at about 2 fold lower compared to those of the recombinant wild type (Figure 42). The %CV for the K_m and V_{max} of rΔK2 were 2.6% and 2.0% respectively. The full length recombinant prothrombin variant with a bovine second kringle (rhBK2) had a K_m (0.91 ± 0.16 μM) about 4 fold higher compared to the wild type and a slightly lower V_{max} (0.66 ± 0.06 U/ml/min);

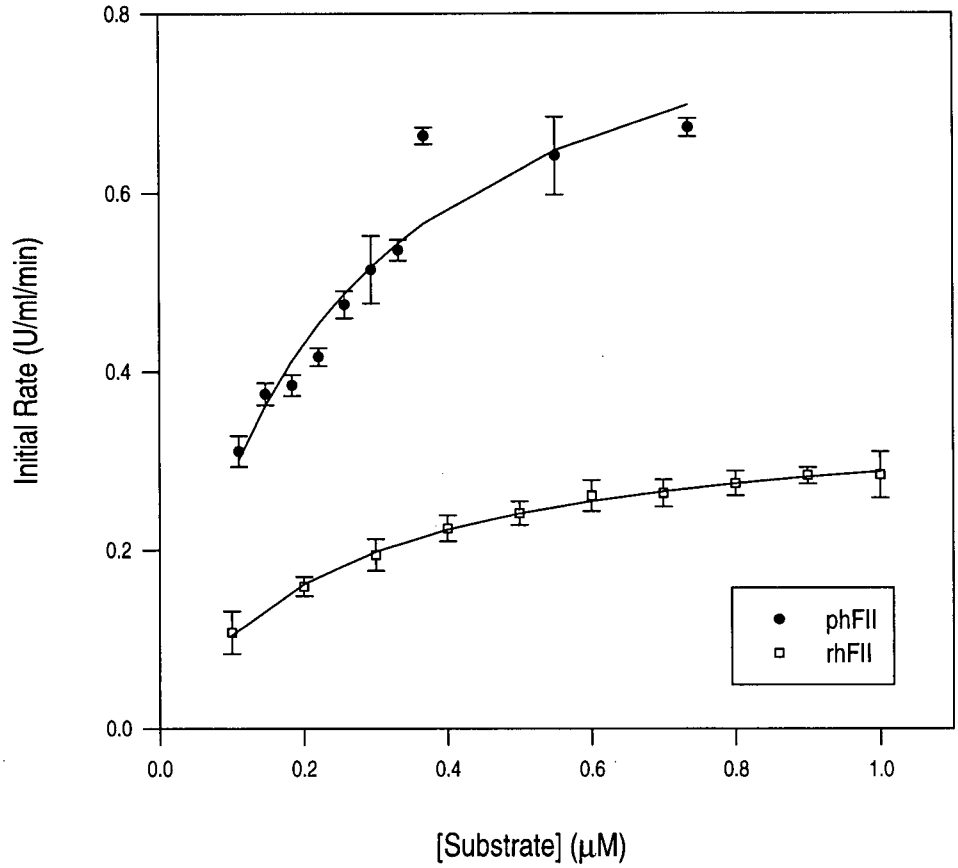


Figure 40. Activation of plasma-derived prothrombin and recombinant prothrombin by the prothrombinase complex. Plasma-derived prothrombin (phFII) and recombinant prothrombin (rhFII) were activated with factor Xa (0.01 nM), calcium ions (5 mM) and PCPS phosphlipid (35 μM) pre-incubated at 37°C in the presence of the cofactor Va (10 nM). Aliquots of the reaction mixture were taken out at two-minute intervals and were subjected to the thrombin activity assay using the chromogenic substrate S-2238. Initial rate of the reaction (units of thrombin produced per ml per min) was plotted against the substrate concentrations and kinetic constants were determined using Michaelis-Menten equation and Sigma Plot non-linear regression analysis. The values and the error bars represented for each protein were from the average of two determinations.

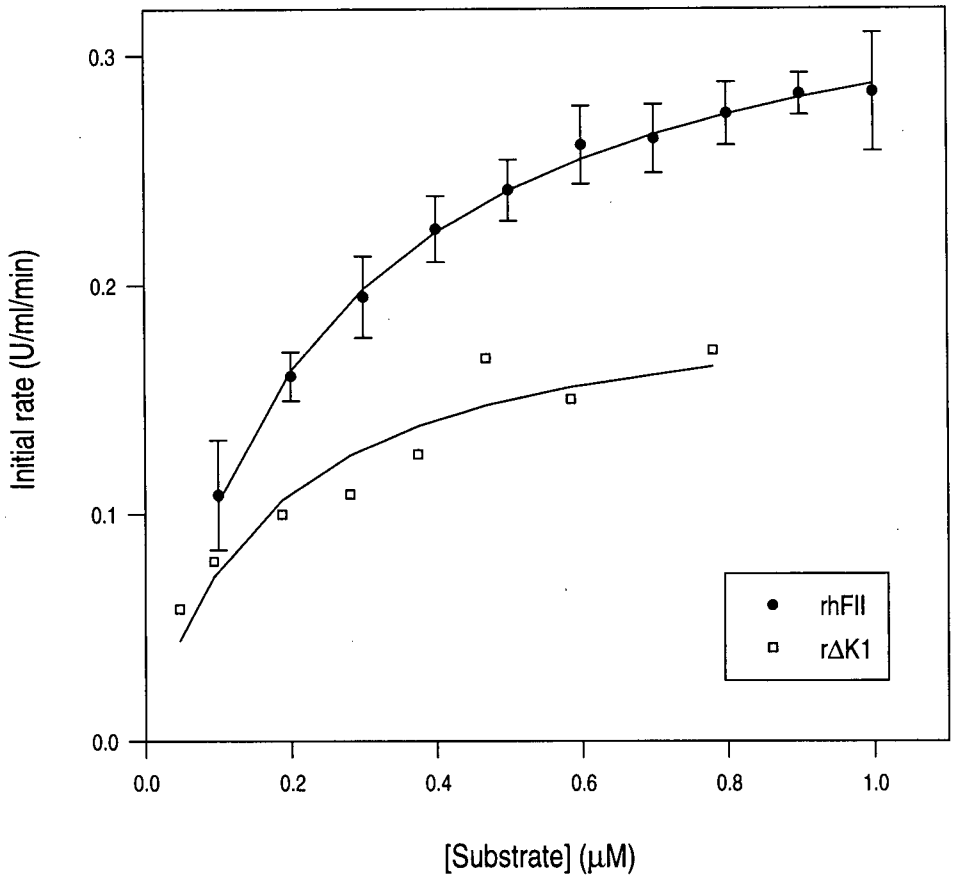


Figure 41. Activation of recombinant prothrombin and the first kringle deleted variant by the prothrombinase complex. Recombinant prothrombin (rhFII) and the variant with the first kringle domain deleted (rΔK1) were activated with factor Xa (0.01 nM), calcium ions (5 mM) and PCPS phosphlipid (35 μM) pre-incubated at 37°C in the presence of the cofactor Va (10 nM). Aliquots of the reaction mixture were taken out at two-minute intervals and were subjected to the thrombin activity assay using the chromogenic substrate S-2238. Initial rate of the reaction (units of thrombin produced per ml per min) was plotted against substrate concentrations and kinetic constants were determined using Michaelis-Menten equation and Sigma Plot non-linear regression analysis. The value represented were from two determinations for rhFII but only one determination was for rΔK1.

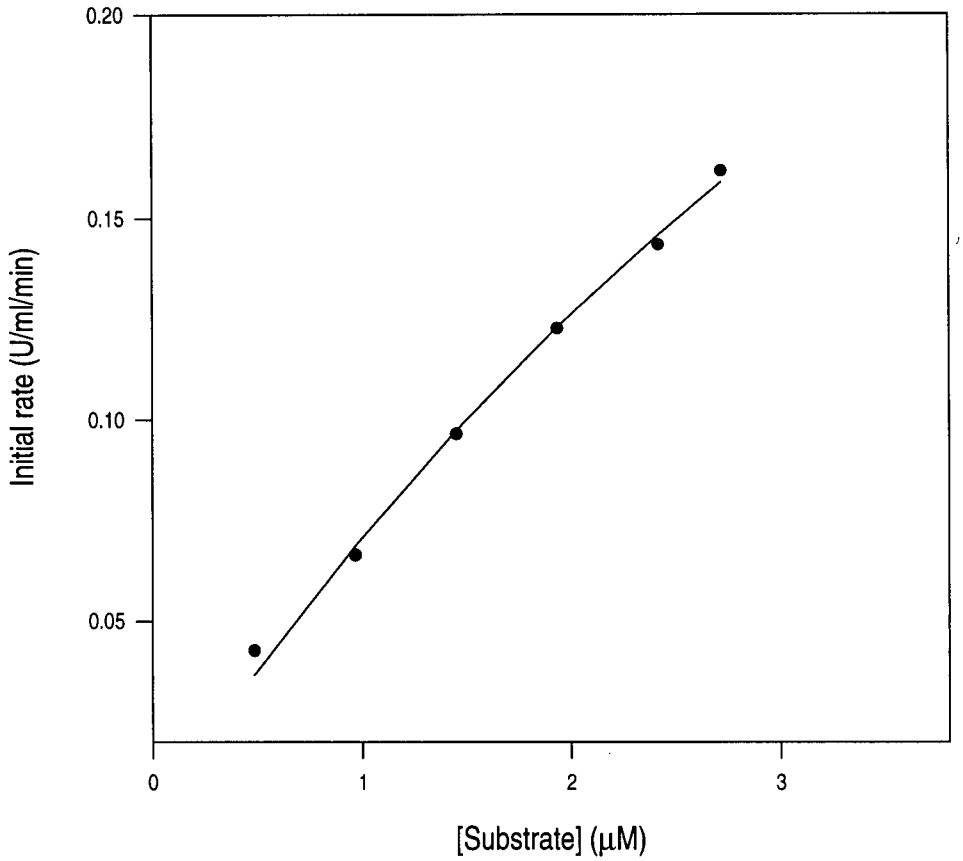


Figure 42. Activation of the second kringle deleted prothrombin variant by the prothrombinase complex. The recombinant prothrombin variant with the second kringle domain deleted (rΔK2) was activated with factor Xa (0.01 nM), calcium ions (5 mM) and PCPS phosphlipid (35 μM) pre-incubated at 37°C in the presence of the cofactor Va (10 nM). Aliquots of the reaction mixture were taken out at two-minute intervals and were subjected to the thrombin activity assay using the chromogenic substrate S-2238. Initial rate of the reaction (units of thrombin produced per ml per min) was plotted against substrate concentrations and kinetic constants were determined using Michaelis-Menten equation and Sigma Plot non-linear regression analysis. The values represented were from a single determination.

