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Cleveland State University

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MOLECULAR MECHANISM OF INCORPORATION OF FACTOR Va INTO PROTHROMBINASE

MELISSA A. BARHOOVER

Bachelor of Science in Chemistry
Bowling Green State University
May, 2000

Submitted in partial fulfillment of requirements for the degree
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This dissertation has been approved for the Department of Chemistry and the College of Graduate Studies by:

Dr. Michael Kalafatis
Dissertation Committee Chairperson

Dr. Lily Ng
Dissertation Committee Member

Dr. Amin Zhou
Dissertation Committee Member

Dr. Harry VanKeulen
Dissertation Committee Member

Dr. Xue-Long Sun
Dissertation Committee Member

Dr. David Anderson
Dissertation Committee Member
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MOLECULAR MECHANISM OF INCORPORATION OF FACTOR Va INTO PROTHROMBINASE

MELISSA A. BARHOOVER

ABSTRACT

The mainstay of the blood coagulation cascade is the formation of the fibrin clot, catalyzed by the serine protease, thrombin. The prothrombinase complex is composed of the enzyme, factor Xa, and the protein cofactor, factor Va, in the presence of divalent metal ions associated on a membrane surface. This complex catalyzes the activation of prothrombin to its active form, thrombin. The enzyme, factor Xa, alone can activate prothrombin by two sequential proteolytic cleavages at Arg$^{271}$ and Arg$^{320}$ resulting in the intermediates, Fragment 1.2 and Prethrombin 2. The overall rate of this reaction is not compatible with survival. On the other hand, the incorporation of an excess of the cofactor, factor Va, into prothrombinase reverses the order of the proteolytic cleavages and increases the catalytic activity of factor Xa by 5 orders of magnitude, making this reaction compatible with survival. While the importance of the contribution of factor Va to the activity of factor Xa for rapid thrombin formation by prothrombinase at the place of vascular injury has been long established, the consequence of the interaction of the cofactor with the members of prothrombinase and the molecular mechanism by which factor Va accelerates prothrombin activation remains an enigma.
The *LONG-TERM* goal of this research is to characterize the physiological mechanism by which the prothrombinase complex promotes blood coagulation with the aim of providing a template for the synthesis of small molecules to be used as anti-coagulants in-vivo.

The *SHORT-TERM* goal of this research is to identify the precise amino acids of the central portion of the factor Va heavy chain involved in its incorporation into the prothrombinase complex and the cofactor function it exerts on the catalytic efficiency of prothrombin activation.

*Our hypothesis is that the incorporation of the factor Va heavy chain is mediated through a binding site for prothrombin and/or thrombin and through a binding site for factor Xa causing factor Xa to express a cryptic exosite for prothrombin which increases the catalytic efficiency of factor Xa.*
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CHAPTER I
INTRODUCTION

1.1 Prevalence of Cardiovascular Disease and Stroke

Heart disease is defined as any disorder of the cardiovascular system (an organ system composed of the heart and blood vessels that move substances to and from cells helping to stabilize body temperature and pH), which affects the heart’s capability to function regularly. Cardiovascular or heart disease is the foremost cause of death in men and women in the United States and is also a major cause of death throughout the rest of the world. It has been approximated that over 61 million Americans have heart disease, and 950 thousand of people with this condition die each year; accounting for almost 40% of all deaths in the United States. In addition, according to the American Heart Association, cardiovascular disease is responsible for more deaths in women than the next six leading causes of death combined.
Moreover, the third leading cause of death in adults in the United States is stroke. Research performed by the American Heart Association shows that nearly 700,000 people suffer from a stroke each year and over 150,000 of these patients die. Today, 5.7 million Americans are stroke survivors suffering from permanent stroke-related disabilities. A thrombotic stroke, a type of ischemic stroke, occurs when a blood clot develops in an artery and gradually narrows the lumen of the artery eventually leading to impeding of blood flow. In time, the clot will break off and partially or totally cut off blood supply to the brain.

1.1.1 Deep Vein Thrombosis

Deep Vein Thrombosis, or DVT, is a condition that results from the formation of a blood clot, or thrombus, located inside a deep vein commonly in the calf or thigh. There are three main causes of DVT: 1, stasis or slowing of blood flow defects, 2, a n disproportion among clot-promoting and clot-preventing factors, and 3, defects found in the vein wall. DVT takes place when the thrombus partially or completely blocks the flow of blood in the deep vein. When the thrombus completely blocks the flow of blood, it causes blood below the blockage to back up and cause more stasis. A major complication associated with DVT is an embolism; which happens when a part of the thrombus breaks off and travels in the blood stream. A pulmonary embolism is when the thrombus travels and becomes lodged in an artery in the lung; blood that goes to the lungs to become oxygenated brings the clot with it, thus blocking the blood flow.

1.1.2 Anti-Coagulant Drug Therapy

It has been demonstrated that heart disease is the number one killer in the United States and has been for almost a century. Survivors of this disease are often
prescribed anti-coagulants as treatment, which are frequently accompanied by serious side effects; including fatal or nonfatal hemorrhage, purple toes syndrome, nausea, loss of appetite, headache, joint or muscle pain, etc. The current anti-coagulants administered are non-specific serine protease inhibitors. Many of the proteases involved in the blood coagulation cascade are serine proteases. In addition, there are serine proteases involved in signaling pathways, enzyme activation, and degradative functions in different cellular or extra cellular compartments.

The most widely used anti-coagulant today is coumadin (warfarin). Coumadin was actually first developed as a rat poison. Coumadin is referred to as a vitamin K antagonist, as it decreases blood coagulation by impeding vitamin K metabolism (1). Vitamin K plays a fundamental role in the activity of vitamin K dependent serine proteases. It operates as a cofactor for the carboxylase catalyzed conversion of certain glutamic acid residues into \( \gamma \)-carboxyglutamic acid during their biosynthesis in the liver. These adapted amino acid residues are required for membrane binding of both enzymes and substrates in order to form the enzymatic complexes necessary for coagulation (2). Coumadin interferes with the \( \gamma \)-carboxylation of vitamin K dependent serine proteases, and the resultant \textit{in vivo} effect is a sequential depression of Factors VII, IX, X and II activities. Since there are so many vitamin K dependent serine proteases involved in the blood coagulation cascade and other pathways; it is, therefore, important to understand the specific regulation of the blood-clotting cascade in order to design a drug that does not have these serious side effects.
1.2 Hemostasis

Hemostasis is the physiological process whereby bleeding is halted (procoagulant) and thrombosis is the formation or presence of a blood clot within a blood vessel or one of the cavities in the heart (anticoagulant). Hemostasis is defined as the equilibrium between procoagulant and anticoagulant systems that function in concert to maintain the fluidity of blood under physiological conditions. The onset of hemostasis is initiated when there is an injury to the vasculature and several steps occur to stop vessel bleeding. Directly following the injury there is an immediate response that promotes vasoconstriction (narrowing of the lumen of the blood vessel), formation of the platelet plug, blood coagulation, and ultimate healing of the injured vessel. Hemostasis takes place through primary and secondary hemostatic events. Immediately following injury, primary hemostasis begins. When circulating platelets bind to collagen exposed at the site of vasculature injury. Platelets are cell fragments that are produced in the bone marrow and circulate in plasma. The binding of platelets to the site of injury forms the hemostatic plug within seconds after injury. Secondary hemostasis is the complex process of blood coagulation involving the regulated interaction of proteases, zymogens, cofactors, and inhibitors presented to the site of vasculature injury in the blood by platelets, blood cells, and the vessel wall (Figure 1.1).

1.3 Blood Coagulation Cascade

The result of the coagulation cascade is the formation of the fibrin clot. The clot attracts and stimulates the growth of fibroblasts and smooth muscle cells within the vessel wall, and begins the repair process, which ultimately results in the dissolution
Figure 1.1: Schematic of the blood coagulation cascade. Two separate pathways, either the intrinsic pathway or the extrinsic pathway can initiate the cascade. These two pathways merge to a common pathway, the activation of prothrombin to thrombin, leading to the formation of the fibrin plug (taken from www.kingsnake.com).
of the clot, a process defined as fibrinolysis. These events must stay localized to the damaged site or clots may occur throughout the vasculature resulting in the occlusion of blood flow to tissues and organs possibly leading to the damaging effects of heart disease and stroke.

1.3.1 The Extrinsic Pathway

In the Extrinsic pathway (tissue factor pathway), following injury there is exposure of an integral membrane glycoprotein, tissue factor, which then forms a complex with the enzyme factor VIIa. Only a small portion of factor VII circulates in its active form, factor VIIa (3). More factor VIIa is produced by proteolytic cleavage at residue Arg$^{152}$ by thrombin, factor IXa, factor Xa, factor VIIa, and factor XIa (4). The tissue factor/factor VIIa complex initiates the blood coagulation cascade by activating factor Xa. This cell-associated enzymatic complex increases the generation of factor Xa by a factor of 30,000. Factor VIIa also activates factor IX in the presence of tissue factor, providing a connection between the Extrinsic and Intrinsic pathways.

1.3.2 The Intrinsic Pathway

The second pathway of blood coagulation is the Intrinsic pathway (contact pathway). This pathway is initiated when several plasma proteins become activated when exposed to negatively charged membrane surfaces following damage to the vasculature. The assemblage of contact phase components results in conversion of prekallikrein to kallikrein, which in turn activates factor XII to factor XIIa (5). Factor XIIa can then hydrolyze more prekallikrein to kallikrein, establishing a reciprocal activation cascade. Factor XIIa also activates factor XI to factor XIa and leads to the
release of bradykinin, a potent vasodilator, from high-molecular-weight kininogen.

In the presence of Ca^{2+}, factor XIa activates factor IX to factor IXa. Active factor IXa cleaves factor X at an internal arg-ile bond leading to its activation to factor Xa (6).

1.3.3 The Common Pathway

The two pathways meet and become common upon the activation of factor Xa and the formation of the prothrombinase complex. It is the prothrombinase complex, composed of the enzyme (factor Xa) the cofactor (factor Va) and the substrate (prothrombin), in the presence of calcium ions and a membrane surface, that activates prothrombin to thrombin. Even though both the Intrinsic and Extrinsic pathways aid in the production of factor Xa, it has been demonstrated that it is the Intrinsic pathway that produces the bulk of factor Xa necessary for coagulation (7). The prothrombinase complex activates prothrombin to its active form, thrombin. In turn, thrombin will then convert fibrinogen to fibrin to form the clot.

1.3.4 Initiation of Coagulation

When the coagulation cascade is initiated, production of thrombin is said to occur in three discrete phases (3, 8). In the first phase, the initiation phase, when the cascade is started it leads to the formation of various active complexes (extrinsic tenase, intrinsic tenase, and prothrombinase) and low levels of thrombin production are detected. At the end of this phase of coagulation, the propagation phase begins with thrombin generation at much higher levels. Once enough thrombin is produced to form the fibrin clot, the termination phase of coagulation begins. Throughout this phase, inhibition of the coagulation proteases begins due to the inhibitors Tissue Factor Pathway Inhibitor (TFPI) and Anti-thrombin (AT). Feedback inhibition of the
cofactor, factor Va, via the protein C pathway also occurs (9-12). These inhibition mechanisms limit the amount of active thrombin.

1.4 Clinical Consequences of Deficiencies or Mutations in Clotting Cofactors

The importance of cofactors to the blood coagulation cascade is portrayed by the deficiency or mutation of the cofactors, factor V/Va and factor VIII/VIIIa. When mutations occur in these proteins, it results in bleeding disorders.

1.4.1 Factor VIII and Hemophilia

Hemophilia A is the result of a deficiency in factor VIII. A variety of point mutations have been characterized in the factor VIII gene (13). Individuals with these defects suffer prolonged bleeding times and often need infusions of factor VIII. The severity of hemophilia A can range from mild to moderate to severe depending on the levels of factor VIII in plasma. There has been over 150 point mutations identified in the factor VIII gene associated with hemophilia A. Although, 90% of hemophilia cases are caused by deficiencies in factor VIII, hemophilia B is caused by a lack of factor IX and hemophilia C is caused by a lack of clotting factor XI.

1.5 Coagulation Cofactor Factor V and activation by thrombin

Human factor V is a single chain glycoprotein ($M_r = 330,000$) present in plasma at a physiological concentration of 20nM (14). It is composed of five domains; including three A domains, two C domains, and a B domain which connects these regions. Factor V is activated by thrombin following three sequential cleavages at Arg$^{709}$, Arg$^{1018}$, and Arg$^{1545}$ creating a heavy chain ($M_r = 105,000$) and a light chain ($M_r = 74,000$) associated non-covalently by divalent metal ions (Figure 1.2) (15). The heavy chain is composed of two A domains, (residues 1-303 and residues 317-657),
which are connected by a region that is rich in basic amino acids. The remaining portion of the heavy chain, residues 658-709, contains a cluster of acidic amino acids. The light chain also contains an A domain, residues 1546-1877, and two C domains, residues 1878-2036 and 2037-2196, respectively \((16, 17)\) The cleavage rates of Arg\(^{709}\) and Arg\(^{1018}\) are approximately equivalent, while cleavage at Arg\(^{1545}\) is somewhat delayed resulting in high molecular weight intermediates and a delayed appearance of the light chain \((18)\). It is this cleavage at Arg\(^{1545}\) that leads to fully active factor Va, indicating that formation of the light chain is an important step in factor V activation \((19)\).

1.5.1 Factor V Activation by other proteases

Factor V can also be activated by a number of venom proteases. One such protease is an enzyme from Russell’s viper Venom (RVV-V). RVV-V cleaves human factor V at two sites, Arg\(^{1018}\) and Arg\(^{1545}\), to create a heavy chain \((M_r 150,000, residues 1-1018)\) and a light chain \((M_r 74,000, residues 1546-2196)\). This generates factor Va\(_\text{RVV-V}\) which has similar activity as factor V activated by thrombin, factor Va\(_{\text{IIa}}\), and has similar affinity for factor Xa \((20)\). Factor V can also be cleaved by an enzyme from the venom of the snake *Naja nigricollis nigricollis* that cleaves factor V at Asp\(^{697}\), Asp\(^{1509}\), and Asp\(^{1514}\) which produces a heavy chain \((M_r 100,000, residues 1-696)\) and a light chain \((M_r 80,000, residues 1509/1514-2196)\), designated factor Va\(_{\text{NN}}\). Factor Va\(_{\text{NN}}\) has 60-80% decreased clotting activity compared to factor Va\(_{\text{IIa}}\) and has a decreased affinity for factor Xa with a \(K_d\) of 4nM \((21)\). Factor V activation by the snake venoms have further elucidated the mechanism of factor Va cofactor
Figure 1.2: Schematic of Factor V Activation by Thrombin. Factor V is activated by thrombin following three cleavages at Arg\textsuperscript{709}, Arg\textsuperscript{1018}, and Arg\textsuperscript{1545}. After cleavage of factor V at Arg\textsuperscript{709}, the B-domain remains attached to the A3-domain. Cleavages at both Arg\textsuperscript{709} and Arg\textsuperscript{1018} release a 71,000 kDa-peptide fragment of the B-domain. The remaining part of the B-domain (150,000 kDa) is released upon cleavage at Arg\textsuperscript{1545}.
activity. Previous data have demonstrated that factor \( V_{aNN} \) results in an active factor Va molecule that has an amino acid segment missing from the heavy chain representing a binding site for prothrombin (amino acids 697-709) (22).

### 1.5.2 Factor V Post-translational Modification

The Factor V molecule undergoes several post-translation modifications, including sulfation, phosphorylation, and glycosylation (Figure 1.3). It has been demonstrated that sulfation is important for factor Va function (23). Factor Va is phosphorylated at amino acid residue Ser692 by a membrane associated platelet casein kinase II (CKII); phosphorylation at this residue increases the inactivation rate of the cofactor by APC (24). The Factor Va molecule is phosphorylated at two positions on the light chain by a platelet derived protein kinase C isoform (25, 26). Factor V has several possible N-linked glycosylation sites located on the B region and on the heavy and light chains that aid in secretion of the molecule (16). Additionally, several other blood-clotting factors go through a post-translational modifications of glutamic acid to \( \gamma \)-carboxyglutamic acid by a vitamin K-dependent carboxylase. The \( \gamma \)-carboxyglutamic acid residues of the vitamin K-dependent blood clotting proteins are located at the N-terminal “Gla-domain”; these proteins include prothrombin, protein C, protein S, factor VII, factor IX, and factor X. The Gla-domain is responsible for the membrane-dependent binding of the proteins (27).