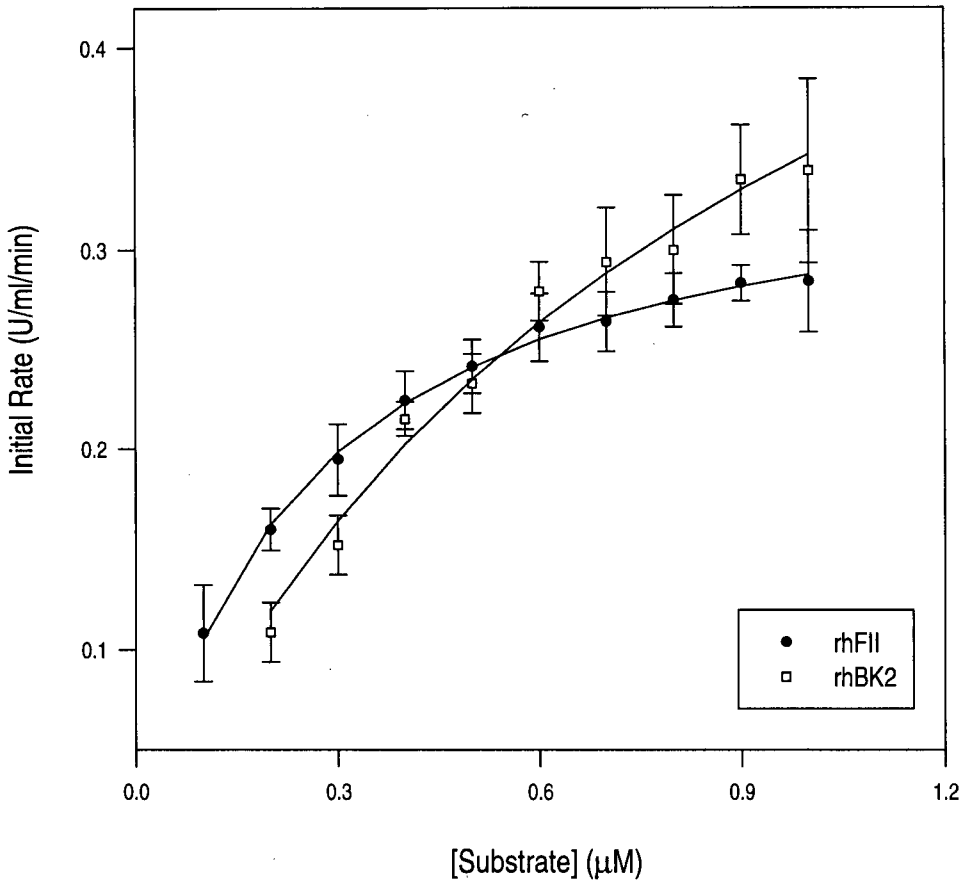


Figure 43. Activation of recombinant human-bovine prothrombin chimera by the prothrombinase complex. Recombinant wild type prothrombin (rhFII) and the prothrombin variant with the second kringle domain substituted by the bovine counterpart (rhBK2) were activated with factor Xa (0.01 nM), calcium ions (5 mM) and PCPS phospholipid (35 μM) pre-incubated at 37°C in the presence of the cofactor Va (10 nM). Aliquots of the reaction mixture were taken out at two-minute intervals and were subjected to the thrombin activity assay using the chromogenic substrate S-2238. Initial rate of the reaction (units of thrombin produced per ml per min) was plotted against substrate concentrations and kinetic constants were determined using Michaelis-Menten equation and Sigma Plot non-linear regression analysis. Values represented for each protein were from the average of two determinations.

the %CV for K_m and V_{max} values were 1.5% and 6.4% respectively (Figure 43).

III. EFFECT OF SYNTHETIC PEPTIDES DERIVED FROM THE SECOND KRINGLE ON PROTHROMBIN ACTIVATION

1. Peptide design

Three peptides were synthesized based on the amino acid sequence of the second kringle of prothrombin corresponding to the three loops of the kringle structure (Table 6). Three-dimensional crystal structure of kringle domains have shown that there were four loops in the kringle region termed loop A, B, C, and D (Figure 44). Sequence alignment of the human prothrombin first and second kringles to bovine sequence showed that the majority of the differences lay in these outer loops of the polypeptide, while the most conserved residues lay in the inner core of the secondary structure (Figure 45). Should the kringle domains play any role in the mediation of protein-protein interactions between prothrombin and factor Xa or factor Va, these outer loops would probably play a significant part in this process. Based on the crystal data and the sequence alignment, three peptides were synthesized corresponding to loop A (residues 15 to 23), loop B and C (residues 25 to 45), and loop D (residues 64 to 71) of the second kringle domain of human prothrombin (hK2). These peptides were termed peptide A, peptide BC and peptide D respectively (numbering system using the first cysteine of the kringle as the first residue). To mimic the secondary structure of the loops in the native protein, all terminal residues of the hK2 peptides were changed to cysteine residues so that a disulfide linkage could be formed, thus constraining the peptide in a secondary conformation hopefully similar to that of the native folding. The distances between the C_α-C_α of the end residues for peptides A, BC and D in the crystal structure were 5.13 A°, 6.07 A° and 4.96 A° respectively; these values are within the range reported in literature of 5.6 – 8.1 A° for distances between naturally occurring disulfide bonds (Matsumura et al., 1991).

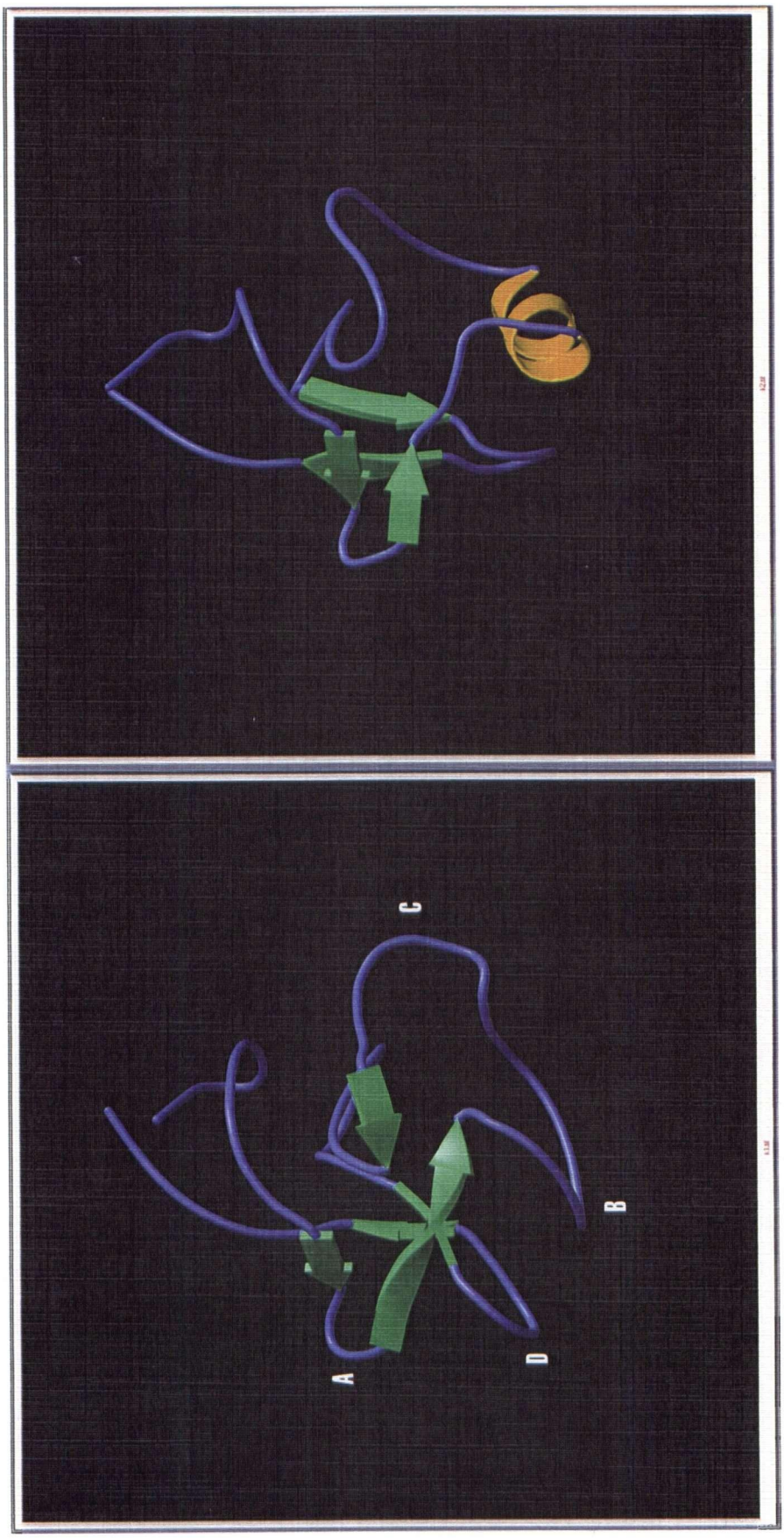
Table 6. Amino acid sequences of synthetic peptides derived from the second kringle of human prothrombin

	Amino acid sequence	Position[a]	Distance between C_α-C_α[b]
PEPTIDE A	**C**-V-T-T-H-G-L-P-**C**	14-22	5.13 A^o
PEPTIDE BC	**C**-A-S-A-Q-A-K-A-L-S-K-H-Q-D-F-N-S-A-V-Q-**C**	25-45	6.07 A^o
PEPTIDE D	**C**-A-G-K-P-G-D-**C**	64-71	4.96 A^o

a numbering system using the first cysteine of the kringle domain as residue number one

b the distance between the alpha-carbons of the disulfide bonded amino acids

Figure 44. Structures of the kringle domains of prothrombin. The figure was from the file "2HPQI" downloaded from the protein database Entrez of the National Centre for Biotechnology Information (website: "http://www.ncbi.nlm.nih.gov/"). The figure was created in Rasmol and modified using Raster3D and MolScript. The polypeptide backbone is shown as a purple line with the alpha helixes as yellow ribbon and the beta sheets as green arrows. There are four loops to each kringle: loop A was the first loop on the left of the structure; loop B was at the bottom-right corner; loop C was at the right; and loop D was at the bottom-left of the domain. The second kringle of prothrombin is the one on the right with the yellow alpha-helix in loop B.