### 1.6 Factor Va binding to factor Xa

Establishment of the factor Xa-factor Va complex involves three steps: 1) binding of factor Xa a to negatively charged membrane surface, 2) binding of factor Va to the same membrane surface, and 3) diffusion of the membrane bound proteins leading to
Figure 1.3: Human Factor V. Shown above is an illustration of the human factor V molecule. The top arrows indicate activation cleavage sites by thrombin, factor Xa, and RVV-V activator. The bottom arrows show the inactivation cleavages by APC and plasmin. The posttranslational modification sites are also indicated. (Taken from Mann and Kalafatis, 2003).
the association of the proteins (28). Factor Xa and factor Va can interact in the absence of a membrane surface with a $K_d$ of 0.8µM and it’s interaction is dependent upon calcium ions (29). However, in the presence of a membrane surface and Ca$^{2+}$, the interaction of factor Xa and factor Va has a $K_d$ of ~1nM (30). It has also been recognized that both chains of factor Va are required for the interaction with factor Xa (31-34). It has been further demonstrated that it is the A1 and A3 domains that are involved in this interaction (34). Factor V also binds to the membrane’s surface to help stabilize this interaction through the C2 and A3 domains of the molecule located on the light chain. It has been established that this interaction is governed by both electrostatic and hydrophobic interactions with the membranes (35-39) Recent kinetic data have shown that the binding of factor Va to factor Xa exposes exosites on the enzyme remote from the active site (40). It allows for more specific interaction with the substrate, prothrombin (41).

1.7 Factor Va binding to prothrombin and/or thrombin

The primary structure of factor Va lends to the ability of the COOH-terminus of the heavy chain to play a role in protein-protein interactions. This region of the cofactor molecule is highly acidic and should be expected to be on the surface of the molecule making it readily available for protein-protein interactions (42). Further, this region also contains tyrosine residues and hirudin-like motifs that have been previously shown to be involved with activation of the pro-cofactor, factor V, as well as cofactor function (23, 43). Hirudin is a naturally occurring peptide in the salivary glands of medicinal leaches. This peptide has anticoagulant activity through it’s ability to specifically inhibit thrombin activity (44). It has been demonstrated that
factor Va interacts with the substrate, prothrombin, via the heavy chain of the molecule (30, 32). Furthermore, a synthetic peptide, amino acid residues 697-709 from the COOH-terminus of the heavy chain of factor Va, was found to interact directly with thrombin agarose, indicating these residues of the cofactor provide a binding site for thrombin and/or prothrombin (45).

1.8 Down Regulation of the Coagulation Cascade

Once enough thrombin is produced to stop bleeding by the formation of the fibrin clot, it then has the ability to participate in the down regulation of the coagulation cascade. Thrombin, therefore, has a dual effect on the cascade; it possesses both coagulant and anti-coagulant properties. When the cofactor thrombomodulin (TM) binds to thrombin, it switches its role form pro-coagulant to anti-coagulant. Now this IIa/TM complex can start the down-regulation of the blood coagulation cascade by activating Protein C (PC) to its active form, Activated Protein C (APC); IIa/TM is the enzymatic complex capable of this activation.

1.8.1 Thrombin/Thrombomodulin Activation of Protein C

Thrombomodulin is an endothelial cell surface glycoprotein cofactor that is composed of 559 amino acids and contains five domains: an NH₂-terminal domain (residues 1-224), six Epidermal Growth Factor (EGF)-like domains (residues 225-461), a 34 amino acid portion that includes eight hydroxyamino acids, a hydrophobic membrane-spanning domain (residues 497-520), and a cytoplasmic tail domain (residues 521-559) (46). Protein C (PC) is a vitamin K-dependent serine protease zymogen that circulates in plasma at a concentration of 60nM. PC is composed of a heavy chain and light chain with Mᵣ of 41,000 and 21,000, respectively. The chains
are connected through a disulfide bond (47). For the activation of Protein C, the enzyme, IIa, and the substrate, PC, need the function of a cofactor for the reaction to work in a physiological efficient manner. For PC, the cofactor is Ca\(^{2+}\). It has been demonstrated that Ca\(^{2+}\) binds to the 70-80 loop of the protease domain on the zymogen form of PC (48). Upon activation, PC is converted to its active form, APC, by cleavage at Arg\(^{169}\) of the heavy chain by the IIa/TM complex in the presence of Ca\(^{2+}\) (47).

**1.8.2 Activated Protein C Inactivation of Coagulation Cofactors**

Once activated, APC down regulates the coagulation cascade by inactivating the protein cofactor, factor Va, by sequential proteolysis (49, 50). Another vitamin K-dependent, Gla-domain containing protein, Protein S, serves as a cofactor to APC (51, 52). The cofactor function of protein S is weak in purified systems, only enhancing the APC inactivation of factor Va by a factor of two (53). APC inactivates factor Va following proteolytic cleavages at Arg\(^{306}\), Arg\(^{506}\), and Arg\(^{679}\) (Figure 1.4). It has been shown that proteolytic cleavage at Arg\(^{506}/Arg^{679}\) results in a factor Va molecule with a 10-fold decrease in its affinity for factor Xa. These same cleavages results in the elimination of its interaction with prothrombin. APC can also inactivate the procofactor, factor V. APC cleaves the pro-cofactor in the presence of a membrane surface at residues Arg\(^{306}\), Arg\(^{506}\), Arg\(^{679}\), and Lys\(^{994}\). It is the first cleavage at Arg\(^{306}\) that is the inactivating cleavage of the procofactor (54). In addition, APC also can inactivate factor VIIIa; however, APC cleavage does not appear to be essential to inactivation because factor VIIIa inactivation occurs spontaneously following the dissociation of the A2 domain (55-57).
1.8.3 Factor $V^{\text{LEIDEN}}$

The importance of cofactors to the blood coagulation cascade is specifically portrayed by the clinical consequences of mutations to factor V/Va. One of the most prevalent mutations, occurring in 5% of the normal population in Western Countries, is the factor $V^{\text{LEIDEN}}$ mutation. It was discovered in 1993 by Dahlback et al. when they observed that plasma from several individuals has a slower response to APC inactivation (58). Factor $V^{\text{LEIDEN}}$ is a result of a single point mutation at amino acid residue 1691 of $G \rightarrow A$, resulting in a factor V molecule with an $\text{Arg}^{506} \rightarrow \text{Gln}$ substitution (59). Arg$^{506}$ is the initial cleavage site for APC on the heavy chain of human factor Va and makes possible the following lipid-dependent inactivation cleavage at Arg$^{306}$. This amino acid replacement results in a cofactor that is unable to be cleaved at residue 506 by APC. The resistance of factor $V^{\text{LEIDEN}}$ to APC inactivation has been shown to be a major risk for venous thrombosis (60). Individuals homozygous for this mutation have an 80-fold higher risk for thrombosis than individuals that possess the normal factor V gene, and individuals that are heterozygous for factor $V^{\text{LEIDEN}}$ have a 7-fold higher risk for thrombosis than healthy individuals. The inability of factor $V^{\text{LEIDEN}}$ to be cleaved at residue 506 results in a slower rate of cleavage at Arg$^{306}$ and Arg$^{679}$ by APC compared to normal plasma factor Va. The slower rate of cleavage has been shown to be a result of the delayed cleavage at Arg$^{506}$, suggesting that the cleavage at Arg$^{506}$ is necessary for the efficient inactivation of the cofactor, factor Va, by APC (61).
Figure 1.4: Diagram of Factor Va Inactivation by APC. APC inactivates factor Va by 3 proteolytic cleavages. First at Arg506, followed by cleavage at Arg306 and Arg679. These cleavages lead to an inactive cofactor.
1.8.4 Factor V Cambridge and Factor V Hong Kong

In 1998, two new mutations in factor V were discovered, factor V Hong Kong (factor V\textsuperscript{HG}) and factor V Cambridge (factor V\textsuperscript{CAM}) (62, 63). Both mutations result in the substitution of amino acid residue 306, Gly for FV\textsuperscript{HG} and Thr for FV\textsuperscript{CAM}. It was suspected that a mutation at Arg\textsuperscript{306} would cause a severe thrombotic state, since it is cleavage at Arg\textsuperscript{306} in factor Va by APC that causes complete inactivation of the cofactor. On the contrary, preliminary evidence has shown that neither factor V\textsuperscript{HG} nor factor V\textsuperscript{CAM} appears to be correlated with an increased risk for thrombosis (64, 65). Additional evidence is needed to further elucidate the mechanism of these mutations in factor V.

1.9 Prothrombin

Prothrombin is composed of five domains: an N-terminal $\gamma$-carboxyglutamic acid (Gla) domain, an aromatic amino acid stack domain, two kringle domains, and a C-terminal serine protease domain (Figure 1.5). It has been demonstrated that the Gla domain, which contains ten $\gamma$-carboxyl-glutamic acid residues, is responsible for its calcium binding properties (66, 67). The Gla domain is also responsible for binding of most vitamin K-dependent proteins to the phospholipid membrane surface. It has shown that prothrombin and thrombin contain two electropositive exosites, anion binding exosite I (ABE-I) and anion binding exosite II (ABE-II). They are responsible for the functions of these molecules. Studies have shown that ABE-I binds to thrombomodulin (68), fibrinogen (69), PAR1 (70), the COOH-terminal of hirudin peptides (71), and heparin cofactor II (72). ABE-II interacts with the protease...
**Figure 1.5: Schematic of Prothrombin activation.** Prothrombin is composed of ten \( \gamma \)-carboxyglutamic residues (hatched), two kringle domains (checkered), and a serine protease domain (black). Pathway I shows cleavage by the factor Xa and a membrane surface. Pathway II shows cleavage by the prothrombinase complex (Taken from Bukys et al. 2006).
nexin (73). Both exosites have been shown to bind factor V and factor VIII (74). It has also been demonstrated that pro-exosite I of the zymogen, prothrombin, interacts directly with factor Va in the prothrombinase complex (75, 76).

1.9.1 Prothrombin Activation by Prothrombinase

The activation of prothrombin can occur through two pathways. In Pathway I, which the enzyme alone activates prothrombin, following first cleavage at Arg$^{271}$ giving Fragment 1.2 and Prethrombin-2 as intermediates (Figure 1.3). The second proteolytic cleavage at Arg$^{320}$ produces the active thrombin molecule and Fragment 1.2. (77-79). Overall, this reaction is slow and is not compatible with survival. In Pathway II, incorporation of the cofactor, factor Va, into the prothrombinase complex reverses the order of these proteolytic cleavages. It cleaves first at Arg$^{320}$ to produce an active intermediate, Meizothrombin, and it cleaves secondly at Arg$^{271}$ to give thrombin and Fragment 1.2. Thus, the incorporation of the cofactor, factor Va, into the prothrombinase complex increases the catalytic efficiency of the enzyme alone by five orders of magnitude (80, 81). The high increase in the turnover number is due to two things: (1) an 100-fold decrease in the $K_m$ as a result of the interaction with the membrane surface localizing the concentration of the substrate and (2) a 3,000-fold increase in the $k_{cat}$ of the enzyme as the result of the incorporation of factor Va into the prothrombinase complex (82, 83).

1.10 New Evidence of Two Equilibrating Forms of Prothrombinase

There have been many studies investigating the mechanism by which coagulation factor Va enhances the rate of prothrombin activation. It has been demonstrated that prothrombin interacts with prothrombinase at exosites that are located at a spatially
distinct site from the active site of factor Xa and that these exosites on prothrombin may perhaps arbitrate substrate recognition and cleavage (75, 84). Obtained data have also illustrated that it is the heavy chain of factor Va that interacts with the fragment-2 domain of the substrate prothrombin (84-86). Recent data have shown that an interaction exists between factor Va and proexosite I of prothrombin (76). Additionally, x-ray crystallographic evidence of prothrombin-2 has shown that the residues preceding the cleavage site at Arg^{320} must go through massive rearrangement in order to properly dock in the active site of factor Xa (87). New research has demonstrated that the ordered steps of prothrombinase on the two activating cleavages in prothrombin occurs from the hindering effects of the substrate bound in two separate conformations through comparable exosite binding interactions to a single form of prothrombinase (88). This group has also provided evidence that ratcheting of the substrate, prothrombin, from the inactive zymogen conformation to the active proteinase conformation forces the ordered presentation of the two cleavage sites at Arg^{271} and Arg^{320} to the active site of the enzyme leading to the sequential activation cleavages on prothrombin by prothrombinase (89). However, another group has proposed a mechanism that shows there are two equilibrating forms of prothrombinase involved in prothrombin activation; the model depicts two forms of the enzyme each recognizing one of the prothrombin cleavage sites. The authors concluded that the kinetics of prothrombin activation follow a “ping-pong”-like mechanism (81). Further research needs to be performed to resolve the exact mechanism of prothrombin activation by the prothrombinase complex.
1.11 Reference List


CHAPTER II
THE ESSENTIAL CONTRIBUTION OF ASP\textsuperscript{334} AND TYR\textsuperscript{335} FROM FACTOR Va HEAVY CHAIN TO THE CATALYTIC EFFICIENCY OF PROTHROMBINASE

2.1 Abstract

Incorporation of factor Va into prothrombinase results in a 300,000-fold acceleration of the catalytic efficiency of prothrombinase as compared to the reaction catalyzed by factor Xa alone. We have demonstrated that amino acids E\textsuperscript{323}, Y\textsuperscript{324}, E\textsuperscript{330}, and V\textsuperscript{331} from factor Va heavy chain are crucial for the binding of factor Xa and are required for coordinating the spatial arrangement of enzyme and substrate directing prothrombin cleavage. We have also demonstrated that amino acid region 332-336 contains residues that are involved in cofactor function. In the present study using overlapping peptides from this region we have identified amino acid residues
DY as important contributors for factor Va cofactor activity. Site-directed mutagenesis was used to study the effect of these amino acids on the catalytic efficiency of prothrombinase. We have constructed recombinant factor V molecules with the mutations D→K and Y→F (factor V^KF) and D→A and Y→A (factor V^AA). These molecules were transiently expressed and purified to homogeneity. Kinetic studies showed that while factor V^KF and factor V^AA had a K_D for factor Xa similar to the K_D observed for wild type factor Va, the clotting activity of the mutant molecules was significantly impaired and the k_cat of prothrombinase assembled with factor V^KF and factor V^AA was notably reduced. The second order rate constant of prothrombinase assembled with factor V^KF and factor V^AA, for prothrombin activation, was 8-fold and 12-fold lower, respectively, than the second order rate constant for the same reaction catalyzed by prothrombinase assembled with the wild type molecule. We have also created two quadruple mutants, factor V^FF/KF (E323F/Y324F and D334K/Y335F), factor V^MI/KF (E330M/V331I and D334K/Y335F), factor V^FF/AA (E323F/Y324F and D334A/Y335A), and factor V^MI/AA (E330M/V331I and D334A/Y335A). Prothrombinase assembled with factor V^KF showed a 47-fold decrease in the second order rate constant while under similar experimental conditions prothrombinase assembled with factor V^MI/KF had a 400-fold decrease in the second order rate constant. The quadruple mutants containing the D334A/Y335A mutations also has decreased second order rate constants, factor V^KF and factor V^AA had 16-fold and 27-fold decreased values, respectively. Time courses studying prothrombin activation by gel electrophoresis demonstrated that prothrombinase
assembled with all these mutants had reduced rates for prothrombin activation. The data demonstrate that amino acid 334-335 from factor Va do not participate in the interaction with factor Xa but are rather required for the rearrangement of enzyme and substrate necessary for efficient catalysis.
2.2 Introduction.

The mainstay of the hemostasis process is the generation of thrombin, which in turn promotes the assembly of the fibrin plug following vascular injury. The prothrombinase complex is composed of the enzyme, factor Xa, and the protein cofactor, factor Va, associated in the presence of divalent metal ions on a membrane surface (1, 2). This complex catalyzes the activation of prothrombin to its active form, thrombin. Prothrombin circulates in blood as an inactive zymogen (Mr = 72,000) at a physiological concentration of 1.4µM. The enzyme, factor Xa, alone can activate prothrombin by two sequential proteolytic cleavages at Arg271 and Arg320 resulting in the intermediates, fragment 1•2 and prethrombin 2 (3, 4). The overall rate of this reaction is not compatible with survival. On the other hand, the incorporation on the cofactor, factor Va, into prothrombinase reverses the order of the proteolytic cleavages and increases the catalytic activity of factor Xa by 5 orders of magnitude (5). The first cleavage at Arg320, which is strictly dependent on the incorporation of factor Va into prothrombinase, produces the intermediate meizothrombin, which has reduced clotting activity compared to thrombin, but it has normal amidolytic activity. Further cleavage at Arg271 generates thrombin and fragment 1•2 (6, 7). The regulation of this key step in the blood coagulation cascade has been under intense investigation for many years.

Plasma factor V circulates as an inactive protein cofactor of Mr 330,000(8). The inactive zymogen protein cofactor, factor V, cannot participate in the prothrombinase complex and aid in prothrombin activation. Factor V must first be activated to factor Va by thrombin through 3 sequential proteolytic cleavages at Arg709, Arg1018, and
Arg^{1545} to produce a heavy chain, composed of amino acid residues 1-709 (M, 105,000) and a light chain composed of amino acid residues 1546-2196 (M, 74,000) (3). Both chains of factor Va have been shown to be required for interaction of the cofactor with factor Xa (9, 10). While the binding site(s) on the light chain remain to be identified, several binding sites for factor Xa on the factor Va heavy chain have been delineated. Griffin et al. have demonstrated that binding sites for factor Xa on factor Va are located within amino acid residues 311-325 (11) and 493-506 (12).

Using synthetic peptides we have demonstrated that amino acid region 323-331 of the factor Va heavy chain contain critical amino acid residues responsible for the direct interaction of the cofactor with factor Xa (13). Our laboratory’s initial results also demonstrated that amino acid sequence ^{332}IWDYA^{336}, which is continuous to the factor Xa binding site, was also involved in the inhibition of prothrombinase activity.

The present study was undertaken to understand the precise contribution of amino acid residues 332-336 of the factor Va heavy chain to the catalytic efficiency of prothrombinase.

After sufficient thrombin production for the formation of the fibrin clot, inactivation of the coagulation cascade commences. Factor Va is inactivated by APC in the presence of a membrane surface by three sequential cleavages at Arg^{306}, Arg^{506}, and Arg^{679} resulting in the dissociation of the A2 domain from the rest of the molecule (14, 15). Factor Va can also be inactivated by plasmin in the presence of a membrane surface. Plasmin is a serine protease with a broad range of substrates. In addition to the cleavage of fibrin, it also is capable of activation or inactivation of blood coagulation proteins. Plasmin cleaves factor Va at Lys^{309}, Lys^{310}, Arg^{313}, and
Arg\textsuperscript{348} which also results in the dissociation of the A2 domain of factor Va (Figure 4) (16). Further analysis of the inactivation fragments of factor Va by plasmin demonstrated that amino acid residues 307-348 are critical for factor Va cofactor function. Specifically, a peptide spanning this amino acid region is a good inhibitor of factor V/Va clotting activity (16). Additionally, this region of factor Va is also thought to be important for cofactor function since it is 82% conserved between human (17), bovine (18), mouse (19), and porcine (20).

To further understand the importance of amino acid region 307-348 of the factor Va heavy chain, eight overlapping peptides (designated AP1-AP8) were synthesized and characterized for inhibition of prothrombinase activity (Figure 2.1) (13). Studies showed that peptide AP4’, containing amino acid residues 323-331, is partially responsible for the interaction of factor Xa with factor Va. Accordingly, this peptide was studied extensively and it was determined that within these nine amino acids, some or all the residues are critical for the interaction of factor Va heavy chain with factor Xa in the prothrombinase complex. Furthermore, these residues are necessary for factor Va cofactor function (13). In addition, analysis of the peptides also revealed that under the experimental conditions employed (4nM factor Va and 10nM factor Xa pre-incubated with 100µM peptides), peptides AP3-AP6 were all found to inhibit prothrombinase activity (13). Particularly, it was found that amino acid region \textsuperscript{332}IWDYA\textsuperscript{336} might contribute to the inhibitory potential of prothrombinase activity. These studies lead to the hypothesis that a small amino acid region of factor Va heavy chain is enough to interact with factor Xa and increase its ability to activate prothrombin in the absence of the whole cofactor. This region includes amino acid
region $^{332}$IWDYA$^{336}$. In addition to kinetic data obtained with the peptides, structural analysis of factor Va has been performed. It was found that the amino acid residues under investigation are located on the surface of the molecule ($^{13}$).
We have recently demonstrated that amino acid region 307-348 of factor Va heavy chain is critical for cofactor function. This region was broken down into 8 overlapping peptides. Kinetic analysis revealed that amino acid region 323-331, designated AP4', is a noncompetitive inhibitor of prothrombinase with respect to thrombin. Thus, the peptide interferes with the Va-Xa interaction. In other words, AP4' contains a binding site for factor Xa on the heavy chain of the cofactor. In addition, amino acid region 317-326, designated as AP3, was found to be a non-competitive inhibitor of prothrombinase with respect to thrombin. (Figure taken from: Kalafatis, M and Beck, D.O. (2002) *Biochemistry* 41, 12715-12728.)

<table>
<thead>
<tr>
<th>Peptide</th>
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<td>TREQRRHK</td>
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<tr>
<td>AP8</td>
<td>NMDKKYRSQH</td>
</tr>
</tbody>
</table>

**Figure 2.1 Peptides From the Central Portion of Factor Va Heavy Chain.** We have recently demonstrated that amino acid region 307-348 of factor Va heavy chain is critical for cofactor function. This region was broken down into 8 overlapping peptides. Kinetic analysis revealed that amino acid region 323-331, designated AP4', is a noncompetitive inhibitor of prothrombinase with respect to thrombin. Thus, the peptide interferes with the Va-Xa interaction. In other words, AP4' contains a binding site for factor Xa on the heavy chain of the cofactor. In addition, amino acid region 317-326, designated as AP3, was found to be a non-competitive inhibitor of prothrombinase with respect to thrombin. (Figure taken from: Kalafatis, M and Beck, D.O. (2002) *Biochemistry* 41, 12715-12728.)
2.3 Experimental Procedures

2.3.1 Materials and Reagents

Diisopropyl-fluorophosphate (DFP), O-phenylenediamine (OPD)-dihydrochloride, N- [2-Hydroxyethyl] piperazine-N’-2-ethanesulfonic acid (Hepes), Trizma (Tris base), and Coomassie Blue R-250, were purchased from Sigma (St. Louis, MO). Factor V-deficient plasma was from Research Protein Inc. (Essex Junction, VT). Secondary anti-mouse and anti-sheep IgG coupled to peroxidase were purchased from Southern Biotechnology Associates Inc. (Birmingham, AL). L-α-phosphatidylserine (PS) and L-α-phosphatidylcholine (PC) were from Avanti Polar Lipids (Alabaster, AL). Chemiluminescent reagent ECL\(^+\) and Heparin–Sepharose were from AmershamPharmacia Biotech Inc. (Piscataway, NJ). Normal reference plasma and chromogenic substrate Spectrozyme-TH were from American Diagnostica Inc. (Greenwich, CT). Thromboplastin reagent was purchased from Organon Teknika Corp. (Durham, NC). EDC (1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide Hydrochloride) was from Pierce Biotechnology (Rockford, IL). Dansylarginine-N-(3-ethyl-1, 5-pentanediyl) amide (DAPA), human factor Xa, human thrombin, and human prothrombin was purchased from Haematologic Technologies Inc. (Essex Junction, VT). Factor V cDNA was from American Type Tissue Collection (ATCC# 40515 pMT2-V, Manassas, VA). All restriction enzymes were from New England Biolabs (Beverly, MA). All molecular biology and tissue culture reagents, specific primers, and medium were from Gibco, Invitrogen Corp. (Grand Island, NY) or as indicated. Human factor V monoclonal antibodies (αHFV\(_{IC}\)#17 and αHFV\(_{LC}\)#9) and
monoclonal antibody αHFV#1 coupled to Sepharose were provided by Dr. Kenneth G. Mann (Department of Biochemistry, University of Vermont, Burlington, VT).

2.3.2 EDC Cross-linking of Heavy Chain Peptides

To investigate if peptides from factor Va heavy chain, N42R, AP3, AP5, AP6, and IWDYA interact with factor Xa, chemical cross-linking with EDC (1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide Hydrochloride) was used. Factor Xa with the active site blocked, FXa-EGR, was incubated by itself or with a 25 molar excess of each peptide, in the presence of lipids and the chemical cross-linker, EDC, for two hours at ambient temperature (37°C). The protein was then separated by reducing SDS-PAGE, followed by staining with Coomassie Blue to visualize the heavy chain (Mr = 42,000) and the light chain (Mr = 16,5000) of factor Xa.

2.3.3 Inhibition by Factor Va Heavy Chain Peptides

The ability of the heavy chain peptides to inhibit prothrombinase function in the absence of factor Va was investigated in an assay with purified reagents. The peptides, P15H, AP5, AP5m, and IWDYA, were pre-incubated with factor Xa. The reaction mixture containing prothrombin (350nM), DAPA (750nM), and PCPS (10µM) was incubated in the dark for 20min, prior to the start of assays. At selected time intervals, an aliquot (1800µL) of the reaction mixture was added to the cuvette and the baseline was monitored on a Perkin-Elmer LS-50B Luminescence Spectrometer with λex = 280nm, λem = 550nm, and a 500nm long-pass filter in the emission beam. The data obtained were immediately analyzed with the software FL WinLab. The reaction was started upon the addition of factor Xa plus peptide (concentrations ranging from 0-500µM) to a final concentration of 10nM factor Xa.
The fluorescence intensity due to the formation of thrombin from activation of prothrombin and its complexation with DAPA was monitored with time. The results were plotted as % cofactor activity versus peptide concentration.

2.3.4 Prothrombin Activation by Heavy Chain Peptides Analyzed by Gel Electrophoresis

Peptides from the heavy chain of factor Va were assessed for their ability to activate prothrombin in the absence of factor Va. Plasma factor Va and the peptides, 50µM N42R, 100µM P15H, 100µM AP3, 100µM AP5, 100µM AP5m, 100µM AP6, 100µM IWDYA and factor Xa alone were pre-incubated with 1nM factor Xa, 20µM PCPS, and 3µM DAPA in TBS, Ca2+, pH 7.40 for 5 minutes at room temperature. A zero point was taken and 1.4µM prothrombin was added to start the reaction. Aliquots were removed at the following time points, 0.5, 1, 3, 5, 7, 10, 12, 15, 20, 30, 45, 60, 75, 90, 105, 120, 150, 180 diluted in two volumes of 0.2M glacial acetic acid. In order to compare prothrombin activation between the different peptides and plasma factor Va, time points were taken at the following times, 3min and 30min for plasma factor Va, and one hour for the peptides and factor Xa alone and diluted in two volumes of 0.2M glacial acetic acid. The samples were dried in a centrifrap and reconstituted in 0.1M Tris base, pH 6.8, 1% SDS, 1% β-mercaptoethanol and heated for exactly 75 seconds at 95°C. A total of 5µg of total protein was loaded per lane and analyzed by SDS-PAGE with a 9.5% acrylamide gel followed by staining with Coomassie Blue.
2.3.5 Construction of Recombinant Factor V Molecules

Recombinant factor \( V^{MI} \) and factor \( V^{FF} \) molecules (having the mutations 323-324 (E323F, Y324F) and 330-331 (E330M, V331I)) were constructed in a two-stage PCR method and characterized as previously described (21). Factor \( V^{KF} \) was obtained in the same manner; briefly, factor \( V^{KF} \) was constructed using the mutagenic primers, 5’-C ATT TGG \textbf{AAG} \textbf{TTT} GCA CCT G-3’ (forward) and 5’-C AGG TGC \textbf{AAA} \textbf{CTT} CCA AAT G-3’ (reverse) (bold underlined letters identify the mutated bases). The resulting amplicon was sub cloned into the vector pGEM-T. The PGEM-T plasmid was digested with restriction enzymes; \( Bsu361 \) and \( Xcm1 \), to remove the factor \( V \) insert and the insert was ligated into pMT2-FV at the same restriction sites. The remaining recombinant plasmids were constructed using Stratagene’s QuikChange® XL Site-Directed Mutagenesis Kit. Factor \( V^{AA} \) was constructed with the primers 5’-GAG GAA GTC ATT TGG \textbf{GCC} \textbf{GCC} GCA CCT GTA ATA- 3’ (forward) and 5’-TAT TAC AGG TGC \textbf{GGC} \textbf{GGC} CCA AAT GAC TTC CTC-3’ (reverse). Factor \( V^{MI/KF} \) was constructed with the primers 5’- GAA TAC TTC ATT GCT GCA GAG \textbf{ATG} GTC ATT TGG \textbf{AAG} \textbf{TTT} GCA CCT GTA ATA-3’ (forward) and 5’-TAT TAC AGG TGC \textbf{AAA} \textbf{CTT} CCA AAT GAC \textbf{CAT} CTC TGC AGC AAT GAA GTA TTC-3’ (reverse). Factor \( V^{FF/KF} \) was constructed with the primers 5’-CAG ATT TGG \textbf{TTT} TTC TT TCC ATG TGG GCA GAG GAA GTC ATT TGG \textbf{AAG} \textbf{TTT} GCA CCT GTA ATA-3’ (forward) and 5’-TAT TAC AGG TGC \textbf{AAA} \textbf{CTT} CCA AAT GAC TTC CTC TGC AGC AAT GAA \textbf{AAA} \textbf{CCA} AAT CTT CAT GTG-3’ (reverse). Factor \( V^{MI/AA} \) was constructed with the primers 5’- GAA TAC TTC ATT GCT GCA GAG \textbf{ATG} GTC ATT TGG \textbf{GCC} \textbf{GCC} GCA CCT GTA
ATA-3’ (forward) and 5’-TAT TAC AGG TGC **GAC** GGC CCA AAT GAC CAT CTC TGC AGC AAT GAA GTA TTC-3’ (reverse). Factor V^{FF/AA} was constructed with the primers 5’-CAC ATG AAG ATT TGG **TTT** TTC TTC ATT GCT GCA GAG GAA GTC ATT TGG **GCC GCC** GCA CCT GTA ATA-3’ (forward) and 5’-TAT TAC AGG TGC AA A **CTT** CCA AAT GAC TTC CTC TGC AGC AAT GAA GAC CAT CCA AAT CTT CAT GTG-3’ (reverse). Factor V^{I332A} was constructed with the primers 5’-GCT GCA GAG GAA GTC **GCC** TGG GAC TAT GCA CCT GTA ATA-3’ (forward) and 5’-TAT TAC AGG TGC ATA GTC CCA **AGC** GAC TTC CTC TGC AGC-3’ (reverse). Factor V^{W333A} was constructed with the primers 5’-GCT GCA GAG GAA GTC ATT **GCC** GAC TAT GCA CCT GTA ATA-3’ (forward) and 5’-TAT TAC AGG TGC ATA GTC **GCC** AAT GAC TTC CTC TGC AGC-3’ (reverse). The Scheme of the recombinant factor V molecules is shown in Figure 2.2. The mutations were confirmed by DNA sequencing (DNA Analysis Facility, Cleveland State University).

### 2.3.6 Transient Transfection of Recombinant Factor V Molecules

The COS-7L cell line was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and the antibiotics streptomycin (100µg/ml) and penicillin (100IU/ml) in an atmosphere of 5% CO₂, 95% air, and 37°C. The purified wild type and mutated plasmids will be transfected into the COS-7L cells with Fugene 6 (Roche Diagnostics) according to the manufacturer’s instructions. After 48 hours of incubation, the cells were washed twice with PBS buffer and 6ml of VP-SPM medium supplemented with 4mM L-glutamine will be added. Following 24 hours the medium was harvested and fresh VP-SPM medium...
was added. Harvesting of protein was repeated for 3-4 consecutive days and the harvest medium was stored at -80°C. The harvest medium was concentrated using a Cole Parmer, Masterflex L/S with MW=30,000 Vivaflow 50 membrane to a volume of 5-15mLs. Then, 2mM DFP was added and the protein was allowed to sit ice for half an hour to overnight before purifying the recombinant protein.

2.3.7 Purification of Recombinant Factor V Molecules

The concentrated recombinant protein was centrifuged at 5,000 rpm for 5 minutes to remove any cellular debris. The protein was purified on a 2ml column of monoclonal antibody αhFV#1 coupled to sepharose. The column was first equilibrated with TBS plus 5mM Ca\(^{2+}\), pH 7.40 (TBS, Ca\(^{2+}\)) (all buffers were filtered before use). The medium containing recombinant protein was added to the column and 0.5ml fractions were collected. The column was washed with 12ml of TBS, Ca\(^{2+}\) and eluted with 20mM Tris Base, 2M NH\(_3\)Cl, pH 7.40. The absorbance of the collected fractions was recorded at 280 nm on a HITACHI U-2000 spectrophotometer and clotting activity monitored cofactor activity. Fractions containing activity were pooled and dialyzed against TBS, Ca\(^{2+}\), pH 7.40 for 2 hours at 4°C. The purified protein was stored at -80°C in small aliquots to avoid repeated freeze thaw cycles. The activity and the integrity of the recombinant factor V molecules were confirmed by clotting assays using factor V deficient plasma and western blotting with monoclonal and polyclonal antibodies.
Figure 2.2 Schematic of recombinant factor V molecules from the central portion of the heavy chain. Model of factor V gene depicting the residues that have been mutated, E$^{323}$, Y$^{324}$, E$^{330}$, V$^{331}$, D$^{334}$, Y$^{335}$, I$^{332}$, and W$^{333}$. 
2.3.8 Determination of Factor Va Clotting Activity of the Recombinant Molecules

The cofactor activity of the recombinant molecules was measured in a clotting assay using factor V-deficient plasma following activation of the cofactor molecules by thrombin (10 min, 37°C). The values were measured on a ST art 4 Analyzer Coagulation Instrument (Diagnostica Stago, Parisippany, NJ) and the values were standardized to the percentage of control. A linear semi-log graph was created using known concentrations of plasma factor Va as a control and the specific activity of each recombinant factor Va molecule was calculated (units/mg).

2.3.9 Gel Electrophoresis and Western Blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses were carried out using 4-12% gradient gels or 9.5% gels following reduction with 2% β-mercaptoethanol according to the methods of Laemmli (1970). The protein was transferred to polyvinylidene difluoride (PVDF) membranes following the described method of Towbin et al. (1979). Factor Va heavy and light chains were probed with the appropriate antibodies and visualized with chemiluminescence; or, the protein was visualized by staining with Coomassie Brilliant Blue R-250, followed by destaining in a solution of methanol, acetic acid, and water.