C
B
A
D

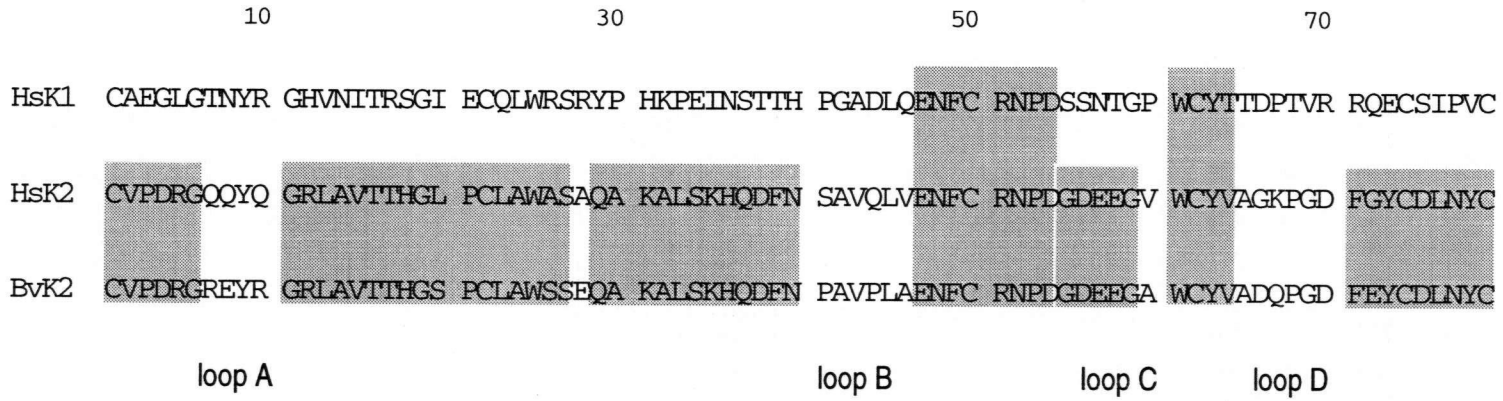


Figure 45. Amino acid sequence alignment of kringle domains of human and bovine prothrombin. The amino acid sequences of human prothrombin first kringle (HsK1) and second kringle (HsK2) were compared together and with the sequences of the second kringle domain of bovine prothrombin (BvK2). Shaded areas denoted the sequence identity of the three domains. The regions A, B, C and D were the outer loops of the kringle domain as determined from the crystal structure.

2. Air oxidation of disulfide linkages of K2 peptides

The peptides were synthesized, purified by HPLC and the molecular weights were characterized by mass spectrometry. To promote disulfide bond formation, each peptide (1 mg) was stirred vigorously in 50 ml of a NH_4OH solution pH 9.0 for 48 hr (pH tested occasionally) (Annis et al., 1997). The formation of intra-peptide disulfide linkage catalyzed by air (O_2) oxidation was enhanced by carrying out the reaction in a large volume of solution and at diluted concentration of peptide. After the oxidizing period, the peptide was lyophilized and dried down to a powder. The powder was resuspended in 20 mM Tris.Cl pH 8.0 before being used in the prothrombin activation assay.

3. Effect of K2 peptides on the activation of prothrombin

3.1. The effect of K2 peptides measured by a one-stage clotting assay

The effects of the disulfide-linked peptides were studied using the prothrombin activation assays by factor Xa and by the prothrombinase complex. Plasma derived prothrombin was the substrate and was activated using these two assays in the absence and presence of the various peptides. Before the peptides were subjected to these assays, the peptides were subjected to a one-stage clotting assay to determine the effective inhibition range if there is any for each peptide. Each peptide was co-incubated with plasma prothrombin (20 μg/ml) at various concentrations and the clotting activity of the mixture was measured. The one-stage clotting assay was similar to the one described in chapter 3. Briefly, to the mixture of prothrombin and peptide (50 μl) was added an equal volume of prothrombin-deficient plasma and incubated at 37°C for 2 min. To activate clotting, 100 μl of a thromboplastin-calcium solution was added. The time for a visible clot was recorded. Based on this information, the effective concentration of each peptide used for the prothrombinase assay was estimated. As shown in Figure 46, all three peptides at high concentrations (between 5.75 to 16.3 μM) prolonged the time required for a recorded visible clot with peptide BC showing the most effect even at a lower concentration of around 1 μM. Based on this result, it seemed that all three peptides inhibited the clotting time of prothrombin as measured by this assay.

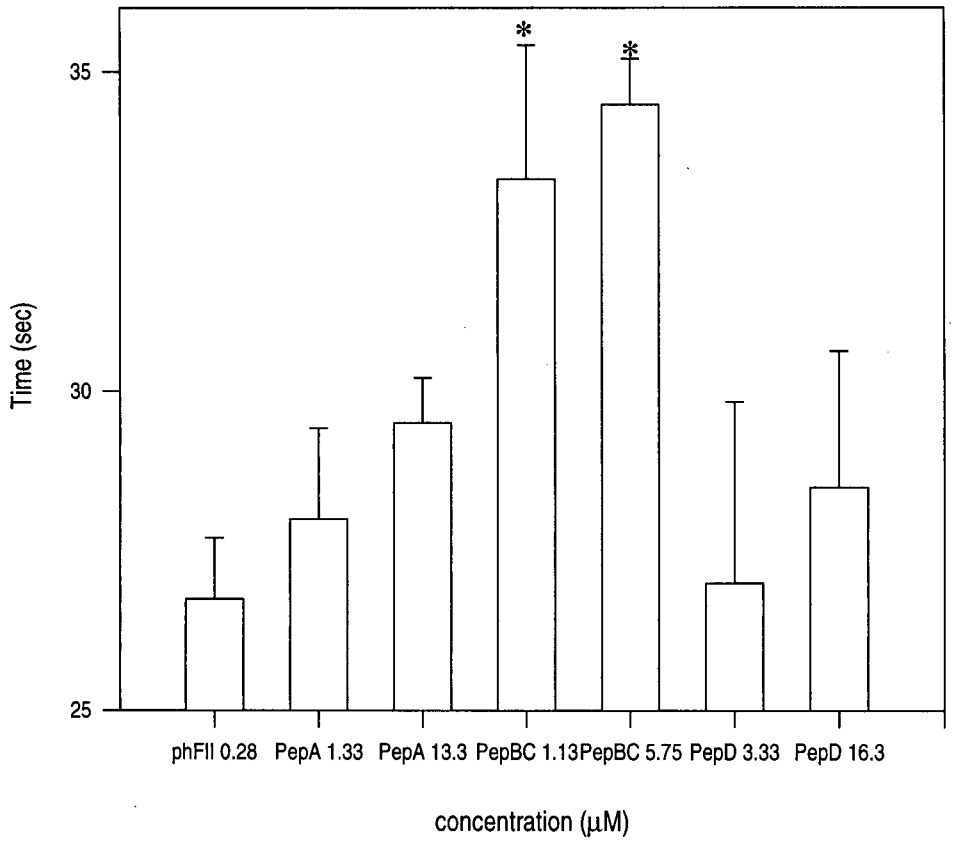


Figure 46. Effects of synthetic K2 peptides on prothrombin activation as measured by a one-stage clotting assay. Plasma prothrombin (20 μg/ml) in the presence of various concentrations of the synthetic K2 peptides A, BC and D was subjected to a one-stage clotting assay using prothrombin-deficient plasma and thromboplastin-calcium. An aliquot of the protein and peptide mixture (50 μl) was incubated with equal volume of prothrombin-deficient plasma at 37°C for 2 min, followed by the addition of the thromboplastin-calcium solution (100 μl). The time for a visible clot was recorded manually. The values represented were from three determinations. Only peptide BC (*) had values statistically different from the others ($P<0.05$).

3.2. *Effect of K2 peptides on factor Xa-activation of prothrombin*

Plasma derived prothrombin was used as the substrate for the activation assays. The reactions were carried out as described above in the presence of the synthetic peptides at various concentrations. However, the thrombin activity assay was carried out in microtitre plates and the rate of A405 nm changes were measured via a plate reader instead of a quartz cuvette as described in section II. Thus another thrombin activity standard plot was required. To establish a thrombin activity standard plot, 50 µl of thrombin at various dilutions was mixed with 50 µl of thrombin assay buffer (TAB) (20 mM Tris.Cl pH 7.4 containing 150 mM NaCl, 20 mM EDTA and 0.1 % albumin) in a microtitre plate. The mixture was incubated in a 37°C incubator oven for 5 min, followed by the addition of 100 µl of 0.5 mM S-2238 substrate. The absorbance was monitored at 405 nm for up to 10 min by a microplate reader. The rate of absorbance increase over time (in minute) was determined and plotted with the concentration of thrombin used (Figure 47). The dynamic linear range for thrombin activity as measured by this method was from 0-12 nM in concentration for thrombin. The equation for the linear response was determined from figure to be $y = 0.005x - 0.0005$ with the value for $R^2 = 0.9977$. Kinetics analysis of recombinant prothrombin in the presence of K2 peptides was characterized using this standard curve to determine the amount of thrombin produced.

Plasma prothrombin was activated by factor Xa in the presence of calcium ions and PCPS (75:25) phospholipid. The initial rate of thrombin appearance was plotted against the concentration of the substrate (prothrombin) and the kinetic constants were determined from Michaelis-Menten equation using non-linear regression analysis from the Sigma Plot program. The results were compared with the activation of prothrombin in the presence of the synthetic K2 peptides at various concentrations. The apparent kinetic constants were listed in Table 7. The K_m and V_{max} for plasma-derived prothrombin were 2.67 ± 0.69 µM and 0.52 ± 0.09 µM respectively. In the presence of peptide A (Figure 48), and peptide D (Figure 49), there seemed to be a minimal effect on the activation of prothrombin by these two peptides judging from the small differences in the amount of thrombin produced. In addition, upon close inspection of the activation profile, the initial rate of activity at low concentrations of substrate was practically identical both in the absence and presence of these