2.3.10 Factor Va Titrations

The ability of the recombinant factor V molecules to assemble in the prothrombinase complex and bind to the enzyme was measured in a discontinuous assay described in detail elsewhere (22). In short, all recombinant factor V molecules were activated with thrombin (10 min. at 37°C). Reaction mixtures contained PCPS
vesicles (20µM), DAPA (3µM), factor Xa (varying concentration), and recombinant factor V species in Reaction Buffer (varying concentration) HEPES, 0.15M NaCl, 50nM CaCl₂, 0.01% Tween-20, pH 7.40). For the practical calculation of the $k_d$ between the factor Va molecules and factor Xa, assays were performed in the presence of limiting factor Xa concentration (15pM) and varying concentrations of the recombinant factor Va species (25pM to 5nM). A zero point was taken and the reaction was started upon the addition of 1.4µM prothrombin. At the following time points 10, 20, 30, and 60 seconds, aliquots of the reaction mixture were removed and diluted in 2 volumes quench solution (20nM HEPES, 0.15M NaCl, 50nM EDTA, 0.1% PEG 8000, pH 7.40) in a 96-well sample plate. The rate of thrombin generation was measured using a chromogenic substrate, Spectrozyme TH (0.4nM), which probes for thrombin generation. The initial rate of thrombin generation was analyzed with Prizm (GraphPad) software.

### 2.3.11 Prothrombin Titrations

The capability of the recombinant factor Va molecules to assemble into the prothrombinase complex, binding to the substrate prothrombin, and the rate of catalytic efficiency was measured. For the determination of the kinetic constants of prothrombinase assembly, $K_m$ and $k_{cat}$, experiments were executed with a limiting amount of factor Xa (5pM) in the presence of a fixed amount of the various recombinant factor Va molecules (10nM), PCPS vesicles (20µM), and DAPA (3µM) in Reaction Buffer. In order to compensate for the variation in the $k_d$ between the different recombinant molecules, the amount of factor Va needed to saturate factor Xa was calculated as described below. A zero point was taken and the reaction was
started with varying amounts of the substrate prothrombin (25nM to 4µM). Aliquots were removed at the time points 20, 40, 60, and 120 seconds and the reaction was stopped in 2 volumes Quench Buffer. The rate of thrombin generation was measured using a chromogenic substrate, Spectrozyme TH (0.4nM), which probes for thrombin generation. The initial rate of thrombin generation was analyzed with Prizm (GraphPad) software.

2.3.12 Calculation of Factor Xa Saturation by Recombinant Factor Va Molecules

To order to compare prothrombinase function assembled in the presence of the various mutant recombinant molecules we have first calculated the $k_d$ of each species for factor Va as described above by titrating a fixed amount of factor Xa with increased concentrations of factor Va as described above. Once the value of the $k_d$ of the interaction of each species of factor Va with factor Xa was determined, the amount of recombinant factor Va required to completely saturate factor Xa and provide similar amount of enzyme (prothrombinase) when using various factor Va recombinant molecules (between 95% - >99% saturation) can be calculate as abundantly described in the literature (22). Briefly, the $k_d$ for the factor Va-factor Xa interaction is given by the equation:

$$K_d = \frac{[Va_F] \cdot [Xa_F]}{[Va \cdot Xa]}$$ (1)

The maximum amount of factor Xa saturated with factor Va formed under the conditions used can be verified by replacing in equation (1) $[Va_F]$ and $[Xa_F]$ by:

$$[Va_F] = [Va_T] - [Va \cdot Xa]$$ (2)

$$[Xa_F] = [Xa_T] - [Va \cdot Xa]$$ (3)
where, \([Va]_F \) and \([Xa]_F \) are the concentrations of free factor Va and factor Xa, while
\([Va]_T \) and \([Xa]_T \) are the total concentrations of factor Va and factor Xa respectively. The final concentration of factor Xa within the mixture was 1nM and the maximum amount of factor Xa saturated with factor Va formed under the above conditions was extrapolated from the following equation directly.

\[
X_{a\text{bound}} = \frac{\left[ n \cdot [Va]_T + [Xa]_T + K_d \right] - \sqrt{\left[ n \cdot [Va]_T \right]^2 + [Xa]_T^2 + K_d^2} - 4 \cdot n \cdot [Va]_T \cdot [Xa]_T}{2}
\]

(4)

with the \(K_d \) for the bimolecular interaction between factor Va and factor Va on a phospholipid surface calculated from the functional titration described above and the concentration of \([Va]_T \) modified as appropriate (53). Throughout all experiments the assumption was \( n = \) moles of factor Xa bound/mole of factor Va at saturation; throughout this study \( n = 1 \); the stoichiometry of the factor Va-factor Xa interaction was fixed at 1.

2.3.13 Calculation of Additive Effect of the Mutations on Prothrombinase Function

The change in transition-state stabilization free energy, which measures the effect of the mutations in the cofactor of the prothrombinase complex have on the catalytic site of the enzyme, was calculated for the double mutants as extensively described previously (22). In brief, the perturbation to the function of prothrombinase assembled with wild type factor Va (state A) caused by a mutation in factor Va (state B) affecting the transition state can be defined in general as follows:

\[
\Delta\Delta G_B = \Delta G_B - \Delta G_A \quad \text{(Eq. 1)}
\]
and since prothrombinase activity (assembled with each of the recombinant factor Va proteins) is being measured against the same substrate (prothrombin), the transition-state stabilization free energy ($\Delta \Delta G_{A \rightarrow B}^{\dagger}$) during catalysis induced by a mutation in factor Va can be determined from the following equation:

$$\Delta \Delta G_{A \rightarrow B}^{\dagger} = \frac{RT \ln ((k_{\text{cat}}/K_m)_B/(k_{\text{cat}}/K_m)_A)}{2}$$

(Eq. 2)

where $R$ is the universal gas constant ($2 \text{ cal} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$), $T$ is the absolute temperature ($298 \text{ K}$ in the experiments presented here), $k_{\text{cat}}$ is the turnover number, and $K_m$ is the Michaelis Menten constant of the reaction. Once calculated, these values can be used to determine $\Delta \Delta G_{\text{int}}$, which reflects the exchange in free energy between the amino acids side chains manipulated in this study (E323F, Y324F, E330M, V331I, D334K, and Y335F) by the following equation:

$$\Delta \Delta G_{\text{int}} = \Delta \Delta G_{A \rightarrow B/C}^{\dagger} - (\Delta \Delta G_{A \rightarrow B}^{\dagger} + \Delta \Delta G_{A \rightarrow C}^{\dagger})$$

(Eq. 3)

A positive value of $\Delta \Delta G_{\text{int}}$ indicates that the interaction of these amino acid side chains reduce the catalytic efficiency of prothrombinase, negative value demonstrates that the mutations are better for prothrombinase, resulting in an increase in the catalytic efficiency of the enzyme. A value of zero would indicate no effect.

2.3.13 Prothrombin Activation Measured by Gel Electrophoresis

Prothrombin was incubated in a reaction mixture containing the following: 20µM PCPS, 50µM DAPA, and 10nM of factor Va$^{\text{WT}}$, factor Va$^{\text{KF}}$, and factor Va$^{\text{AA}}$ and 30nM of factor Va$^{\text{FF/KF}}$, factor Va$^{\text{MI/KF}}$, factor Va$^{\text{FF/AA}}$, and factor Va$^{\text{MI/AA}}$ (activated with thrombin) in TBS, Ca$^{2+}$. A zero point was taken and the addition of 1 nM factor Xa marked the start of the reaction. Aliquots of the reaction mixture will be removed at selected time points (0:20, 0:40, 1:00, 1:20, 1:40, 2:00, 2:20, 2:40, 3:00, 3:20,
3:40, 4:00, 5:00, 6:00, 10:00, 20:00, 30:00 and 60:00 minutes) and added to two volumes 0.2M glacial acetic acid. The samples will be dried in a centrivap and reconstituted in 0.1M Tris base, pH 6.8, 1% SDS, 1% β-mercaptoethanol and heated for exactly 75 seconds at 95°C. A total of 5µg of total protein will be loaded per lane and analyzed on 9.5% SDS-PAGE followed by staining with Coomassie Blue.
2.4 Results

2.4.1 Inhibition of Prothrombinase Function by Synthetic Peptides from the Central Portion of Factor Va Heavy Chain.

Our laboratory has previously shown that a peptide representing amino acid residues 323-331 inhibits factor Va cofactor activity (13). We have extensively characterized the important amino acids from this region by site directed mutagenesis (21). Our research group has also shown that under the experimental conditions employed, while peptides encompassing region 327-336 (AP5) and 332-341 (AP6) of factor Va heavy chain also inhibit factor Va cofactor activity, the pentadecapeptide P15H (representing amino acids 337-351 of factor V) did not have any effect on factor Va activity and was a good negative control (13). Our laboratory have thus hypothesized that the overlapping sequence between AP5 and AP6 (amino acid motif 332IWDYA336) may contain amino acids responsible for the inhibition of prothrombinase.

To investigate if the peptides from the heavy chain of factor Va, N42R, AP3, AP5, and AP6 were able to directly interact with factor Xa, chemical cross-linking with EDC was used. Active site blocked, factor Xa (factor Xa-EGR) was incubated with a 25 molar excess of the peptides in the presence of lipids and the cross-linker EDC. The results show in Figure 2.3, that the peptide N42R was able to bind to both the heavy chain (M_r = 42,000) and the light chain (M_r = 16,5000) of factor Xa in a spot outside of the active site of the enzyme (lane one depicts factor Xa-EGR alone, and lane two shows factor Xa-EGR plus N42R). Under the same experimental conditions, the peptides AP4, AP5, and AP6 did not display the chemical cross-
linking with EDC due to the low molecular weight of the peptides there was not enough of a difference in the molecular weight between factor Xa-EGR and factor Xa-EGR-cross-linked (data not shown). These results demonstrates that peptides from the heavy chain of factor Va are able to directly bind to factor Xa in a spot distant from the active site of the enzyme.

To investigate the role of these amino acids as it pertains to prothrombinase assembly and function, we have initiated a series of experiments using peptides from this region. The peptides were assayed for their ability to inhibit prothrombinase activity in assay that uses purified reagents and a fluorescent thrombin inhibitor (Figure 2.4). The data demonstrate that AP5 inhibits prothrombinase with an IC<sub>50</sub> of 15 µM (Figure 2.4, filled diamonds) while the control peptide (P15H) had no effect on prothrombinase activity under the conditions employed even at concentrations as high as 500 µM (Figure 2.4, closed circles). Complete inhibition of prothrombinase by AP5 occurred at 200 µM peptide. The K<sub>i</sub> of inhibition calculated from the IC<sub>50</sub> was 8 µM.

In view of these results we have synthesized an additional peptide (IWDYA, I5A) and assayed its capability to inhibit prothrombinase activity. I5A inhibited cofactor activity, although not completely. Even at 500 µM peptide there was still approximately 25% cofactor activity remaining (Figure 2.4, closed squares). The data demonstrated that AP6 and IWDYA have similar K<sub>i</sub> values of 64 µM and 69 µM respectively. The similar K<sub>i</sub> values between AP6 and IWDYA suggest that the inhibitory effect of the peptides is caused by the shared amino acid sequence<sup>332IWDYA<sup>336</sup>. To understand the effect of the amino acid sequence of this
pentapeptide on cofactor activity we have synthesized another peptide with the sequence DY replaced by KF (AP5\textsuperscript{DY→KF}, AP5m) and assayed the peptide for prothrombinase inhibition. AP5m had only a weak effect on prothrombinase function with ~60% cofactor activity remaining at concentrations of peptide as high as 500 µM (Figure 2.4, closed triangles), with a K\textsubscript{i} value of ~530 µM. These data indicate that residues 334-335 are important for the expression of the inhibitory potential of AP5. Taken together, the results indicate that 1) peptide AP5 from the central portion of the factor Va heavy chain is a potent inhibitor of factor Va cofactor activity; and 2) residues 334-335 from factor Va heavy chain have a crucial role for optimum expression of prothrombinase activity.

Next, the ability of the peptides to accelerate the activation of prothrombin by factor Xa was investigated. The peptides were pre-incubated with prothrombin, DAPA, and lipids in TBS, Ca\textsuperscript{2+}, pH 7.40. Factor Xa was used to start the reaction and the peptides were analyzed over a three-hour time course as described in Experimental Procedures. Control experiments with plasma factor Va and factor Xa alone show the expected results. Factor Xa alone cleaves first at Arg\textsuperscript{271} showing the intermediates Fragment 1.2 and Prethrombin-2 followed by cleavage at Arg\textsuperscript{320} producing thrombin (Figure 2.5) and the addition of factor Va reverses the order of these cleavages, cleaving first at Arg\textsuperscript{320} to produce the intermediate meizothrombin followed by cleavage at Arg\textsuperscript{271} to produce thrombin (Figure 2.5). The addition of the control peptide, P15H, does not have an effect on prothrombin activation as compared to factor Xa alone (Figure 2.5). Addition of the heavy chain peptides, N42R and AP3 show an increase in prothrombin disappearance and thrombin generation as compared
Figure 2.3 EDC Cross-Linking. To investigate if a peptide from factor Va heavy chain, N42R, interacts with factor Xa, chemical cross-linking with EDC was used. Factor Xa with the active site blocked, FXa-EGR, was incubated by itself (Lane 1) or with a 25 molar excess of N42R (Lane 2), in the presence of lipids. Reducing SDS-PAGE, followed by staining with Coomassie Blue, then separated the protein. The results show that N42R was cross-linked to FXa-EGR on both the heavy ($M_r = 42,000$) and light chains ($M_r = 16,500$).
**Figure 2.4 Peptide Inhibition.** Increasing concentrations of AP5, P15H, I5A, and AP5^{DY→KF} were preincubated with factor Xa and assayed for prothrombinase activity as described in the “Experimental Procedures” section. The final concentration of factor Xa in the mixture was 10 nM. P15H ({filled circles}) represents the control peptide, amino acids 337-351 of human factor Va heavy chain. AP5 ({filled diamonds}) represents amino acid region 327-336. AP5^{DY→KF} ({filled triangles}) represents amino acid residues 327-336 with residues 334-335 mutated from DY to KF. IWDYA ({filled squares}) is amino acid residues 332-336 of human factor Va heavy chain. The concentration of peptide given on the x axis represents its final concentration in the prothrombinase mixture. The data represent the average of the results found in three independent experiments. The apparent inhibition constant (K_i) reported in the text is the value calculated from the formula: IC_{50}=K_i(1+S_0/K_m), where K_m is the Michaelis-Menten constant of the reaction in the absence of inhibitor, S_0 is the concentration of prothrombin used, and IC_{50} is the half maximal inhibition of prothrombinase by a given peptide.
<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC$_{50}$ (µM)</th>
<th>$K_i$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP5</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>AP5$_{DY→KF}$</td>
<td>1000</td>
<td>530</td>
</tr>
<tr>
<td>AP6</td>
<td>120</td>
<td>64</td>
</tr>
<tr>
<td>IWDYA</td>
<td>130</td>
<td>69</td>
</tr>
</tbody>
</table>

**Table 2.1 Peptide Inhibition Constants.** The IC$_{50}$ values for AP5, AP5$_{DY→KF}$, AP6, and IWDYA were calculated from inhibition experiments (Figure 2.3). The IC$_{50}$ value is the concentration of the peptide at 50% inhibition.
to factor Xa alone. In addition, the peptides AP5 and AP6 also show an increase in prothrombin activation, but not to the extent of N42R and AP3 (Figure 2.5). On the other hand, AP5m, which as the DY→KF substitution, does not show any increase in prothrombin activation as compared to factor Xa alone; indicating that residues 334DY335 are important to factor Va cofactor activity (Figure 2.5). In order to compare the prothrombin activation scheme by the different heavy chain peptides, one time point was chosen for each peptide and control and the results were run on the same gel (Figure 2.6). The results clearly show that peptides from the factor Va heavy chain, N42R and AP3, accelerate Pathway I of prothrombin cleavage compared to factor Xa alone; demonstrated by increased Prethrombin-2 accumulation indicative of first cleavage at Arg271. The factor Va reaction displays the expected fragments of prothrombin activation following Pathway II, accumulation of Fragment 1.2A (meizothrombin under reducing conditions) followed by thrombin generation. This result suggests that small portion of factor Va represented by the peptides analyzed here increases the activity of the enzyme, factor Xa, alone and that it must be another portion of the cofactor that accelerates the factor Va directed prothrombin activation pathway.

2.3.3 Confirmation Amino Acid Residues 334-335 are the Important Residues From Region 332-336 From Factor Va Heavy Chain.

To ascertain that amino acid residues 334-335 from the IWDYA peptide are responsible for the effect observed, recombinant molecules were made at residues 332 (factor V1332A) and 333 (factor V333A). These recombinant molecules were assessed for their ability to assemble in the prothrombinase complex and bind the enzyme,
Figure 2.5 Prothrombin Activation by Factor Va Heavy Chain Peptides. The peptides (50µM N42R, 100µM P15H, 100µM AP3, 100µM AP5, 100µM AP6, and 100µM AP5m) and plasma factor Va (10nM) were allowed to pre-incubate with factor Xa (1nM) in the presence of PCPS (20µM) and DAPA (3µM) and the reaction was started by the addition of prothrombin (1.4µM) as described in “Experimental Procedures”. Each experiment was done in triplicate with different dilutions of peptides and one representative gel is shown.
Figure 2.6 Comparing Prothrombin Activation Between Factor Va Heavy Chain Peptides. Plasma factor Va and the peptides, 50µM N42R, 100µM P15H, 100µM AP3, and factor Xa alone were pre-incubated with 1nM factor Xa, 20µM PCPS, and 3µM DAPA in TBS, Ca2+, pH 7.40 for 5 minutes at room temperature. Aliquots were removed at the following time points, 3min and 30min for plasma factor Va, and one hour for the peptides and factor Xa alone as described in “Experimental Procedures”.
factor Xa, in a discontinuous assay. The results show similar $k_d$ values for factor V$^{I332A}$ and factor V$^{W333A}$, indicating these residues are not involved in the binding of the cofactor to the enzyme in the prothrombinase complex (Figure 2.7, Table 2.2). Next, the capability of factor V$^{I332A}$ and factor V$^{W333A}$ to assemble in the prothrombinase complex and bind the substrate, prothrombin, and the catalytic efficiency was measured in a discontinuous assay. Similarly, these results show that these residues are not involved in substrate binding as indicated by similar $K_m$ values and catalytic efficiencies (Figure 2.8, Table 2.2). Taken together, these results indicate that amino acid residues 332-333 from the central portion of factor Va heavy chain, specifically within the peptide IWDYA, are not involved in cofactor function. Thus, it can be deduced that it is definitely amino acid residues 334-335 that are important for the expression of inhibitory potential of amino acid region IWDYA.

2.4.3 Transient Expression and activation of recombinant human factor V molecules.

We next employed a recombinant protein scheme to further evaluate the importance of amino acid residues 334-335 of factor Va heavy chain to cofactor activity. We used one charge reversal mutation (D334K) and one conservative mutation (Y335F) to obtain recombinant factor V$^{KF}$. In addition, we also mutated residues 334-335 to alanines. We have also combined these resulting recombinant mutant molecules with previously characterized recombinant proteins, factor V$^{MI}$ and factor V$^{FF}$ that are deficient in factor Xa binding. We have thus obtained the quadruple mutants, factor V$^{MI/KF}$, factor V$^{FF/KF}$, factor V$^{MI/AA}$, and factor V$^{FF/AA}$ (21). We first analyzed the recombinant proteins for clotting activity and the results are
shown in Table 2.3. In a two-stage clotting assay, recombinant wild type factor Va displayed normal clotting times, compared to plasma derived factor Va at a concentration of 2.5nM (580-700 U/mg). Conversely, while factor Va^KF^ and factor Va^AA^ had a reduced clotting activity under similar experimental conditions (57-100 U/mg), the quadruple mutants, factor Va^{MI/KF}, factor Va^{FF/KF}, factor Va^{MI/AA}, and factor Va^{FF/AA} were severely impaired in their clotting activities (≤8 U/mg). These results demonstrate that while mutation of amino acids residues 334-335 severely impairs clotting activity, these mutations alone are not enough to completely abolish factor Va clotting activity. However, combining these mutations with mutations at the extremities of the previously described factor Xa binding site (22), results in cofactor molecules that are deficient in their clotting activity.

2.4.4 The Effect of Recombinant Factor Va molecules on Prothrombin Activation by prothrombinase.

The capability of the recombinant factor Va molecules to incorporate into prothrombinase and activate prothrombin was investigated by gel electrophoresis. Prothrombinase assembled with purified recombinant wild type factor Va displayed the normal activation pattern of prothrombin by the prothrombinase complex as compared to plasma derived factor Va (Figure 2.9, top panel) with initial cleavage at Arg^{320} producing fragment 1•2-A. This fragment is rapidly consumed following cleavage at Arg^{371} to produce fragment 1•2 and the active product, thrombin. Activation of prothrombin by prothrombinase assembled with purified factor Va^KF^ and factor Va^AA^ showed delayed thrombin formation as evidenced by delayed formation fragment 1•2-A and B chain of thrombin. Even after the one-hour time
Figure 2.7: Factor Va Titrations to determine the affinity of the recombinant factor Va molecules for factor Xa. Thrombin generation experiments were carried out as described under “Experimental Procedures”. Prothrombinase complex assembled with varying concentrations (30 pM to 5 nM) of recombinant purified wild type factor Va is depicted by filled squares, purified recombinant factor Va<sup>1332A</sup> by filled inverted triangles, and purified factor Va<sup>W333A</sup> by filled circles. The solid lines represent a nonlinear regression fit of the data using Prizm GraphPad software for a one binding site model. Titrations were performed in triplicate with at least four different preparations of purified and partially purified protein.
Figure 2.8 Prothrombin Titrations to determine the kinetic parameters of prothrombinase assembled with the various recombinant factor Va species. Thrombin generation experiments were carried out as described under “Experimental Procedures” by varying the substrate concentration (25 nM to 4 μM) with 5 pM of factor Xa saturated with the factor Va species. Prothrombinase complex assembled with recombinant purified wild type factor Va (filled squares), purified recombinant factor VaI332A (filled inverted triangles), and purified factor VaW333A (filled circles). The data shown are the average of five different titrations performed in triplicate with at least four different preparations of purified and partially purified proteins.
### Table 2.2 Kinetic Constants

The dissociation constants of recombinant factor Va species for plasma derived factor Xa were calculated at limiting concentrations of the enzyme as described in “Experimental Procedures”. The $K_m$ constants were determined at described in “Experimental Procedures” using limiting amounts of the enzyme and varying concentrations of substrate.

<table>
<thead>
<tr>
<th>Factor Va Species</th>
<th>$K_d$ (nM)</th>
<th>$K_m$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor Va$^{WT}$</td>
<td>0.267</td>
<td>0.228</td>
</tr>
<tr>
<td>Factor Va$^{I332A}$</td>
<td>0.331</td>
<td>0.397</td>
</tr>
<tr>
<td>Factor Va$^{W333A}$</td>
<td>0.368</td>
<td>0.348</td>
</tr>
</tbody>
</table>
point the fragment 1•2-A still persist (Figure 2.9, middle and bottom panel, respectively). Under similar experimental conditions factor $V_{a}^{M_{I/KF}}$, factor $V_{a}^{F_{F/KF}}$, factor $V_{a}^{M_{I/AA}}$, and factor $V_{a}^{F_{F/AA}}$ were unable to activate prothrombin, even following a 3-hour incubation period (Figure 2.10). These results indicate that substitution of amino acid residues 334-335 of the factor Va heavy chain has a damaging consequence on factor Va cofactor activity, resulting in delayed prothrombin activation by factor Xa within prothrombinase. Overall these results indicate that amino acid residues 334-335 are essential for optimal conditions of prothrombin activation by prothrombinase.

2.4.5 Kinetic Analyses of Prothrombinase assembled with Recombinant Factor Va.

The capability of the recombinant factor Va molecules to be incorporated in prothrombinase was investigated using an assay employing purified reagents. We have purified the recombinant wild type cofactor and mutants factor $V_{a}^{K_{F}}$ and factor $V_{a}^{A_{A}}$ and compared the kinetic data obtained with these molecules to the data obtained with prothrombinase assembled with the partially purified factor Va molecules following activation by thrombin. No significant differences were observed when comparing the numbers obtained with the two different recombinant factor Va molecules (Figure 2.11, Table 2.4). These data demonstrate that the partially purified cofactor displays similar kinetic behavior as the purified cofactor molecule. Under similar experimental conditions, purified factor $V_{a}^{K_{F}}$ and factor $V_{a}^{A_{A}}$ displayed a comparable $K_D$ value to the wild type molecule (Table 2.3).
<table>
<thead>
<tr>
<th>Species</th>
<th>Two-Stage Clotting Time</th>
<th>Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor Va\textsuperscript{plasma}</td>
<td>15.5±1.10</td>
<td>698</td>
</tr>
<tr>
<td>Factor Va\textsuperscript{WT}</td>
<td>18.6±0.81</td>
<td>597</td>
</tr>
<tr>
<td>Factor Va\textsuperscript{KF}</td>
<td>34.5±1.23</td>
<td>68</td>
</tr>
<tr>
<td>Factor Va\textsuperscript{AA}</td>
<td>32.1±1.36</td>
<td>79</td>
</tr>
<tr>
<td>Factor Va\textsuperscript{FF/KF}</td>
<td>59.6±2.36</td>
<td>≤8</td>
</tr>
<tr>
<td>Factor Va\textsuperscript{FF/AA}</td>
<td>56.3±1.78</td>
<td>≤8</td>
</tr>
<tr>
<td>Factor Va\textsuperscript{MI/KF}</td>
<td>62.1±3.12</td>
<td>≤8</td>
</tr>
<tr>
<td>Factor Va\textsuperscript{MI/AA}</td>
<td>60.4±2.65</td>
<td>≤8</td>
</tr>
</tbody>
</table>

**Table 2.3 Clotting Times and Activities of Recombinant Factor Va Molecules.**

The clotting times and activity were determined as described in ‘Experimental Procedures’. Factor V-deficient plasma was used and the recombinant proteins were analyzed at a final concentration of 2.5nM.
Figure 2.9 Prothrombin Activation Analysis by Gel Electrophoresis. Recombinant factor Va molecules, factor Va\textsuperscript{WT}, factor Va\textsuperscript{KF}, and factor Va\textsuperscript{AA} were incubated with prothrombin, PCPS vesicles, and DAPA as detailed in the “Experimental Procedures” section at a final concentration of 10 nM. Factor Xa was added to start the reaction to a final concentration of 1 nM. Aliquots were withdrawn at given time intervals and treated as described in the “Experimental Procedures”. The legends to the right of the gels indicate the prothrombin activation fragments: II (prothrombin), F1•2A (Fragment 1•2-A chain), F1•2 (fragment 1•2), and B (B chain of thrombin). Experiments were performed with least three preparations of purified proteins and one representative gel is shown.
Figure 2.10 Prothrombin Activation Analysis by Gel Electrophoresis With The Quadruple Mutants. Recombinant factor Va molecules, factor Va\(^{\text{WT}}\), factor Va\(^{\text{FF/KF}}\), factor Va\(^{\text{FF/AA}}\), factor Va\(^{\text{FF/AA}}\), and factor Va\(^{\text{MI/AA}}\) were incubated with prothrombin, PCPS vesicles, and DAPA as detailed in the “Experimental Procedures” section at a final concentration of 30 nM. Factor Xa was added to start the reaction to a final concentration of 1nM. Aliquots were withdrawn at given time intervals and treated as described in the “Experimental Procedures”. The legends to the right of the gels indicate the prothrombin activation fragments: II (prothrombin), F1•2A (Fragment 1•2-A chain), F1•2 (fragment 1•2), and B (B chain of thrombin). Experiments were performed with at least three preparations of purified proteins and one representative gel is shown.
Conversely, the quadruple mutant recombinant factor Va molecules showed significant decreases in the $k_d$ values. The affinities of factor $V_{a}^{\text{MI/KF}}$, factor $V_{a}^{\text{FF/KF}}$, factor $V_{a}^{\text{MI/AA}}$, and factor $V_{a}^{\text{FF/AA}}$ were approximately 6-fold, 14-fold, 19-fold, and 10-fold respectively lower than the affinity of the wild type cofactor molecule for factor Xa. These latter data are in good agreement with our recent results, and demonstrate that amino acids 334-335 do not participate in the interaction of the cofactor with factor Xa (21).

Figure 2.12 shows the initial steady-state rate of prothrombin activation as a function of substrate concentration. The average kinetic constants obtained in several experiments using at least four different preparations of each recombinant cofactor molecule, are provided in Table 2.3. The data show that all mutant recombinant factor Va species have a small but significant effect on the $K_m$ of the reaction as compared to the wild type cofactor (Figure 2.12 and Table 2.4). While our recent data using prothrombinase assembled with factor $V_{a}^{\text{FF}}$, factor $V_{a}^{\text{MI}}$, and factor $V_{a}^{\text{FF/MI}}$ demonstrated similar $K_m$ values to the data obtained with prothrombinase assembled with the plasma and the wild type recombinant factor Va molecules (~0.15 µM, ref), all the data obtained using the mutant factor Va molecules containing the $334\text{DY}^{335}\rightarrow \text{KF}$ substitution, have a 4-fold higher $K_m$ value than prothrombinase assembled with the wild type cofactor (Table 2.4). The data suggest that prothrombinase assembled with these mutant molecules is impaired in its interaction with the substrate. Furthermore, prothrombinase assembled with purified factor $V_{a}^{\text{KF}}$ and factor $V_{a}^{\text{AA}}$ was characterized by a ~1.6-fold and 2-fold decrease, respectively in the $k_{cat}$ of the reaction (Figure 2.13). Furthermore, prothrombinase assembled with
Figure 2.11 Factor Va Titrations to determine the affinity of the recombinant factor Va molecules for factor Xa. Thrombin generation experiments were carried out as described under “Experimental Procedures”. Prothrombinase complex assembled with varying concentrations (30 pM to 5 nM) of recombinant partially purified wild type factor Va (filled squares), purified recombinant factor Va\textsuperscript{WT} (open squares), partially purified factor Va\textsuperscript{KF} (filled triangles), purified factor Va\textsuperscript{KF} (open triangles), purified factor Va\textsuperscript{AA} (open circle), partially purified factor Va\textsuperscript{FF/KF} (open inverted triangle), partially purified Va\textsuperscript{ML/KF} (filled diamonds), partially purified factor Va\textsuperscript{MI/AA} (filled circles), and partially purified factor Va\textsuperscript{FF/AA} (open diamonds). The solid lines represent a nonlinear regression fit of the data using Prizm GraphPad software for a one binding site model. Titrations were performed in triplicate with at least four different preparations of purified and partially purified protein.
Figure 2.12 Prothrombin Titrations to determine the kinetic parameters of prothrombinase assembled with the various recombinant factor Va species. Thrombin generation experiments were carried out as described under “Experimental Procedures” by varying the substrate concentration (25 nM to 4 µM) with 5 pM of factor Xa saturated with the factor Va species. Prothrombinase complex assembled with recombinant partially purified wild type factor Va (filled squares), purified recombinant factor Va<sup>WT</sup> (open squares), partially purified factor Va<sup>KF</sup> (filled triangles), purified factor Va<sup>KF</sup> (open triangles), purified factor Va<sup>AA</sup> (open circle), partially purified factor Va<sup>FF/KF</sup> (open inverted triangle), partially purified Va<sup>M/KF</sup> (filled diamonds), partially purified factor Va<sup>M/AA</sup> (filled circles), and partially purified factor Va<sup>FF/AA</sup> (open diamonds). The data shown are the average of five different titrations performed in triplicate with at least four different preparations of purified and partially purified proteins.
Table 2.4 Kinetic Constants of Recombinant Factor Va Molecules. The dissociation constants of recombinant factor Va species for plasma derived factor Xa were calculated at limiting concentrations of the enzyme as described in “Experimental Procedures”. The $K_m$ constants were determined at described in “Experimental Procedures” using limiting amounts of the enzyme and varying concentrations of substrate.

<table>
<thead>
<tr>
<th>Factor Va Species</th>
<th>$K_d$ (nM)</th>
<th>$K_m$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor Va\textsuperscript{WT}</td>
<td>0.238</td>
<td>0.164</td>
</tr>
<tr>
<td>Factor Va\textsuperscript{WT} (P)</td>
<td>0.389</td>
<td>0.134</td>
</tr>
<tr>
<td>Factor Va\textsuperscript{KF}</td>
<td>0.554</td>
<td>0.577</td>
</tr>
<tr>
<td>Factor Va\textsuperscript{KF} (P)</td>
<td>0.574</td>
<td>0.420</td>
</tr>
<tr>
<td>Factor Va\textsuperscript{AA} (P)</td>
<td>0.598</td>
<td>0.625</td>
</tr>
<tr>
<td>Factor Va\textsuperscript{FF/KF}</td>
<td>1.29</td>
<td>0.661</td>
</tr>
<tr>
<td>Factor Va\textsuperscript{FF/AA}</td>
<td>2.51</td>
<td>0.215</td>
</tr>
<tr>
<td>Factor Va\textsuperscript{MI/KF}</td>
<td>5.49</td>
<td>0.593</td>
</tr>
<tr>
<td>Factor Va\textsuperscript{MI/AA}</td>
<td>4.56</td>
<td>0.374</td>
</tr>
</tbody>
</table>
the quadruple mutant molecules, factor \( \text{Va}^{\text{MI/KF}} \) and factor \( \text{Va}^{\text{FF/KF}} \), demonstrated a significant decrease in the \( k_{\text{cat}} \) of the enzyme with prothrombinase assembled with factor \( \text{Va}^{\text{MI/KF}} \) always being the most impaired in its catalytic efficiency (Table 2.4). Overall the data demonstrate that substitution of amino acids 334-335 in factor Va has a profound effect on the catalytic efficiency of prothrombinase, decreasing the second order rate constant of the reaction by ~6-fold. The inability of prothrombinase assembled with factor \( \text{Va}^{\text{KF}} \) to function optimally can be explained by both the inability of factor Xa to efficiently convert prothrombin to thrombin because of diminished productive collisions (difference in \( k_{\text{cat}} \)) and because of impaired prothrombinase-substrate interaction (increase in the \( K_m \)).