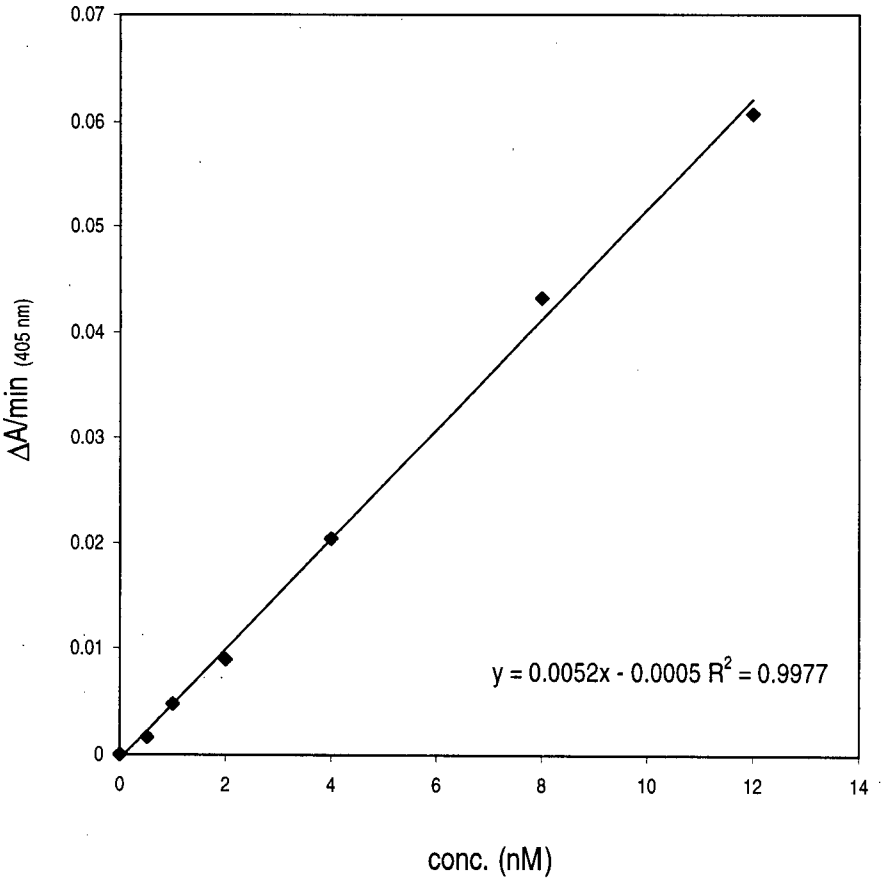


Figure 47. Thrombin activity standard curve for peptide study. 50 µl of thrombin at various dilutions was mixed with 50 µl of thrombin assay buffer (20 mM Tris.Cl pH 7.4 containing 150 mM NaCl, 2 mM EDTA and 0.1 % albumin). The mixture was incubated in a 37°C water bath for 5 min, followed by the addition of 100 µl of 0.5 mM S-2238 substrate. The solution was transferred immediately to a microtitre plate and the absorbance was monitored at 405 nm for 10 min. The rate of absorbance increase over time (in minute) was determined and plotted with the concentration of enzyme used. Best-fit curve was determined using a linear regression analysis.

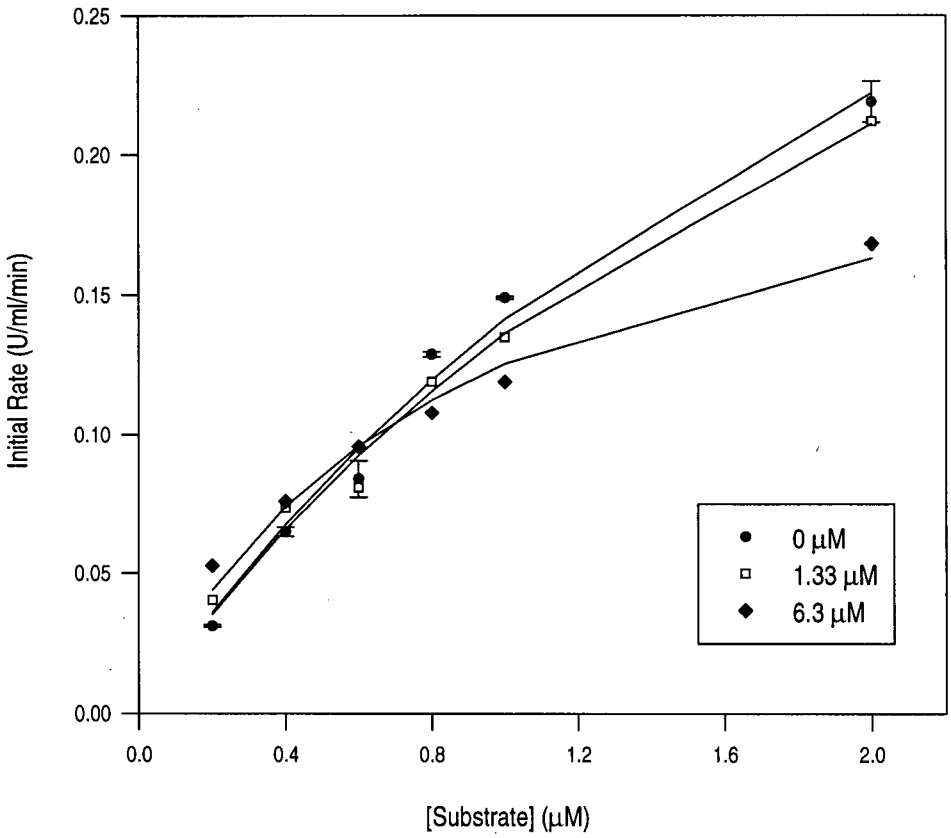


Figure 48. Effect of peptide A on the activation of prothrombin by factor Xa, calcium and PCPS phospholipid. Plasma-derived prothrombin in the presence of various concentrations of peptide A was activated with factor Xa (5 nM), calcium ions (5 mM) and PCPS phosphlipid (35 μM) pre-incubated at 37°C. Aliquots of the reaction mixture was taken out at 12, 25 and 40 min intervals and were subjected to the thrombin activity assay using the chromogenic substrate S-2238. Initial rate of the reaction (units of thrombin produced per ml per min) was plotted against substrate concentrations and kinetic constants were determined using Michaelis-Menten equation and Sigma Plot non-linear regression analysis. The values represented for all experiments in the presence of the peptide were from a single determination.

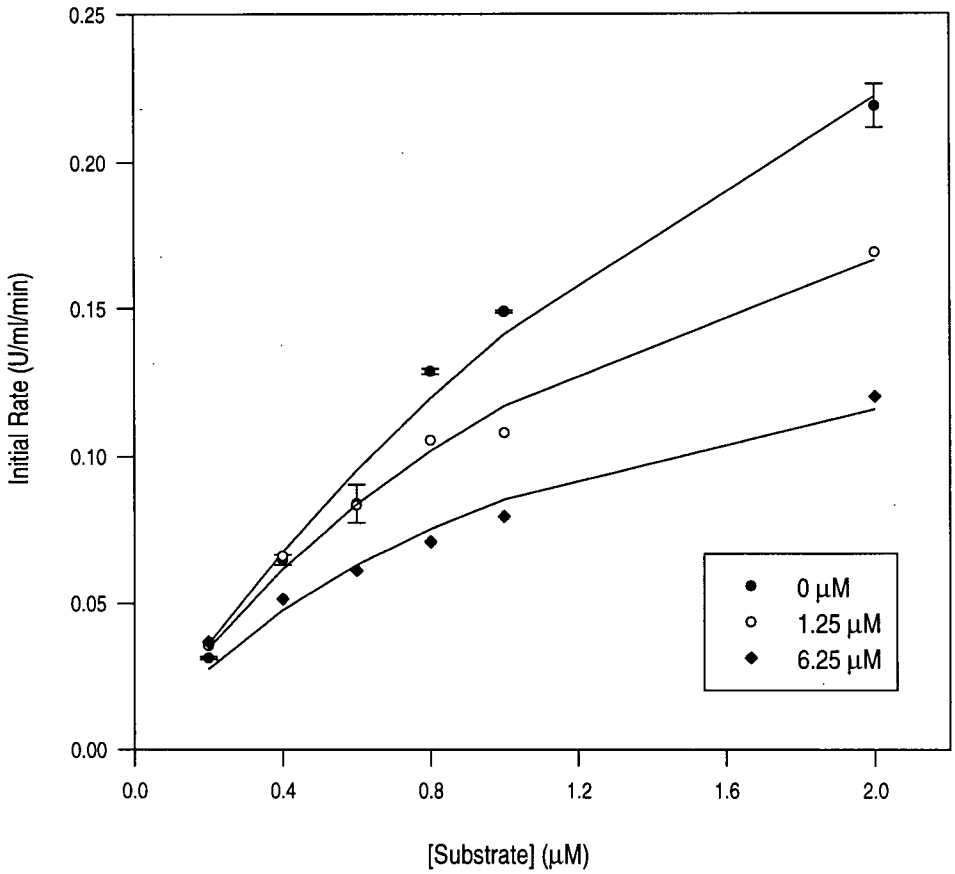


Figure 49. Effect of peptide D on the activation of prothrombin by factor Xa, calcium and PCPS phospholipid. Plasma-derived prothrombin in the presence of various concentrations of peptide D was activated with factor Xa (5 nM), calcium ions (5 mM) and PCPS phosphlipid (35 μM) pre-incubated at 37°C. Aliquots of the reaction mixture was taken out at 12, 25 and 40 min intervals and were subjected to the thrombin activity assay using the chromogenic substrate S-2238. Initial rate of the reaction (units of thrombin produced per ml per min) was plotted against substrate concentrations and kinetic constants were determined using Michaelis-Menten equation and Sigma Plot non-linear regression analysis. The values represented for all experiments in the presence of the peptide were from a single determination.

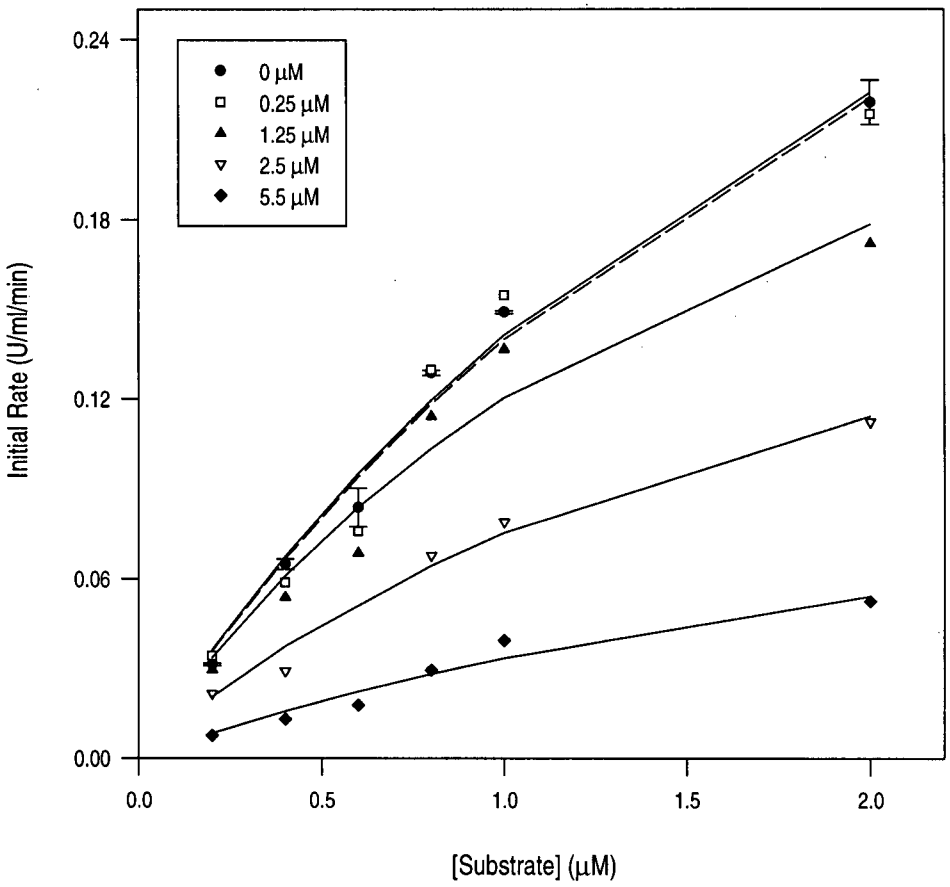


Figure 50. Effect of peptide BC on the activation of prothrombin by factor Xa, calcium and PCPS phospholipid. Plasma-derived prothrombin in the presence of various concentrations of peptide BC was activated with factor Xa (5 nM), calcium ions (5 mM) and PCPS phosphlipid (35 μM) pre-incubated at 37°C. Aliquots of the reaction mixture was taken out at 12, 25 and 40 min intervals and were subjected to the thrombin activity assay using the chromogenic substrate S-2238. Initial rate of the reaction (units of thrombin produced per ml per min) was plotted against substrate concentrations and kinetic constants were determined using Michaelis-Menten equation and Sigma Plot non-linear regression analysis. The values represented for all experiments in the presence of the peptide were from a single determination.

Table 7. Kinetic constants of prothrombin activation by factor Xa, Ca(II) and PCPS phospholipid in the presence of the synthetic K2 peptide BC.

Concentration (μM)[a]	Parameter[b]	Value (μM) ± StdErr[c]
0	V_{max}	0.519 ± 0.091
	K_m	2.666 ± 0.690
0.25	V_{app}	0.519 ± 0.148
	K_{app}	2.711 ± 1.129
1.25	V_{app}	0.342 ± 0.088
	K_{app}	1.843 ± 0.765
2.5	V_{app}	0.235 ± 0.048
	K_{app}	2.108 ± 0.687
5.5	V_{app}	0.142 ± 0.056
	K_{app}	3.236 ± 1.803

[a] prothrombin was activated by factor Xa (5 nM), calcium ions (5 mM) and PCPS phospholipid (35 μM) in the presence of various concentrations of peptide BC (μM)

[b] values represented for each peptide were from a single determination

[c] values were determined from the Michaelis-Menten equation using Sigma Plot program

two peptides. In contrast, peptide BC had a significant effect on the activation of prothrombin by factor Xa, calcium and PCPS phospholipid. As shown in Figure 50, increasing concentration led to increasing inhibition of prothrombin activation or thrombin activity. The effective range of peptide BC inhibition started at 2.5 μM around the same concentration as the substrate (2.2 μM) and increased to almost complete inhibition at 5.5 μM, only two fold higher in concentration. The inhibition effect of peptide BC did not demonstrate a competitive nature, in that increasing concentration of peptide led only to a reduced catalytic rate and minimal changes in K_m values (Table 7). Thus peptide BC seemed to be a non-competitive inhibitor of prothrombin activation by factor Xa. The effective K_i for peptide BC was calculated to be 2.19 ± 0.20 μM (Table 9), at about the same range as the K_m value for the activation of prothrombin by factor Xa and calcium-PCPS phospholipid complex.

3.3. Effect of K2 peptides on prothrombinase activation of prothrombin

Plasma prothrombin was activated by factor Xa, calcium ions and PCPS (75:25) phospholipid in the presence of the cofactor Va (the prothrombinase complex). The initial rate of thrombin appearance was plotted against the concentration of the substrate (prothrombin) and the kinetic constants were determined from Michaelis-Menten equation using non-linear regression analysis from the Sigma Plot program. The results were compared with the activation of prothrombin in the presence of the synthetic K2 peptides at various concentrations. The apparent kinetic constants were listed in Table 8. The K_m and V_{max} for plasma-derived prothrombin activated by the prothrombinase complex were 0.71 ± 0.21 μM and 1.38 ± 0.18 μM respectively (Figure 51). In the presence of increasing concentration of peptide A at 1.3, 2.5 and 6.3 μM, the V_{app} of the reaction progressively decreased while the K_{app} remained relatively unchanged. Thus peptide A seemed to affect only the apparent catalytic rate of the activation of prothrombin. The effect was minimal at the lower doses (1.3 and 2.5 μM) but more prominent at the higher concentration dose of 6.7 μM (Figure 51). Both peptides BC (Figure 52) and D (Figure 53) had similar inhibitory effects on the activation of prothrombin by the prothrombinase complex. They both inhibited the catalytic rate of the reaction similar to peptide A, showing effectiveness even at lower doses, while the apparent K_m appeared unchanged. In particular, peptide BC seemed to be

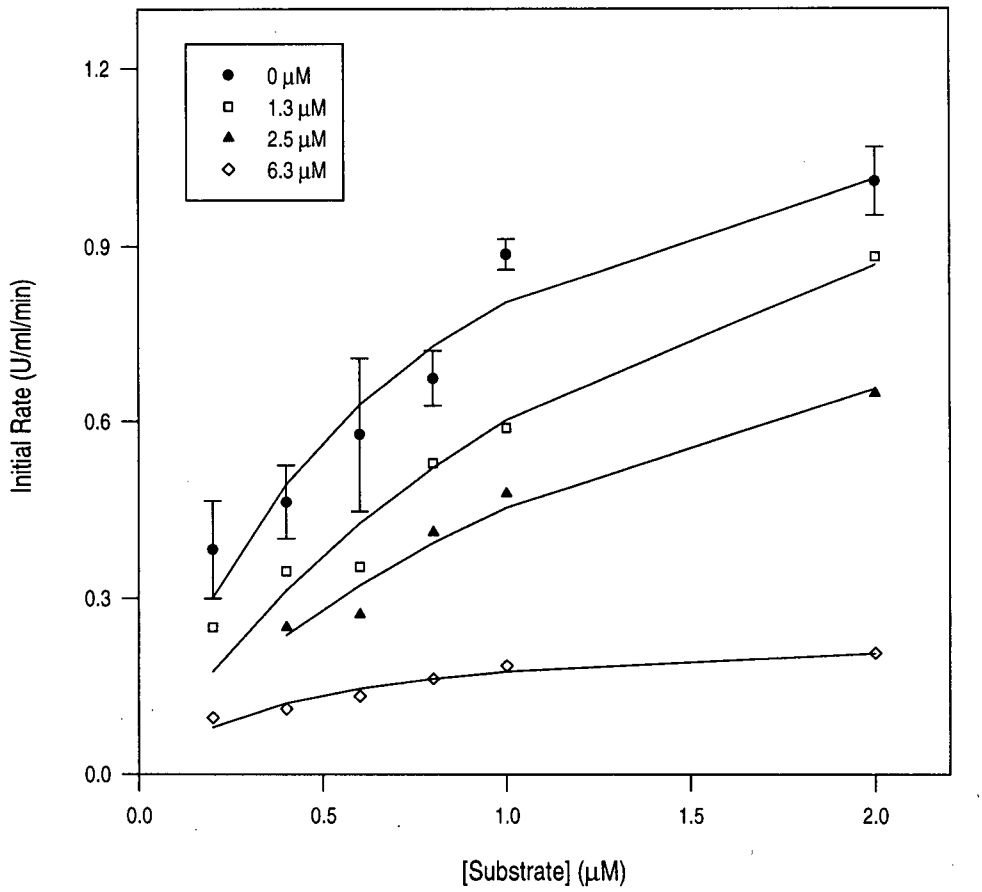


Figure 51. Effect of peptide A on prothrombin activation by the prothrombinase complex. Plasma-derived prothrombin in the presence of the synthetic peptide A at various concentrations was activated with factor Xa (0.01 nM), calcium ions (5 mM) and PCPS phosphlipid (35 μM) pre-incubated at 37°C in the presence of the cofactor Va (10 nM). Aliquots of the reaction mixture were taken out at two-minute intervals and were subjected to the thrombin activity assay using the chromogenic substrate S-2238. Initial rate of the reaction (units of thrombin produced per ml per min) was plotted against substrate concentrations and kinetic constants were determined using Michaelis-Menten equation and Sigma Plot non-linear regression analysis. The values represented for all experiments in the presence of the peptide were from a single determination.

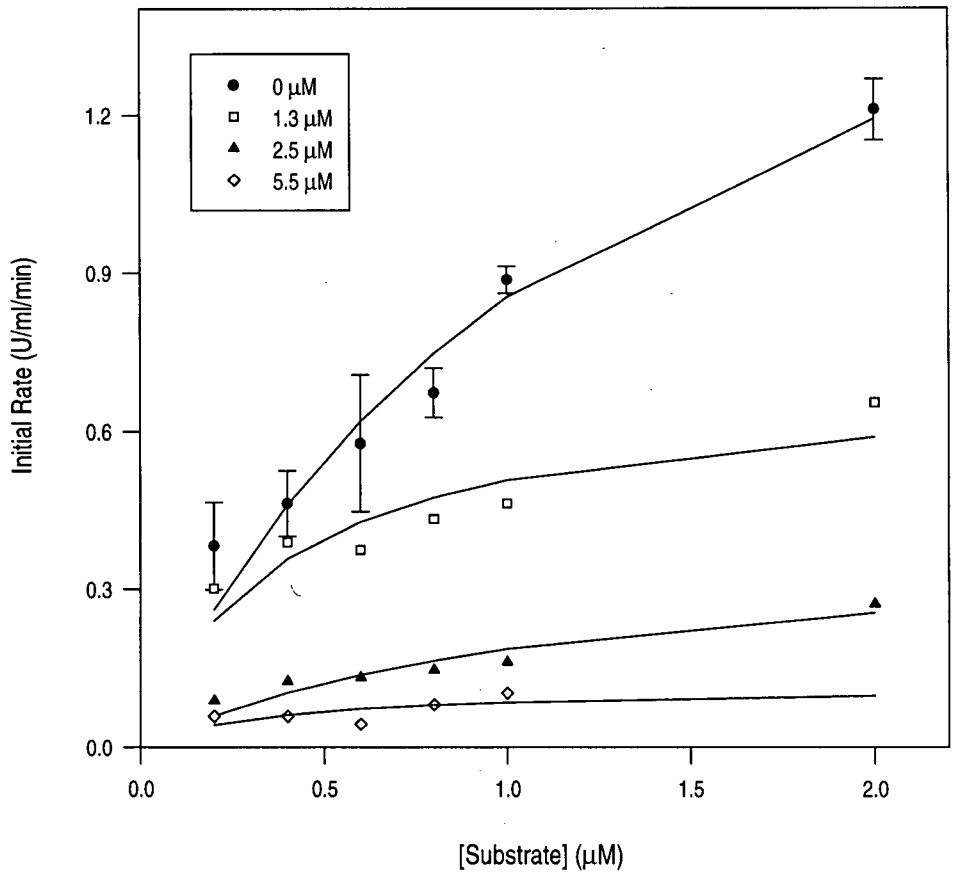


Figure 52. Effect of peptide BC on prothrombin activation by the prothrombinase complex. Plasma-derived prothrombin in the presence of the synthetic peptide BC at various concentrations was activated with factor Xa (0.01 nM), calcium ions (5 mM) and PCPS phosphlipid (35 µM) pre-incubated at 37°C in the presence of the cofactor Va (10 nM). Aliquots of the reaction mixture were taken out at two-minute intervals and were subjected to the thrombin activity assay using the chromogenic substrate S-2238. Initial rate of the reaction (units of thrombin produced per ml per min) was plotted against substrate concentrations and kinetic constants were determined using Michaelis-Menten equation and Sigma Plot non-linear regression analysis. The values represented for all experiments in the presence of the peptide were from a single determination.