2.4.6 Additive Effect of the mutations on prothrombin catalysis.

Prothrombinase is composed of factor Va and factor Xa assembled on a membrane surface in the presence of divalent metal ions. We can thus assume that prothrombinase is an enzyme composed of two subunits: a catalytic subunit (factor Xa) and a regulatory subunit (factor Va). Any perturbation in the interaction between the two subunits or any perturbations in the interaction of prothrombinase with the substrate caused by a mutation may influence (modify) the stability of the catalytic site of the enzyme. The kinetic data showed that combining the DY→KF mutations in the regulatory subunit of prothrombinase with the mutations in either of the other two factor Xa-binding sites previously described result in a dramatic decrease of the catalytic efficiency of prothrombinase. Thus, the consequences of mutations in factor Va affecting factor Xa catalytic efficiency and their additivity can be measured relative to the change in transition-state stabilization free energy (\( \Delta \Delta G^\\) of the
Figure 2.13 Graphical Representation of Catalytic Efficiencies of the Recombinant Factor Va Molecules. The values of $k_{\text{cat}}$ were determined using the $k_d$'s and the quadratic equation to determine the saturation concentration of the various recombinant factor Va species.
Figure 2.14 Graphical Representation of Second Order Rate Constants of Recombinant Factor Va Molecules. The second order rate constants were determined by dividing the $k_{\text{cat}}$ by the $K_m$. 
enzyme as previously established (22). To quantify the interaction between the mutations and their synergistic effects on prothrombinase function, we have calculated the difference in free energy of the transition state analog (ΔΔG_int) for each of the double mutant according to Equations 2 and 3 (described in the experimental procedures section and we have also constructed a thermodynamic cycle for cleavage of prothrombin by prothrombinase (Figure 2.14). The positive value of ΔΔG_int for the combination of mutations indicates that mutation of amino acids in the heavy chain of factor Va are disruptive and detrimental to the catalytic activity of prothrombinase. Moreover, the nearly 2-fold increase in the ΔΔG_int for prothrombinase assembled with factor Va^{Ml/KF} compared with the ΔΔG_int obtained for prothrombinase assembled with factor Va^{FF/KF} is in agreement with the kinetic data, and our recent results denoting the critical contribution of amino acids 330-331 to prothrombinase assembly and function (21).
Figure 2.15 Additive Effect of Recombinant Proteins on Prothrombin Catalysis.

$\Delta \Delta G_{\text{int}}$ is the energy of interaction between the side chains of $^{323}$EF$^{324}$ and $^{334}$DF$^{335}$ and $^{330}$EF$^{331}$ and $^{334}$DF$^{335}$ of factor Va heavy chain and was calculated as described under “Experimental Procedures”.

\[
\begin{align*}
\text{Factor Va}^{\text{WT}} & \quad +1.06 \text{ kcal/mol} \quad \text{Factor Va}^{\text{KF}} \\
\text{Factor Va}^{\text{FF}} & \quad +2.12 \text{ kcal/mol} \quad \text{Factor Va}^{\text{FF/KF}} \\
\text{Factor Va}^{\text{MI/KF}} & \quad \Delta \Delta G_{\text{int}} = +2.40 \text{ kcal/mol} \\
\text{Factor Va}^{\text{FF/AA}} & \quad \Delta \Delta G_{\text{int}} = +0.34 \text{ kcal/mol} \\
\text{Factor Va}^{\text{MI/AA}} & \quad \Delta \Delta G_{\text{int}} = +0.51 \text{ kcal/mol}
\end{align*}
\]
2.5 Discussion

Collectively, through a systematic approach using both kinetic studies with synthetic peptides and recombinant proteins, our data demonstrate that amino acid residues D\textsuperscript{334} and Y\textsuperscript{335} are crucial for optimum re-arrangement of enzyme and substrate required for efficient catalysis of prothrombin by prothrombinase. These residues are conserved throughout evolution, being identical in human, bovine and porcine species, indicating their importance to factor Va cofactor function.

The mechanism of inhibition of prothrombinase function by AP5, AP5\textsuperscript{DY→KF}, IWDYA, and P15H was investigated by assessing factor Va cofactor activity in the presence of increasing concentrations of synthetic peptide. Our previous data suggested that amino acid region \textsuperscript{332}IWDYA\textsuperscript{336} may contribute to the activity of prothrombinase (22). The present data show that the IWDYA motif shared by AP5 and AP6 contains the amino acids accountable for the inhibitory effect of the peptides. This is indicative by the similar K\textsubscript{i} values of peptides AP6 and IWDYA.

AP5 has an 8-fold lower K\textsubscript{i} value than AP6 and I5A, because it also contains amino acids Glu\textsuperscript{330} and Val\textsuperscript{331}, which have been previously shown to be involved in factor Xa binding (21). Analysis of the mode of inhibition of prothrombinase by AP5 suggests that the peptide is a mixed type inhibitor and interacts with both prothrombinase and prothrombinase bound to the substrate (prothrombin). Thus, while it is possible that AP5 interacts with prothrombinase in the presence and absence of prothrombin, the possibility that AP5 also binds prothrombin when the enzyme is in complex with the substrate must be kept in mind. Finally, within the peptide sequence IWDYA, amino acids DY are crucial for its function, since
substitution of these two amino acids by KF results in the almost complete loss of the inhibitory potential of AP5 (AP5\textsuperscript{DY→KF}).

Site directed mutagenesis was next used to assess the importance of amino acids 334-335 from factor Va heavy chain during prothrombin catalysis. The data show that mutating these amino acids results in a factor Va molecule that when incorporated into a prothrombinase produces a prothrombinase with altered \( K_m \) and \( k_{cat} \). In addition, the study of prothrombin cleavage by prothrombinase by gel electrophoresis assembled with factor Va\textsuperscript{KF} and factor Va\textsuperscript{AA} demonstrated approximately an 8-fold lower rate of prothrombin cleavage than prothrombinase assembled with the wild type cofactor molecule. Since the \( K_D \) of factor Va\textsuperscript{KF} and factor Va\textsuperscript{AA} is similar to the \( K_D \) of the wild type molecule, these data like the kinetic data strongly suggest that amino acids 334 and 335 from factor Va heavy chain are involved in the interaction of the enzyme (prothrombinase) with the substrate (prothrombin). It has been further confirmed that it is indeed amino acid residues, 334-335, by recombinant protein data with factor Va\textsuperscript{I332A} and factor Va\textsuperscript{W333A}. These recombinant proteins displayed similar kinetic activity when compared to the wild type molecule.

Our data reveal that, although amino acid substitution at amino acid residues 334-335, is enough to produce an effect on cofactor function, it takes substitution at four amino acids (323-324 and 334-335 or 330-331 and 334-335) in order to completely abolish factor Va cofactor activity. The kinetic data show that the quadruple factor Va mutants, factor Va\textsuperscript{MI/KF}, factor Va\textsuperscript{FF/KF}, factor Va\textsuperscript{MI/AA}, and factor Va\textsuperscript{FF/AA}, have a reduced affinity for the enzyme factor Xa, having 5- to 20-fold increased \( k_d \) values. In addition, these recombinant proteins have a 10-fold or greater decrease in their
catalytic efficiencies. On the other hand the quadruple mutants do no have any impairment in their ability to bind the substrate, prothrombin. The $K_m$ of all the reactions is the same. Since factor $Va^{MI/KF}$, factor $Va^{FF/KF}$, factor $Va^{MI/AA}$, and factor $Va^{FF/AA}$ are only damaged in their interaction with factor Xa, it can be concluded that the cofactor, factor Va, plays a role in prothrombinase assembly and function by rearranging the enzyme into the most favorable position to cleave two distant sites (36Å) on the substrate (22, 23).

The thermodynamic data presented herein imply that mutation at residues Glu$^{323}$ and Tyr$^{324}$ or Glu$^{330}$ and Val$^{331}$ together with Asp$^{334}$ and Tyr$^{335}$ have a collective effect on prothrombin activation. The data also suggest that the side chains of these amino acids act synergistically to stabilize the transition state complex of the reaction. However, it cannot be ruled out that the change in free energy following mutation at the specified amino acids is not due to a conformation change of the protein.

Collectively, the data demonstrate that amino acids 334-335 of factor Va play a crucial role for the expression of factor Va cofactor activity and demonstrated the cofactor requirement for the efficient rearrangement of enzyme (factor Xa) and substrate (prothrombin) within prothrombinase required for efficient catalysis. Thus, one more time our data strongly suggest that factor Va directs (regulates) catalysis of prothrombin by factor Xa within prothrombinase.
2.6 Reference List


CHAPTER III
THE LONG-RANGE RELATIONSHIP BETWEEN IMPORTANT AMINO ACID REGIONS OF FACTOR VA HEAVY CHAIN

3.1 Abstract

The prothrombinase complex catalyzes the activation of prothrombin to thrombin. The incorporation of the cofactor, factor Va, into the prothrombinase complex increases the catalytic efficiency of the enzyme by five orders of magnitude. Thus, the proper interaction of factor Va with the members of the prothrombinase complex results in timely thrombin formation at the place of vascular injury. We have previously shown that amino acid region 695DYDYQ699 of the COOH-terminus of the heavy chain of factor Va plays an important role during factor V activation and is required for optimal prothrombinase function. We have also demonstrated that amino acid region 334DY335 are required for optimum activity of prothrombinase. In
addition, we have structural data demonstrating that residues 334-335 and 695-699 of factor Va are in close proximity on the surface of the heavy chain of the cofactor. In order to assess the effect of these residues we created recombinant factor Va molecules, combining mutations at amino acid residues 334-335 and 695-698 as follows: factor V$^{3K}$ (334DY→KF335 and 695DYDY→KFKF698), factor V$^{K4}$ (334DY→KF335 and 695DYDY→AAAA698), and factor V$^{6A}$ (334DY→AA335 and 695DYDY→AAAA698). These recombinant factor V molecules were transiently expressed into COS7 cells, purified to homogeneity by affinity chromatography, and assessed for cofactor activity in the presence of saturating concentrations of recombinant factor Va and limiting concentrations of factor Xa. SDS-PAGE and Western Blotting verified the integrity of the recombinant factor Va molecules with monoclonal antibodies specific to the heavy and light chains. Kinetic analysis revealed that factor V$^{3K}$, factor V$^{K4}$, and factor V$^{6A}$ had a reduced affinity for the enzyme, factor Xa, when compared to the affinity of the wild type molecule for the enzyme. In addition, prothrombinase assembled with a saturating concentration of factor V$^{3K}$ had a 10-fold reduced second order rate constant for prothrombin activation compared to the value obtained with prothrombinase assembled with the wild type molecule. On the other hand, prothrombinase assembled with factor V$^{K4}$ and factor V$^{6A}$ had approximately 1.5-fold reduced second order rate constant. Overall, the data demonstrate that the inability of prothrombinase assembled with factor V$^{3K}$, factor V$^{K4}$, and factor V$^{6A}$ to function optimally can be explained by the inability of factor Xa to efficiently convert
prothrombin to thrombin because of diminished productive collisions due to defective interaction between factor Xa and prothrombin.
3.2 Introduction

Thrombin is necessary for continued existence and is formed following activation of its inactive precursor, prothrombin, by the prothrombinase complex. The prothrombinase complex is composed if the enzyme, factor Xa and the protein cofactor, factor Va, assembled on a membrane surface in the presence of divalent metal ions. Even though, factor Xa alone can activate prothrombin, the rate of this reaction is not compatible with survival. On the other hand, incorporation of the cofactor into the prothrombinase complex increases the catalytic efficiency of prothrombin activation by 5 orders of magnitude, thus providing the physiological pathway for thrombin production. This increase is believed to be the result of a 100-fold decrease in the $K_m$ and a 3,000-fold increase in the $k_{cat}$ of the enzyme when compared to the enzyme, factor Xa, alone. The decrease in the $K_m$ seems to be the outcome of a tighter interaction of the prothrombinase complex with the membrane surface, which results in higher local concentrations of the enzyme and the increase in the $k_{cat}$ of prothrombinase can be credited to the productive interaction between factor Va and factor Xa thus causing conformational changes in the enzyme complex (1-3).

It has long been established that factor Va is essential for suitable and specific activation of prothrombin, however the exact molecular mechanism by which factor Va exerts its cofactor function is still under investigation. Many independent laboratories have established the beneficial interaction of factor Va within the prothrombinase complex. In addition, the interaction of the heavy chain of factor Va with prothrombin involves anion binding exosite I of prothrombin has also been demonstrated (4-7).
It has been previously demonstrated by our laboratory that the COOH terminus of the factor Va heavy chain is important for factor V activation and essential for optimal cofactor activity (8). Specifically, it has been demonstrated that amino acid region 680-709 is important for these functions (9-11). This was demonstrated by using a factor V molecule activated with the purified protease from the venom of the snake *Naja nigricollis nigricollis* (NN), which cleaves factor V at Asp<sup>697</sup>, Asp<sup>1509</sup>, and Asp<sup>1514</sup> to produce factor V<sub>NN</sub> composed of a Mr 100,000 heavy chain (amino acid residues 1-696) and a Mr 80,000 light chain (amino acid residues 1509/1514-2196) (11). It has been demonstrated by our research group that factor V<sub>NN</sub> has reduced clotting activity and an increased K<sub>d</sub> value of 4 nM when compared to factor V activated with thrombin (fV<sub>IIa</sub>) (K<sub>d</sub> ~0.5nM) (12). Further experiments showed that when factor V<sub>IIa</sub> is additionally cleaved by NN, there is a 60-80% reduction in cofactor activity. This reduction in activity has been hypothesized to be a direct result from the loss of crucial amino acids of the heavy chain that are released when cleaved by NN. This region of the cofactor is highly acidic in nature and contains several tyrosine residues that have been shown to have potential to be involved in factor V activation by thrombin and proper cofactor function (13). Our laboratory has further characterized the importance of this region of factor Va by a series of experiments beginning with peptide studies. Five overlapping peptides, designated HC1-HC5, were constructed and characterized; specifically, HC3 (consisting of amino acids 690-699) and HC4 (containing residues 695-704), were found to inhibit prothrombinase activity with IC<sub>50</sub> values of 12 and 10 µM, respectively (14). Both peptides were found to competitively inhibit prothrombinase with K<sub>i</sub> values of 6.3 µM for HC3 and
5.3 µM for HC4. It was found that the overlapping region between peptides HC3 and HC4, amino acid motif $^{695}$DYDYQ$^{699}$, is a potent inhibitor of prothrombinase function with an IC$_{50}$ of 1.6 µM (8). This region was further assessed by making a recombinant factor V molecule with the mutations Asp$^{695}$ → Lys, Tyr$^{696}$ → Phe, Asp$^{697}$ → Lys, and Tyr$^{698}$ → Phe, designated factor V$^{KFKF}$. Data demonstrated that factor V$^{KFKF}$ could only be partially activated by thrombin and was deficient in its interaction with prothrombin. However, activation by RVV-V and factor Xa proceeded at a normal rate despite the impaired cofactor activity(8).

As demonstrated in Chapter II, we have discovered residues from the central portion of the heavy chain of factor Va to contain a binding site for factor Xa. Specifically, we have shown that amino acid residues 334-335 are crucial for cofactor function. Analysis of peptides encompassing this amino acid region shows inhibitory potential with respect to prothrombin activation; specifically the peptide AP5 with the amino acid substitution DY → KF is unable to inhibit prothrombin activation, indicating that it is residues 334-335 that are required for cofactor function. In addition, recombinant protein analysis demonstrates that factor Va$^{KF}$ and factor Va$^{AA}$ have similar affinities for factor Xa as indicated by their similar $k_d$ values compared to factor Va$^{WT}$. However, these molecules are impaired in their ability to activate prothrombin compared to the wild type cofactor, gel electrophoresis analysis shows sustained prothrombin and delayed thrombin appearance. We also demonstrated that combining mutations at residues 334-335 with mutations at residues 323-324 and 330-331 result in cofactor molecules that are completely diminished in their ability to effectively assemble in the prothrombinase complex and activate prothrombin. These
results suggest that mutation at more than two amino acid residues is required to have a profound effect on cofactor function. The present study was undertaken to understand the importance of two distinct important regions of factor Va heavy chain when acting in concert on prothrombinase assembly and function.
3.3 Experimental Procedures

3.3.1 Materials and Reagents

Diisopropyl-fluorophosphate (DFP), O-phenylenediamine (OPD)-dihydrochloride, N-[2-Hydroxyethyl] piperazine-N’-2-ethanesulfonic acid (Hepes), Trizma (Tris base), and Coomassie Blue R-250, were purchase from Sigma (St. Louis, MO). Factor V-deficient plasma is from Research Protein Inc. (Essex Junction, VT). Secondary anti-mouse and anti-sheep IgG coupled to peroxidase were purchased from Southern Biotechnology Associates Inc. (Birmingham, AL). L-α-phosphatidylserine (PS) and L-α-phosphatidylcholine (PC) were from Avanti Polar Lipids (Alabaster, AL). Chemiluminescent reagent ECL+ and Heparin-Sepharose were from Amersham Pharmacia Biotech Inc. (Piscataway, NJ). Normal reference plasma and chromogenic substrate Spectrozyme-TH were from American Diagnostica Inc. (Greenwich, CT). Thromboplastin reagent was purchased from Organon Teknika Corp. (Durham, NC). Dansylarginine-N-(3-ethyl-1, 5-pentanediyl) amide (DAPA), human factor Xa, human thrombin, and human prothrombin were purchased from Haematologic Technologies Inc. (Essex Junction, VT). Factor V cDNA was from American Type Tissue Collection (ATCC# 40515 pMT2-V, Manassas, VA). All restriction enzymes were from New England Biolabs (Beverly, MA). All molecular biology and tissue culture reagents, specific primers, and medium were from Gibco, Invitrogen Corp. (Grand Island, NY) or as indicated. Human factor V monoclonal antibodies (αHFV<sub>HC</sub>#17 and αHFV<sub>LC</sub>#9) and monoclonal antibody αHFV#1 coupled to Sepharose were provided by Dr. Kenneth G. Mann (Department of Biochemistry, University of Vermont, Burlington, VT).
Figure 3.1 Schematic of Recombinant Factor Va Molecules. This scheme displays the mutant recombinant factor V molecule constructed.
3.3.2 Construction of Recombinant Factor V Molecules

Factor V^KFKF and factor V^AAAA were constructed previously by our laboratory (8). Recombinant factor V molecules, Factor V^3K, factor V^KF/4A, and factor V^6A were constructed using Stratagene’s QuikChange® XL Site-Directed Mutagenesis Kit. Factor V^3K was constructed using the mutagenic primers, 5'-C ATT TGG AAG TTT GCA CCT G-3' (forward) and 5'-C AGG TGC AAA CTT CCA AAT G-3' (reverse), to insert the 334DY→KF335 mutations on factor V^KFKF as a template (bold underlined letters identify the mutated bases). To construct factor V^KF/4A these same primers were used and factor V^AAAA was used as the template in the PCR reaction. Factor V^6A was constructed with the primers, 5'-GAG GAA GTC ATT TGG GCC GCC GCA CCT GTA ATA- 3' (forward) and 5'-TAT TAC AGG TGC GGC GGC CCA AAT GAC TTC CTC-3’ (reverse), with factor V^AAAA as the template in the PCR reaction. The mutations were confirmed by DNA Sequencing (DNA Analysis Facility, Cleveland State University). Figure 3.1 shows a schematic of the recombinant factor V molecules.

3.3.3 Transient Transfection of Recombinant Factor V Molecules.

The COS-7L cell line will be maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and the antibiotics streptomycin (100µg/ml) and penicillin (100IU/ml) in an atmosphere of 5% CO₂, 95% air, and 37°C. The purified wild type, factor V^3K, factor V^KF/4A, and factor V^6A plasmids were transfected into the COS-7L cells with fugene 6 (Roche Diagnostics) according to the manufacturer’s instructions. In short, 4µg of DNA, 300µL of serum free media, and 27µL of fugene 6 per cell culture plate was pre-incubated for 15-45 minutes at room
temperature. The mixture was then added drop-wise to the culture plate. After 48 hours of incubation, the cells will be washed twice with PBS buffer and 6ml of VP-SPM media supplemented with 4mM L-glutamine will be added. Following 24 hours the media will be harvested and fresh VP-SPM media will be added. Harvesting of protein will be repeated for 3-4 consecutive days and the harvest media will be stored at -80°C. The harvest media will be concentrated using a Cole Parmer, Masterflex L/S with MW=30,000 Vivaflow 50 membrane to a volume of 5-15mLs. Then, 2mM DFP will be added and the protein allowed will on ice for half an hour before purifying the recombinant protein.

3.3.4 Purification of Recombinant Factor V Molecules

The concentrated recombinant protein was centrifuged at 5,000 rpm for 5 minutes to remove any cellular debris. The protein was purified on a 2ml column of monoclonal antibody αhFV#1 coupled to sepharose. The column was first equilibrated with TBS plus 5mM Ca²⁺, pH 7.40 (TBS, Ca²⁺) (all buffers were filtered before use). The recombinant protein media was added to the column and 0.5ml fractions were collected. The column was washed with 12ml of TBS, Ca²⁺ and eluted with 20mM Tris Base, 2M NH₄Cl, pH 7.40. The absorbance of the collected fractions was recorded at 280 nm on a HITACHI U-2000 spectrophotometer and clotting activity monitored cofactor activity. Fractions containing activity were pooled and dialyzed against TBS, Ca²⁺, pH 7.40 for 2 hours at 4°C. The purified protein was stored at -80°C in small aliquots to avoid repeated freeze thaw cycles. The activity and the integrity of the recombinant factor V molecules were confirmed.
by clotting assays using factor V deficient plasma and western blotting with monoclonal and polyclonal antibodies.

3.3.5 Determination of Factor Va Clotting Activity of the Recombinant Molecules

The cofactor activity of the recombinant molecules was measured in a clotting assay using factor V-deficient plasma following activation of the cofactor molecules by thrombin (10 min, 37°C). The values were measured on a STAart 4 Analyzer Coagulation Instrument (Diagnostica Stago, Parisippany, NJ) and the values were standardized to the percentage of control. A linear semi-log graph was created using known concentrations of plasma factor Va as a control and the specific activity of each recombinant factor Va molecule was calculated (units/mg).

3.3.6 Gel Electrophoresis and Western Blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses were carried out using 4-12% gradient gels or 9.5% gels following reduction with 2% β-mercaptoethanol according to the methods of Laemmli (1970). The protein was transferred to polyvinylidene difluoride (PVDF) membranes following the described method of Towbin et al. (1979). Factor Va heavy and light chains were probed with the appropriate antibodies and visualized with chemiluminescence; or, the protein was visualized by staining with Coomassie Brilliant Blue R-250, followed by destaining in a solution of methanol, acetic acid, and water.

3.3.7 Factor Va Titrations

The ability of the recombinant factor V molecules to assemble in the prothrombinase complex and bind to the enzyme was measured in a discontinuous
assay described in detail elsewhere (15). In short, all recombinant factor V molecules were activated with thrombin (10 min. at 37°C). Reaction mixtures contained PCPS vesicles (20µM), DAPA (3µM), factor Xa (varying concentration), and recombinant factor V species in Reaction Buffer (varying concentration) HEPES, 0.15M NaCl, 50nM CaCl₂, 0.01% Tween-20, pH 7.40). For the practical calculation of the K_D between the factor Va molecules and factor Xa, assays were performed in the presence of limiting factor Xa concentration (15pM) and varying concentrations of the recombinant factor Va species (25pM to 5nM). A zero point was taken and the reaction was started upon the addition of 1.4µM prothrombin. At the following time points 10, 20, 30, and 60 seconds, aliquots of the reaction mixture were removed and diluted in 2 volumes quench solution (20nM HEPES, 0.15M NaCl, 50nM EDTA, 0.1% PEG 8000, pH 7.40) in a 96-well sample plate. The rate of thrombin generation was measured using a chromogenic substrate, Spectrozyme TH (0.4nM), which probes for thrombin generation. The initial rate of thrombin generation was analyzed with Prizm (GraphPad) software.

3.3.8 Prothrombin Titirations

Next, the capability of the recombinant factor Va molecules to assemble into the prothrombinase complex, bind to the substrate prothrombin, and the rate of catalytic efficiency was measured. For the determination of the kinetic constants of prothrombinase assembly, K_m and k_cat, experiments were executed with a limiting amount of factor Xa (5 pM) in the presence of a fixed amount of the various recombinant factor Va molecules at their saturation concentrations (10 nM to 20 nM), PCPS vesicles (20 µM), and DAPA (3 µM) in Reaction Buffer. A zero point was
taken and the reaction was started with varying amounts of the substrate prothrombin (25 nM to 4 µM). Aliquots were removed at the time points 20, 40, 60, and 120 seconds and the reaction was stopped in 2 volumes Quench Buffer. The rate of thrombin generation was measured using a chromogenic substrate, Spectrozyme TH (0.4 nM), which probes for thrombin generation. The initial rate of thrombin generation was analyzed with Prizm (GraphPad) software.

3.3.9 Calculation of Factor Xa Saturation by Recombinant Factor Va Molecules

To order to compare prothrombinase function assembled in the presence of the various mutant recombinant molecules we have first calculated the \( k_d \) of each species for factor Va as described above by titrating a fixed amount of factor Xa with increased concentrations of factor Va as described above. Once the value of the \( k_d \) of the interaction of each species of factor Va with factor Xa was determined, the amount of recombinant factor Va required to completely saturate factor Xa and provide similar amount of enzyme (prothrombinase) when using various factor Va recombinant molecules (between 95% - >99% saturation) can be calculate as abundantly described in the literature (15). Briefly, the \( k_d \) for the factor Va-factor Xa interaction is given by the equation:

\[
K_d = \frac{[Va]^F \cdot [Xa]^F}{[Va \bullet Xa]} \quad (1)
\]

The maximum amount of factor Xa saturated with factor Va formed under the conditions used can be verified by replacing in equation (1) \([Va]^F\) and \([Xa]^F\) by:

\[
[Va]^F = [Va]^T - [Va \bullet Xa] \quad (2)
\]

\[
[Xa]^F = [Xa]^T - [Va \bullet Xa] \quad (3)
\]
where, \([Va]_F\) and \([Xa]_F\) are the concentrations of free factor Va and factor Xa, while \([Va]_T\) and \([Xa]_T\) are the total concentrations of factor Va and factor Xa respectively. The final concentration of factor Xa within the mixture was 1nM and the maximum amount of factor Xa saturated with factor Va formed under the above conditions was extrapolated from the following equation directly.

\[
Xa_{\text{bound}} = \frac{\left[ \left( n \cdot Va \right)_T + \left( Xa \right)_T + K_d \right]}{2} - \sqrt{\left[ \left( n \cdot Va \right)_T + \left( Xa \right)_T + K_d \right]^2 - \left[ 4 \cdot n \cdot [Va]_T \cdot [Xa]_T \right]}
\]

(4)

with the \(K_d\) for the bimolecular interaction between factor Va and factor Va on a phospholipid surface calculated from the functional titration described above and the concentration of \([Va]_T\) modified as appropriate (53). Throughout all experiments the assumption was \(n = \) moles of factor Xa bound/mole of factor Va at saturation; throughout this study \(n = 1\); the stoichiometry of the factor Va-factor Xa interaction was fixed at 1.

3.3.10 Calculation of Additive Effect of the Quadruple or Sextuplet Mutations on Prothrombinase Function

The change in transition-state stabilization free energy, which measures the effect of the mutations in the cofactor of the prothrombinase complex have on the catalytic site of the enzyme, was calculated for the double mutants as extensively described previously (A6). In brief, the perturbation to the function of prothrombinase assembled with wild type factor Va (state A) caused by a mutation in factor Va (state B) affecting the transition state can be defined in general as follows:
and since prothrombinase activity (assembled with each of the recombinant factor Va proteins) is being measured against the same substrate (prothrombin), the transition-state stabilization free energy ($\Delta\Delta G_{A\rightarrow B}^\ddagger$) during catalysis induced by a mutation in factor Va can be determined from the following equation:

$$\Delta\Delta G_{A\rightarrow B}^\ddagger = - RT \ln \left[ \frac{(k_{\text{cat}}/K_m)_B}{(k_{\text{cat}}/K_m)_A} \right]$$  \hspace{1cm} (Eq. 2)

where $R$ is the universal gas constant (2 cal•K⁻¹•mol⁻¹), $T$ is the absolute temperature (298 K in the experiments presented here), $k_{\text{cat}}$ is the turnover number, and $K_m$ is the Michaelis Menten constant of the reaction. Once calculated, these values can be used to determine $\Delta\Delta G_{\text{int}}$, which reflects the exchange in free energy between the amino acids side chains manipulated in this study (D334K or A, Y335F or A, D695K or A, Y696F or A, D697K or A, and Y698F or A) by the following equation:

$$\Delta\Delta G_{\text{int}} = \Delta\Delta G_{A\rightarrow B/C} - (\Delta\Delta G_{A\rightarrow B} + \Delta\Delta G_{A\rightarrow C})$$  \hspace{1cm} (Eq. 3)

A positive value of $\Delta\Delta G_{\text{int}}$ indicates that the interaction of these amino acid side chains reduce the catalytic efficiency of prothrombinase, negative value demonstrates that the mutations are better for prothrombinase, resulting in an increase in the catalytic efficiency of the enzyme. A value of zero would indicate no effect.

### 3.3.10 Prothrombin Activation Measured by Gel Electrophoresis

Prothrombin will be incubated in a reaction mixture containing the following: 20µM PCPS, 50µM DAPA, and 10nM of factor Va$^{\text{WT}}$ and 20nM of factor Va$^{3K}$, factor Va$^{\text{KF}/4A}$, and factor Va$^{6A}$ (activated with thrombin) in TBS, Ca$^{2+}$. A zero point was taken and the addition of 1 nM factor Xa marked the start of the reaction. Aliquots of the reaction mixture will be removed at selected time point (0:20, 0:40,
1:00, 1:20, 1:40, 2:00, 2:20, 2:40, 3:00, 3:20, 3:40, 4:00, 5:00, 6:00, 10:00, 20:00, 30:00 and 60:00 minutes) and added to two volumes 0.2M glacial acetic acid. The samples will be dried in a centrivap and reconstituted in 0.1M Tris base, pH 6.8, 1% SDS, 1% β-mercaptoethanol and heated for exactly 75 seconds at 95°C. A total of 5µg of total protein will be loaded per lane and analyzed on 9.5% SDS-PAGE followed by staining with Coomassie Blue.
2.4 Results

2.4.1 Transient Expression and Activation of Recombinant Factor V Molecules

In order to further assess the importance of residues from the heavy chain of factor Va, specifically, amino acid residues 334-335 and 695-698, recombinant protein technology was used. Mutant recombinant factor Va molecules, factor \( V^{3K} \) (D334K, Y334F, D695K, Y696F, D697K, and Y698F), factor \( V^{KF/4A} \) (D334K, Y335F, D695A, Y696A, D697A, and Y698A), and factor \( V^{6A} \) (D334A, Y335A, D695A, Y696A, D697A, and D698A) were prepared. Recombinant factor \( V^{WT} \) and the mutants were expressed in COS-7 cells, and the resulting proteins were purified to homogeneity on a monoclonal antibody column specific to factor V.

First, the recombinant molecules were assessed for their clotting activity in a two-stage clotting activity assay and the results are shown in Table 3.1. Thrombin activation of factor \( V^{WT} \) resulted in a cofactor with similar clotting activity (597U/mg) to plasma factor Va (data not shown). The mutant recombinant factor Va molecules displayed reduced clotting activities compared to the wild type molecule. Factor \( V^{6A} \) has a 4-fold decrease in the clotting activity (149U/mg), factor \( V^{KF/4A} \) has a 6.5-fold reduction in the clotting activity (93.7U/mg), and factor \( V^{3K} \) is most impaired in its’ clotting activity with a 20-fold decrease (28.6U/mg). The results demonstrate that mutation of the amino acid motif \( DY \rightarrow KF \) has a more detrimental effect that mutation to alanine residues.
Table 3.1 Clotting Times and Activities of Recombinant Factor Va Molecules.
The 2-stage clotting time and clotting activity were determined as outlined by the “Experimental Procedures”.

<table>
<thead>
<tr>
<th>Species</th>
<th>2-Stage Clotting Time</th>
<th>Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor Va(^{WT})</td>
<td>18.63±0.81</td>
<td>597</td>
</tr>
<tr>
<td>Factor Va(^{3K})</td>
<td>47.58±0.93</td>
<td>28.6</td>
</tr>
<tr>
<td>Factor Va(^{K/F4A})</td>
<td>35.76±2.13</td>
<td>93.7</td>
</tr>
<tr>
<td>Factor Va(^{6A})</td>
<td>31.8±0.75</td>
<td>149</td>
</tr>
</tbody>
</table>
3.4.2 Kinetic Analysis of Recombinant Factor Va Molecules

Next, we examined the capability of the recombinant factor Va molecules to assemble in the prothrombinase complex using an assay with purified reagents and a chromogenic substrate to probe for thrombin generation. The assay was performed with conditions of limiting factor Xa concentrations, any deficiency in the ability of the recombinant molecules to act as a cofactor in the enzymatic prothrombinase complex, will reflect the ability of the factor V species to properly bind the enzyme and activate thrombin. Figure 3.2 and Table 3.2 show the results of the kinetic studies. The data demonstrate that under the experimental conditions factor Va\textsuperscript{WT} has the same affinity for the enzyme, factor Xa, as the plasma factor Va counterpart (k\textsubscript{d} = \textasciitilde0.2nM). Conversely, the recombinant factor Va molecules, factor Va\textsuperscript{3K}, factor Va\textsuperscript{KF/4A}, and factor Va\textsuperscript{6A} were impaired in their ability to productively bind the enzyme having, dissociation constants with 7-fold increase for factor Va\textsuperscript{3K} and 10-fold increase for factor Va\textsuperscript{KF/4A} and factor Va\textsuperscript{6A}. These results are interesting because when amino acid residues 334-335 and 695-698 are mutated separately, they do not display deviations in their binding constants. Therefore, it can be concluded that in order to have an effect on the affinity for factor Xa, both amino acid regions are required.