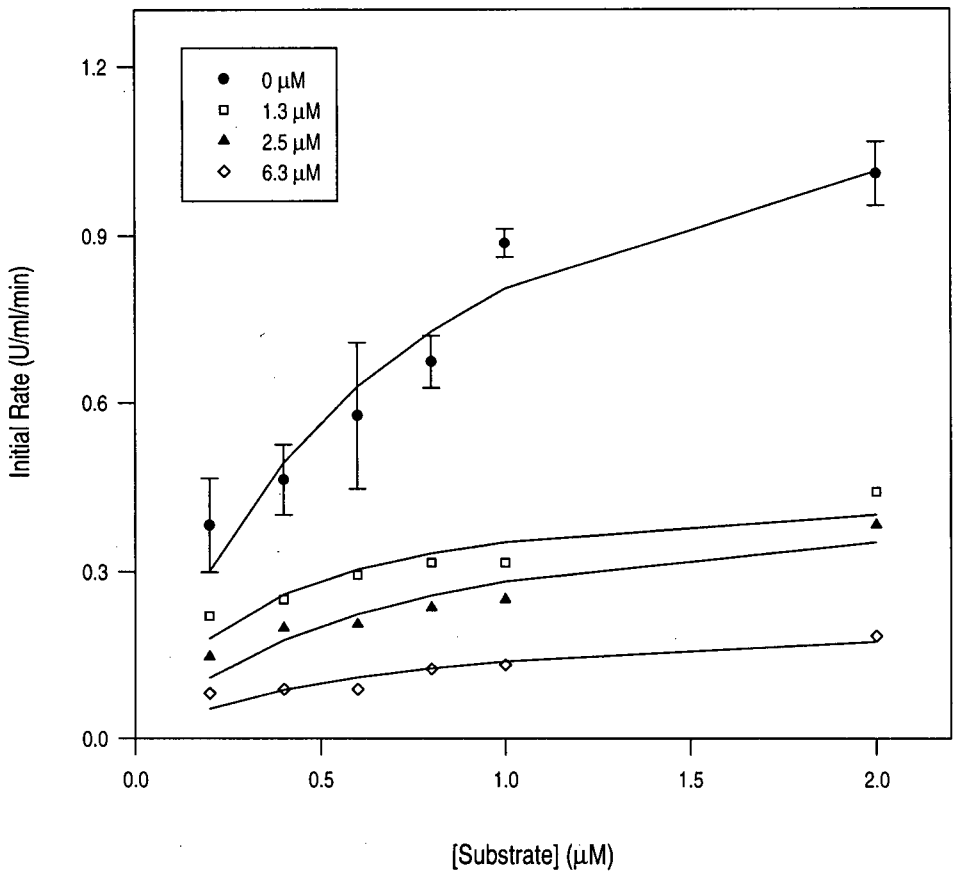


Figure 53. Effect of peptide D on prothrombin activation by the prothrombinase complex. Plasma-derived prothrombin in the presence of the synthetic peptide D at various concentrations was activated with factor Xa (0.01 nM), calcium ions (5 mM) and PCPS phosphlipid (35 µM) pre-incubated at 37°C in the presence of the cofactor Va (10 nM). Aliquots of the reaction mixture were taken out at two-minute intervals and were subjected to the thrombin activity assay using the chromogenic substrate S-2238. Initial rate of the reaction (units of thrombin produced per ml per min) was plotted against substrate concentrations and kinetic constants were determined using Michaelis-Menten equation and Sigma Plot non-linear regression analysis. The values represented for all experiments in the presence of the peptide were from a single determination.

Table 8. Kinetic constants of prothrombin activation by the prothrombinase complex in the presence of the synthetic K2 peptides A, BC and D.

phFII	Parameter	Value	StdErr
	V_{max}	1.377	± 0.181
	K_m	0.715	± 0.212

Parameter[b]	Value ± StdErr	Value ± StdErr	Value ± StdErr
		peptide A[a]	
	1.3 μM	2.5 μM	6.3 μM
V_{app}	1.250 ± 0.134	0.975 ± 0.083	0.249 ± 0.022
K_{app}	0.924 ± 0.203	1.044 ± 0.174	0.430 ± 0.108
		peptide BC[a]	
	1.3 μM	2.5 μM	5.5 μM
V_{app}	0.787 ± 0.131	0.325 ± 0.058	0.155 ± 0.311
K_{app}	0.545 ± 0.230	0.789 ± 0.307	0.615 ± 0.257
		peptide D[a]	
	1.3 μM	2.5 μM	6.3 μM
V_{app}	0.516 ± 0.068	0.468 ± 0.081	0.231 ± 0.045
K_{app}	0.488 ± 0.171	0.655 ± 0.267	0.666 ± 0.300

[a] values represented for each peptide were from a single determination

[b] values were determined from the Michaelis-Menten equation using Sigma Plot program

Table 9. Inhibition constants of the synthetic K2 peptides in the activation of prothrombin by two enzymic complexes

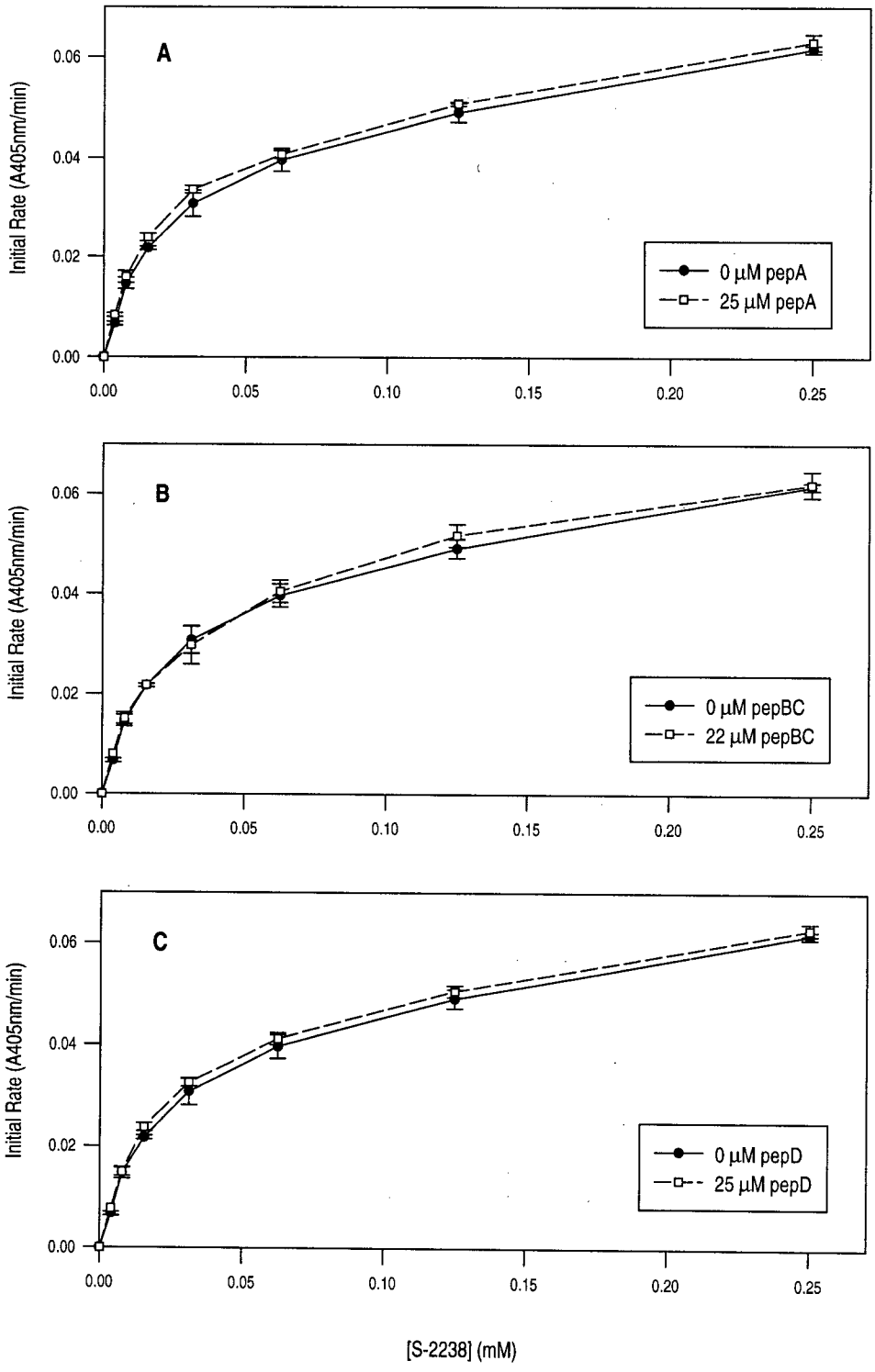
	Activation by factor Xa, calcium and PCPS[a,c]	Activation by the prothrombinase complex[b,c]
	K_i (μM)	K_i (μM)
peptide A	-	6.75 ± 5.73
peptide BC	2.19 ± 0.20	1.07 ± 0.58
peptide D	-	1.11 ± 0.29

[a] substrates were activated with 5 nM factor Xa, 5 mM $CaCl_2$ and 35 μM of PCPS (75:25)

[b] substrates were activated with 0.01 nM factor Xa, 10 nM cofactor Va, 5 mM $CaCl_2$ and 35 μM PCPS (75:25)

[c] deviations were from three determinations

Figure 54. Effect of synthetic K2 peptides on the cleavage of the chromogenic substrate S-2238 by thrombin. Thrombin (3.375 nM) was incubated at37°C in the presence of synthetic peptides at 25 μM each, followed by the addition of the chromogenicsubstrate S-2238. Absorbance at 405 nm was monitored continuously for 10 min at room temperature. Initial rate of the reaction (changes in A405 nm per min) was plotted against substrate concentrations and kinetic constants were determined using Michaelis-Menten equation and Sigma Plot non-linear regression analysis. The values represented were from a single determination. (panel A: peptide A; panel B: peptide BC; panel C: peptide D)


A
Initial Rate (A405nm/min)
0.06
0.04
0.02
0.00
0.00
0.05
0.10
0.15
0.20
0.25
0 μM pepA
25 μM pepA
B
Initial Rate (A405nm/min)
0 μM pepBC
22 μM pepBC
C
Initial Rate (A405nm/min)
0 μM pepD
25 μM pepD
[S-2238] (mM)

the most effective at inhibiting the activation of prothrombin by the prothrombinase complex. The mode of action seemed to resemble that of a non-competitive type of inhibition for these three peptides. The values of K_{app} and V_{app} for the three peptides were summarized in Table 8. The K_i for the non-competitive inhibition of peptides A, BC and D were averaged to be 6.75 ± 5.73, 1.07 ± 0.58 and 1.11 ± 0.29 μM respectively (Table 9).

3.4. Effect of K2 peptides on thrombin activity

The synthetic peptides were assessed for their effects in the activity of the prothrombinase complex and factor Xa. Their effects towards the activity of thrombin, the product of the prothrombin activation by these two enzymic complexes, were investigated to confirm that their observed inhibitory effects were not due to any activity towards thrombin itself, but were directed mainly towards factor Xa and the prothrombinase complex. Thrombin was incubated in the presence of the synthetic K2 peptides at various concentrations, and was subjected to catalytic cleavage of the chromogenic substrate S-2238. Absorbance at 405 nm was monitored in microtitre plates continuously for up to 10 min. A plot of the rate of absorbance changes at 405 nm was plotted against the concentration of the substrate S-2238. There was no detectable effect of the synthetic K2 peptides toward the cleavage of S-2238 by thrombin (Figure 54). Thus the observed inhibitory effects the peptides exhibited against factor Xa and the prothrombinase complex activity were not due to any interaction between the peptides and thrombin.

IV. DISCUSSION

1. The role of the first kringle in the activation of prothrombin

The approach toward understanding the structure-function relationship of the kringle domains in the interactions with the physiologic enzymic complexes (factor Xa, Ca(II) and PCPS complex and the prothrombinase complex) was to study recombinant prothrombin variants that had these kringle domains altered. When the first kringle deleted variant (rΔK1) was activated by the factor Xa, Ca(II) and PCPS complex, the K_m was increased about 9-fold

compared to that of wild type plasma prothrombin, and about 5-fold compared to recombinant prothrombin; while the k_{cat} was about the same for both plasma and recombinant prothrombin. The k_{cat}/K_m for rΔK1 was about 10-fold and 5-fold lower than plasma prothrombin and recombinant prothrombin respectively (Table 4). The differences lay mainly in the value of the Michaelis constant K_m and not in the catalytic rate. Thus these results demonstrated that the first kringle had lower enzyme-substrate specificity toward the factor Xa, Ca(II) and PCPS implying that there was specific interaction between the first kringle and the factor Xa necessary for optimal enzyme-substrate complex formation.

When rΔK1 was activated by the prothrombinase complex, the opposite effect was noticed in that the K_m remained relatively unchanged while the k_{cat} value was reduced by 5-fold and 2-fold compared with those from plasma and recombinant prothrombin respectively. The enzyme-substrate specificity (k_{cat}/K_m) was 3-fold and 2-fold lower compared to that of plasma prothrombin and recombinant prothrombin respectively. These results demonstrated that in the presence of the cofactor Va, the first kringle deleted variant was activated by prothrombinase complex with changes in the catalytic rate. It has similar enzyme-substrate specificity as the wild type recombinant protein. Thus interaction between the first kringle domain and the prothrombinase complex was observed. This result may also reflect the clotting result of rΔK1 measured by a one-stage clotting assay. The variant rΔK1 had 36% clotting activity when compared to plasma prothrombin (100%) and recombinant prothrombin (61%).

2. The role of the second kringle in the activation of prothrombin

The most significant effect was observed in the kinetic constants of the second kringle-deleted variant of prothrombin (rΔK2) in terms of activation by both the factor Xa, Ca(II) and PCPS complex and by the prothrombinase complex. When activated by factor Xa, Ca(II) and PCPS, rΔK2 had an increased K_m as well as a decreased k_{cat}. The K_m value was 9-fold and 5-fold higher compared to those of plasma and recombinant prothrombin respectively, while the K_{cat} value was 1.7-fold lower compared to both. The enzyme-substrate specificity (k_{cat}/K_m) value for rΔK2 was 15-fold and 7-fold lower compared to plasma and recombinant prothrombin respectively (Table 4). These values were similar to those of the first kringle deleted variant and demonstrated as well that the second kringle

domain played important part in the activation with factor Xa, probably even more significant than that of the first kringle domain. Recently, a linear sequence of the second kringle domain of prothrombin has been shown to interact with factor Xa (Taneda et al., 1994). The sequence of the peptide was from residues 36-51 and overlapped with that of peptide BC (residues 25-45). This peptide was immobilized onto microplates and bound onto factor Xa in a calcium and phospholipid-dependent manner. The fact that only peptide BC demonstrated inhibition and nor peptides A and D correlated very well with this observation that this second loop of the second kringle domain had extensive interaction with factor Xa.

In the presence of the cofactor Va, significant changes were observed with respect to both K_m and k_{cat} when rΔK2 was activated by the prothrombinase complex. The K_m was substantially increased to 32-fold higher than the value of plasma prothrombin, and about 29-fold higher than recombinant prothrombin. The catalytic rate k_{cat} was about half that of the plasma protein and about the same that of the recombinant wild type. The enzyme-substrate specificity (k_{cat}/K_m) was about 50-fold lower compared to the plasma wild type, and was 19-fold lower compared to the recombinant wild type protein. This result demonstrated that there was a significantly weaker affinity of rΔK2 toward the prothrombinase enzyme complex. Thus the large differences in the enzyme-substrate specificity values were directly attributed to the absence of the second kringle domain in this protein implying a significant interaction between the second kringle domain and factor Va in the prothrombinase complex. Since rΔK2 had a much lesser affinity to both factor Xa and the prothrombinase complex compared to rΔK1, the clotting activity of rΔK2 would be much less. This prediction was confirmed with the observed reduced clotting activity of rΔK2 (19%) compared to rΔK1 (36%).

The importance of the second kringle domain in the interaction with the prothrombinase complex was further demonstrated in the kinetic constants of rhBK2, a human prothrombin variant with the second kringle replaced with a bovine counterpart. In the factor Xa-dependent activation of rhBK2, the K_m value of rhBK2 was about 2.6-fold higher than that of plasma prothrombin, but practically the same as recombinant prothrombin; the k_{cat} of rhBK2 was slightly lower than both plasma and recombinant wild type prothrombin by a small factor of 1.4-fold difference. In the prothrombinase-dependent

activation, the K_m was slighltly higher at 4-fold difference compared to both plasma and recombinant wild type; while the k_{cat} was only slightly less than the plasma wild type but was slightly higher than the recombinant wild type (Table 4). The results showed that rhBK2 had kinetic properties very similar to that of the recombinant prothrombin and correlated very well also with the observed clotting activity of the two proteins as measured by the one-stage clotting assay. Wild type recombinant prothrombin had 61% clotting activity compared to the plasma wild type (100%) and rhBK2 had 57% clotting activity. The sequence identity of the second kringle domains between the human and bovine species is about 80%; this is a high enough similarity that could confer similar structural property leading to similar functions as was confirmed by the results above. Thus it seemed that the bovine second kringle was an adequate substitute for the human domain in its ability to be activated by both factor Xa and the prothrombinase complex. The interaction between the second kringle domain was demonstrated in the increased K_m of rhBK2 in the prothrombinase complex compared to that of the recombinant wild type (about 4-fold). Despite the fact that the human and bovine kringles probably have identical secondary structures, it is not enough for the bovine kringle to completely replace the human counterpart in terms of specific interaction between prothrombin and the prothrombinase complex, or more specifically the cofactor Va. This specific interaction was further demonstrated in the synthetic peptide inhibition experiments.

3. The role of the loops of the second kringle in the activation of prothrombin

The four loops A, B, C and D of the human second kringle domain of prothrombin were represented in three synthetic peptides A, BC and D. These loops had cysteine residues at the end so that a disulfide linkage can be formed at the ends thereby giving the peptides a constrained secondary conformation to mimic that of the native structure. The synthetic K2 peptides were subjected to prothrombin activation assays by factor Xa, Ca(II) and PCPS complex and by the prothrombinase complex. In the absence of the cofactor Va, loop BC was a non-competitive inhibitor to prothrombin activation by factor Xa in the presence of calcium and PCPS phospholipid with a K_i of 2.19 μM similar to the Km value of prothrombin (2.67 μM). In the presence of the cofactor Va, all three peptides inhibited prothrombin activation by the prothrombinase complex by changing the apparent V_{max}

leaving the K_m unchanged, thus demonstrating a non-competitive inhibition. The most effective peptide was peptide BC having a K_i value (1.07 μM) very similar to the K_m value of the substrate prothrombin (0.72 μM) in the presence of the cofactor Va. In the presence of cofactor Va, peptide D had a K_i value (1.11 μM) also comparable with that of peptide BC, while peptide A had a higher K_i value of 6.75 μM. The fact that the K_i values of these peptides were very close to the K_m value of prothrombin demonstrated that the synthetic K2 peptides were potent inhibitors able to interact quite closely with the enzymic complex of factor Xa and cofactor Va.

Based on the results, it could be inferred that there was extensive interaction between all three loops of the second kringle domain with the prothrombinase complex. Since only peptide BC was shown to have an effect on the activation of prothrombin by factor Xa, Ca(II) and PCPS phospholipid, the inhibitory effect all three peptides directed against the prothrombinase complex implied that the interactions were with the cofactor Va. The nature of the inhibition also confirmed that the interactions were specific to sites removed from the active site of the enzyme complex. This result proved conclusively that the peptides derived from the second kringle domain of prothrombin interacted with the cofactor Va of the prothrombinase complex and inhibited the catalytic rate of the enzyme. The interaction may be a dynamic and complex one since peptide BC, representing loops B and C in the second kringle domain, also showed interaction with factor Xa. In addition, these peptides did not have any effect in the activity of thrombin as shown in the chromogenic substrate S-2238 assays.

CHAPTER 6

CONCLUSIONS

I. PROTHROMBIN and the PROTHROMBINASE COMPLEX

Over the past decade, there have been many studies on the assembly and activity of the prothrombinase complex on the physiologic surfaces of activated platelets and circulating blood cells. The complex components have been examined extensively alone and with respect to each other. The result presented in this study had shown that there were significant roles for the non-enzymatic domains of prothrombin, namely Gla domain and the first and second kringle domains. The Gla domain of prothrombin was shown to be mainly responsible for the calcium-mediated phospholipid binding event preceding the assembly into the prothrombinase complex. The second kringle domain played a more significant role compared to the first kringle domain in that the deletion of this region led to more adverse effect in the ability of prothrombin to be activated. In addition, peptides derived from the loops of the second kringle structure exhibited potent non-competitive inhibition in the activation of prothrombin as well. The second loop and third loop (peptide BC) of the second kringle had been shown to interact with factor Xa as demonstrated in the peptide inhibition experiment and by others (Taneda et al., 1994). In addition, all four loops of the second kringle had extensive interaction with the prothrombinase complex as well. This interaction was inferred to be mainly with cofactor Va and influenced the catalytic efficiency of cofactor Va.

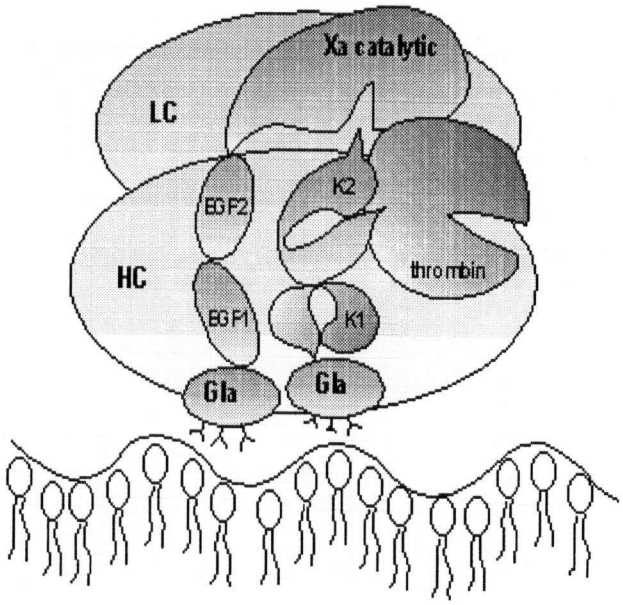
Recently however, other workers had shown that the second kringle domain by itself did not contribute significantly to the ability of cofactor Va to enhance the catalytic efficiency of factor Xa in the prothrombinase complex (Krishnaswamy et al., 1997). In this study, non-covalently linked fragment 2 (the second kringle region and the activating peptide) was shown to have no effect on the kinetics of prethrombin2 (prothrombin without fragment 1 and 2) activation by the prothrombinase complex. This observation seemed to suggest that the interaction of the second kringle with the cofactor Va was not important for its catalytic activity. This seemed controversial at first, but there are several factors that might explain this behavior. Firstly, the kinetics of prothrombin activation is not the same as the kinetics of prethrombin2 activation (about ten fold higher) (Krishnaswamy et al., 1997). Thus the interaction between these two substrates and the prothrombinase complex may be completely different. Secondly, the protease domain of prothrombin has been shown to interact with the second kringle fragment (Liaw et al., 1998; Martin et al., 1997). It would

seem then that the structural features of intact prothrombin with all the domains covalently linked assembled in the prothrombinase complex were very different from that of a polypeptide having only the protease domain. These conformational differences may account for the role of the kringle domain in the interaction with the components of the prothrombinase complex. In addition, the second kringle structure may be too large a polypeptide to be able to interact exactly with factor Xa or with cofactor Va. In contrast, small loops of the kringle such as the synthetic peptides, having a much smaller structure and more defined shapes, might be able to do this more efficiently.

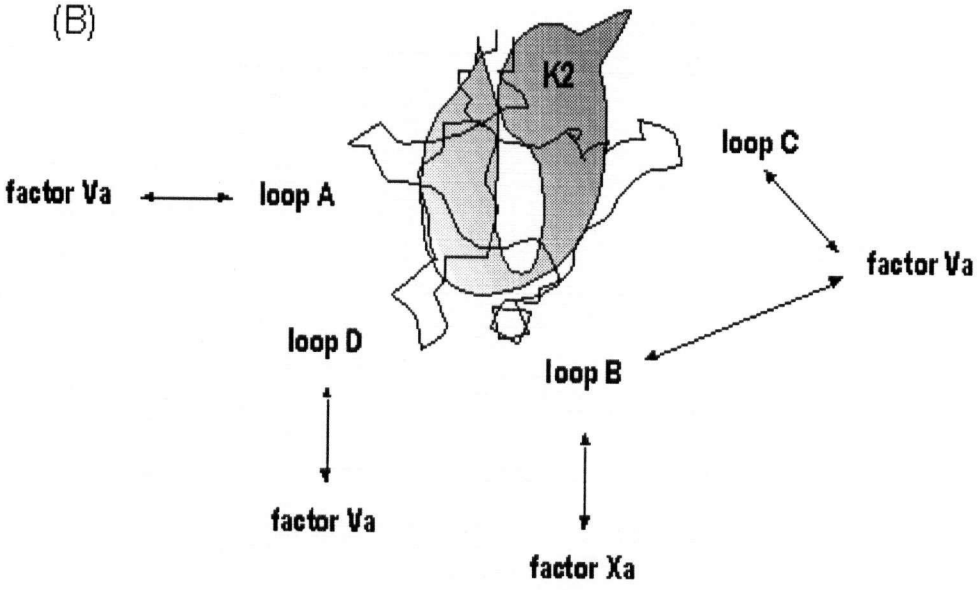
Based on this study and others, a model for the interaction of the prothrombinase complex can be proposed (Figure 55). In the prothrombinase complex, interactions between factor Xa and cofactor Va had been extensively characterized. A region (residues 263-274) of the heavy chain of factor Xa was shown to be the binding site for cofactor Va (Chattopadhyay et al., 1992). Different regions of cofactor Va were characterized to show interaction with factor Xa including both the light chain (Kalafatis et al., 1994; Tracy et al., 1983), and the heavy chain (Heeb et al., 1996; Kalafatis et al., 1994; Kojima et al., 1998). For the heavy chain of cofactor Va, the binding sites involved were primarily in a region between the A1 and A2 domains (residues 311-325) (Kojima et al., 1998), and at a region near the activated protein C cleavage site (residues 493-506) (Heeb et al., 1996). Similarly, prothrombin has been shown previously to interact with factor Va and factor Xa in the prothrombinase complex (Esmon et al., 1974b). In particular, the interaction of the second kringle domain of prothrombin with the prothrombinase complex was demonstrated by study with antibodies (Church et al., 1991), ultracentrifugal binding (Luckow et al., 1989), and through recombinant variants (Kotkow et al., 1995; Kotkow et al., 1993). This study had demonstrated that both the non-enzymatic kringle domains of prothrombin had interaction with factor Xa and cofactor Va, with the second kringle having a more prominent effect. The second kringle was further characterized to exhibit interaction with cofactor Va through its outer loops predominantly the second (loop B) and third loop (loop C). Based on all of the previous observations and the present study, a model for the assembly of the prothrombinase can be proposed (figure 55). Initially, the protease domain of factor Xa complexes with both the heavy and light chain of cofactor Va on the surface of an activated phospholipid membrane, and this interaction leads to a

Figure 55. Proposed model for the assembly of the prothrombinase complex on phospholipid membrane surface. The components of the prothrombinase complex was depicted in panel A: the heavy chain (HC) and light chain (LC) of cofactor Va was shown to interact with the protease factor Xa leading to a conformation optimal for the binding of the substrate prothrombin. The first kringle domain (K1) of prothrombin had limited interaction with factor Xa, while the second kringle domain (K2) had extensive interactions with factor Xa, cofactor Va as well as the protease region of prothrombin (thrombin). The role of the loops of the second kringle domain was demonstrated in panel B: loops A, B, C, and D all had interaction with cofactor Va, while only loops B and C interacts with factor Xa.

(A)


Xa catalytic
LC
K2
EGF2
HC
thrombin
EGF1
K1
Gla
Gla

(B)


K2
loop C
factor Va
factor Va
loop A
loop D
loop B
factor Va
factor Xa

conformational change in the complex favorable for substrate affinity. The substrate prothrombin interacts with the factor Xa:cofactor Vă leading to a catalytically efficient conformation resulting in an enhanced rate of substrate activation. For prothrombin, the interactions with the prothrombinase complex were mediated through the non-enzymatic regions of the protein, namely the first and second kringle domains. The interaction of the non-enzymatic domains of the various proteins in the prothrombinase complex was similarly parallel to that of the tenase complex. In the tenase complex, the protease factor IXa interacts with both the heavy and light chain of the cofactor VIIIa possibly through the A2 region (Fay et al., 1998; Lenting et al., 1996). The interactive sites for factor IXa include both the EGF1 domain and predominantly the catalytic domain (Mathur et al., 1999; Mathur et al., 1997; O'Brien et al., 1995). The role of the EGF2 domain of factor IXa and the EGF domains of factor X remained unknown at this time.

II. Future work

There are still many unknowns in the understanding of the intricate and complex event of the formation of the physiologically important clotting protease thrombin. Knowledge of the assembly and activity of the prothrombinase complex, the physiologic activator of thrombin, leads to clearer insight in this matter. Clearly the non-enzymatic domains of prothrombin play a distinct and significant role in mediating the multiple protein-protein interactions of the components in the prothrombinase complex. To understand this interaction further, the regions involved in both factor Xa and cofactor Va need to be determined. The synthetic peptides could be utilized in binding studies with both purified factor Xa and cofactor Va to probe the respective interacting residues in these proteins. Although the loops of the second kringle was shown to play important role in the activation of prothrombin, the exact residues or determinants responsible for each loop need to be elucidated. One approach is to construct a series of recombinant prothrombin molecules having a second kringle region with a loop different from the wild type and to characterize these variants in a similar systematic manner. In addition, due to theirs potent inhibition, the peptides may serve as useful models for the design and construction of small synthetic inhibitors that could be employed in approaches to anti-coagulation therapy.

CHAPTER 7

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