We further evaluated the effect of the mutations in the recombinant factor Va molecules to assemble in the prothrombinase complex and activate prothrombin using kinetic experiments designed to determine the K\textsubscript{m} and k\textsubscript{cat} values for prothrombinase
**Figure 3.2** Factor Va Titrations. Thrombin generation experiments were carried out as described under “Experimental Procedures”. Prothrombinase complex assembled with varying concentrations (30 pM to 5 nM) of purified recombinant factor Va\(^{WT}\) (*closed squares*), purified factor Va\(^{3K}\) (*filled triangles*), purified factor Va\(^{KF/4A}\) (*closed inverted triangle*), and purified factor Va\(^{6A}\) (*filled circles*). The solid lines represent a nonlinear regression fit of the data using Prizm GraphPad software for a one binding site model. Titrations were performed in triplicate with at least four different preparations of purified and partially purified protein.
function. The experiments were performed in the presence of limiting amounts of factor Xa (5-10pM) and the concentration of the cofactor was kept constant (10-20nM) while the substrate concentration was varied (25nM to 4µM) and the results are displayed in Figure 3.4 and Table 3.2. Under the experimental conditions employed, factor Va \textsuperscript{WT} displays an affinity for the substrate, prothrombin, similar to the plasma counterpart. In contrast the results with the previous experiments showing the decreased affinity for the enzyme, the results of the prothrombin titrations show similar affinities for prothrombin. Next, the catalytic efficiencies were compared in Figure 3.5. These kinetic studies show that factor Va\textsuperscript{3K}, factor Va\textsuperscript{KF/4A}, and factor Va\textsuperscript{6A} have a 6.5, 1.7, and 1.4-fold reduced catalytic efficiency compared to factor Va\textsuperscript{WT}. Conversely, when amino acid region 695-698 alone is mutated, factor Va\textsuperscript{KFKF} and factor Va\textsuperscript{AAAA} have 1.4 and 1.2-fold increased catalytic efficiency compared to the wild type molecule. This increased catalytic efficiency can be explained by sustained meizothrombin accumulation (data not shown). Subsequently, the substrate specificities (k\textsubscript{cat}/K\textsubscript{m}) were compared between the recombinant factor Va molecules and the results are shown in Figure 3.6. These results demonstrate that, similar to the comparisons of k\textsubscript{cat} values, the substrate specificities are also reduced in the recombinant factor Va molecules, factor Va\textsuperscript{3K} (4.5-fold reduced value), factor Va\textsuperscript{KF/4A} (2-fold reduced value), and factor Va\textsuperscript{6A} (1.6-fold reduced value). While the sextuplet mutants display decreased substrate affinity, the quadruple 695-698 mutants display an increase; factor Va\textsuperscript{KFKF} and factor Va\textsuperscript{4A} both have approximately a 1.5-fold increase in the specificity.
Figure 3.3 Prothrombin Titrations. Thrombin generation experiments were carried out as described under “Experimental Procedures” by varying the substrate concentration (25 nM to 4 µM) with 10 pM of factor Xa saturated with the factor Va species. Prothrombinase complex assembled with recombinant purified wild type factor Va (filled squares), purified factor Va^3K (filled triangles), purified factor Va^KF/4A (filled inverted triangles), and purified factor Va^6A (closed circles). The data shown are the average of five different titrations performed in triplicate with at least four different preparations of purified and partially purified proteins.
Table 3.2 Kinetic Constants of Recombinant Factor Va Molecules. The dissociation constants of recombinant factor Va species for plasma derived factor Xa were calculated at limiting concentrations of the enzyme as described in “Experimental Procedures”. The $K_m$ constants were determined at described in “Experimental Procedures” using limiting amounts of the enzyme and varying concentrations of substrate.

<table>
<thead>
<tr>
<th>Factor Va Species</th>
<th>$K_d$ (nM)</th>
<th>$K_m$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor Va$^{WT}$</td>
<td>0.238</td>
<td>0.164</td>
</tr>
<tr>
<td>Factor Va$^{3K}$</td>
<td>1.76</td>
<td>0.3032</td>
</tr>
<tr>
<td>Factor Va$^{KF/4A}$</td>
<td>2.46</td>
<td>0.458</td>
</tr>
<tr>
<td>Factor Va$^{6A}$</td>
<td>2.33</td>
<td>0.441</td>
</tr>
</tbody>
</table>
Figure 3.4 Comparison of $k_{\text{cat}}$ Values. The values of $k_{\text{cat}}$ were determined using the $k_d$’s and the quadratic equation to determine the saturation concentration of the various recombinant factor Va species.
Figure 3.5 Comparisons of Second Order Rate Constants. The second order rate constants were determined by dividing the $k_{\text{cat}}$ by the $K_m$. 

Comparison of Second Order Rate Constants

![Bar graph showing comparisons of second order rate constants for different recombinant Factor Va molecules. The x-axis represents the molecules: WT, KFKF, 4A, 3K, KF/4A, 6A. The y-axis represents $k_{\text{cat}}/K_m \times 10^{-7}$ (s$^{-1}$M$^{-1}$). The graph shows a comparison of the rate constants for these molecules.]
3.4.4 Additive Effect of Mutations in the Heavy Chain of Factor Va on Prothrombinase Function

If there is a disturbance in the interaction between factor Xa and factor Va within the prothrombinase complex caused by inserting a mutation it could impact the stability of the catalytic site of the enzyme and can be determined by measuring the transition state stabilization free energy of prothrombin activation as describe in ‘Experimental Procedures’. To determine if the two separate mutation sites on the heavy chain of factor Va interact with each other to affect the catalytic site of factor Xa, thermodynamic cycles were created and the exchange in free energy between the side chains of amino acids residues under investigation \(^{334}\text{DY}^{335}\) and \(^{695}\text{DY}^{698}\) was calculated and the results are shown in Figure 3.7. Factor Va\(^{3K}\) has a \(\Delta\Delta G_{\text{int}}\) value of +1.722 indicating the \(\text{DY} \rightarrow \text{KF}\) substitution of amino acid residues 334-335 and 695-698 do not have an additive effect but are disruptive in nature which results in a slower rate of prothrombin catalysis evidenced by kinetic and gel electrophoretic analysis. On the other hand the substitution of \(\text{DY} \rightarrow \text{AAAA}\) in recombinant proteins factor Va\(^{KF,4A}\) and factor Va\(^{6A}\) do have slightly negative values for \(\Delta\Delta G_{\text{int}}\) (-0.313 and -0.760, respectively) implying that the alanine substitutions are not detrimental to cofactor activity. In conclusion, these mutations appear to create a molecule that is a better cofactor for factor Xa. All together these data imply that the interaction of factor Va heavy chain with factor Xa and prothrombin are fundamental for efficient of prothrombin to thrombin.
Figure 3.6 Additive Effect of Recombinant Proteins on Prothrombin Catalysis. \( \Delta \Delta G_{\text{int}} \) is the energy of interaction between the side chains of \( ^{334}DY^{335} \) and \( ^{695}DY^{698} \) of factor Va heavy chain and was calculated as described under “Experimental Procedures”.
3.4.5 Prothrombin Activation By Prothrombinase Assembled with Factor Va\textsuperscript{WT}, Factor Va\textsuperscript{3K}, Factor Va\textsuperscript{KF/4A}, and Factor Va\textsuperscript{6A}

To test the ability of the recombinant factor Va molecules to successfully assemble in the prothrombinase complex and activate prothrombin gel electrophoresis was used. The cofactor molecules were allowed to pre-incubate with prothrombin in the presence of a membrane surface and calcium and factor Xa was added to start the reaction. The results in Figure 3.8 demonstrate that the prothrombin activation schemes for all cofactor molecules are similar and proceed through the meizothrombin pathway displayed by the presence of fragment 1.2A of prothrombin which is the result of initial cleave at Arg\textsuperscript{320}. The mutant cofactor molecules have a slower rate of prothrombin and fragment 1.2A disappearance. These results indicate that factor Va\textsuperscript{3K}, factor Va\textsuperscript{KF/4A}, and factor Va\textsuperscript{6A} have a slower rate of cleavage at Arg\textsuperscript{271} and the presence of the intermediate meizothrombin is sustained longer than compared to the wild type molecule.
Figure 3.7 Prothrombin Activation by Prothrombinase Assembled with the Recombinant Factor Va Molecules. Recombinant factor Va molecules, factor Va\textsuperscript{WT}, factor Va\textsubscript{3K}, factor Va\textsuperscript{KF/4A} and factor Va\textsubscript{6A} were incubated with prothrombin, PCPS vesicles, and DAPA as detailed in the “Experimental Procedures” section at a final concentration of 10 nM for the factor Va\textsuperscript{WT} and 20 nM for the mutant molecules. Factor Xa was added to start the reaction to a final concentration of 1nM. Aliquots were withdrawn at given time intervals and treated as described in the “Experimental Procedures”. The legends to the right of the gels indicate the prothrombin activation fragments: II (prothrombin), F1\textsubscript{1}•2A (Fragment 1•2-A chain), F1•2 (fragment 1•2), and B (B chain of thrombin). Experiments were performed with least three preparations of purified proteins and one representative gel is shown.
3.5 Discussion

The mechanism by which the cofactor, factor Va, improves the catalytic efficiency of the enzyme, factor Xa, is not well understood. There is an agreement in the literature that in order for prothrombin to bind to the prothrombinase complex, an exosite on factor Xa and/or factor Va must be expressed. Presently we have shown that amino acid residues 334-335 together with 695-698 of the factor Va heavy chain are imperative for factor Xa to efficiently convert prothrombin to thrombin because of diminished productive collisions due to defective interaction with both factor Xa and prothrombin. We have previously demonstrated that residues $^{334}\text{DY}^{335}$ are crucial for optimum re-arrangement of enzyme and substrate required for efficient catalysis of prothrombin by prothrombinase. Mutation of these residues results in a cofactor with impaired catalytic efficiency of prothrombin activation compared to wild type factor Va in a prothrombinase assay with purified reagents and a chromogenic substrate. Our laboratory has also identified factor Xa binding sites on the central portion of the factor Va heavy chain. Mutation of amino acid residues 323 and 324 together with 330 and 331 results in a recombinant molecule, factor Va$^{\text{FF/MI}}$, that is unable to competently activate prothrombin to thrombin through the meizothrombin pathway (15). Our laboratory has also established that residues from the COOH-terminus of factor Va heavy chain are involved in cofactor function. In order to establish if residues 334-335 and 695-698 allosterically work together recombinant protein technology was used. The obtained results demonstrate that only when these residues are combined they have a remarkable effect on cofactor activity. Factor Va$^{3K}$ has a nearly 7-fold reduction in the catalytic efficiency compared to factor Va$^{\text{WT}}$, while
when the amino acid regions are mutated by themselves they have a 1.6-fold decrease and a 1.2-fold increase for factor $\text{Va}^{KF}$ and factor $\text{Va}^{3K}$, respectively. These results support our hypothesis that amino acids 334-335 and 695-698 are on the surface of the factor Va heavy chain and they act in concert for optimal cofactor function and prothrombin activation.

It has been previously demonstrated that the role of factor Va in the prothrombinase complex is to localize factor Xa on the membrane surface and that factor Va-factor Xa binding does not induce a change in the catalytic site of the enzyme toward small synthetic substrates. It has, therefore, been proposed that the factor Va dependent increase in catalytic efficiency of prothrombinase is owed to the stabilization of the prothrombin binding site \((16)\). Our data support this hypothesis because multiple mutations in the heavy chain of the cofactor result in a molecule that has less productive collisions between the enzyme and substrate, therefore resulting in slower prothrombin production.

It has been established that effective prothrombin activation is a result of proexosite-1 on prothrombin interacting with a site on the prothrombinase complex \((7)\). Further studies have shown that a proexosite-1 specific peptide ligand taken form the C-terminal domain of the leech inhibitor, hirudin, is able to inhibit activation of prothrombin in the presence of, but not in the absence, factor Va. The C-terminus of the factor Va heavy chain has a homologous domain to the hirudin peptide, suggesting that it is factor Va in the prothrombinase complex that may provide a binding site for proexosite-1 of prothrombin \((8)\). Our laboratory has demonstrated
that a peptide of factor Va, DYDYQ, with a hirudin-like sequence results in the inhibition of initial cleavage of prothrombin at Arg\textsuperscript{320} by the enzyme (17).

There are conflicting results in the literature regarding the importance of the COOH-terminus of the factor Va heavy chain. One group has published that the C-terminal region of factor Va heavy chain does not contribute to the increased catalytic function of prothrombin activation by prothrombinase (18). Their results demonstrate that recombinant molecules with regions of the carboxyl-terminal of factor Va heavy chain, factor Va\textsuperscript{709} (des710-1545), factor Va\textsuperscript{699} (des700-1545), and factor Va\textsuperscript{692} (des693-1545), and factor Va\textsuperscript{678} (des679-1545), exhibited similar clotting activities to plasma-derived factor Va and had unaltered K\textsubscript{m} and k\textsubscript{cat} values. However, closer interpretation of the published data may indicate some points were overlooked; factor Va\textsuperscript{709}, factor Va\textsuperscript{699}, factor Va\textsuperscript{692} and factor Va\textsuperscript{678} display k\textsubscript{cat} values 1.2-fold, 1.4-fold, 1.3-fold, and 1.5-fold increased values compared to the recombinant factor Va\textsuperscript{WT}. These results are in agreement with our kinetic data with factor Va\textsuperscript{KFKF} that also has a 1.2-fold increased k\textsubscript{cat} value. Our interpretation of these results differ in that we feel this is a significant difference in cofactor function; this observation is further backed by experiments using gel electrophoresis to analyze prothrombin activation. We have shown that recombinant factor Va\textsuperscript{KFKF} displays sustained meizothrombin formation during a one hour time course of prothrombin activation (data not shown).

Overall, our data clearly demonstrate that amino acids from the heavy chain of factor Va, namely residues 334-335 and 695-698, are fundamental to cofactor function and most favorable for prothrombin activation.
3.7 References


11. Bakker, H. M., Tans, G., Thomassen, M. C., Yukelson, L. Y., Ebberink, R.,

33561.

13. Pittman, D. D., Tomkinson, K. N., Michnick, D., Selighsohn, U., and


15. Bukys, M. A., Blum, M. A., Kim, P. Y., Brufatto, N., Nesheim, M. E., and

33, 6538-45.

of Biological Chemistry 281, 39194-39204.

8773-8780.
CHAPTER IV
APC INACTIVATION OF FACTOR Va

4.1 Abstract

The coagulation cascade results in the production of thrombin which will aide in the formation of the fibrin plug to stop bleeding. The prothrombinase complex, composed of the enzyme factor Xa, the cofactor factor Va, and the substrate prothrombin associated on a cell surface in the presence of divalent metal ions, catalyzes the activation of prothrombin to thrombin 300,000-fold more effectively than the enzyme alone. Once enough thrombin is produced, it then participates in the down-regulation of the cascade by activating protein C to its active form, activated protein C (APC). APC down regulates the coagulation cascade by inactivating the protein cofactors, factor Va and factor VIIIa, by proteolysis. APC inactivates factor Va following proteolytic cleavages at Arg306, Arg506, and Arg679. Individuals with a single amino acid substitution at
residue 1691 (G →A) results in a factor V molecule with Arg506 → Gln substitution (designated factor V\textsuperscript{LEIDEN}). These individuals have delayed inactivation of factor Va. The delayed inactivation is due to the inability of factor Va\textsuperscript{LEIDEN} to get cleaved at Arg506 and as a result cleavage at Arg306 is retarded. In order to analyze the effect of APC inactivation on prothrombin activation, a recombinant protein scheme was used to create mutant cofactor molecules with the APC cleavage sites on factor V/Va mutated. Our data demonstrate that APC cleavage at Arg\textsuperscript{306} are not equal in the procofactor and active cofactor protein, with greatly increased clotting times with inactivation of the procofactor and decreased affinity for the enzyme and substrate in the prothrombinase complex. We also validate in the absence of APC inactivation cleavage sites Arg\textsuperscript{506} and Arg\textsuperscript{506} the procofactor and active cofactor are unable to be proteolytically inactivated by APC in the presence of a membrane surface.
4.2 Introduction

The coagulation cascade culminates in the production of thrombin from prothrombin by the prothrombinase complex, i.e. factor Xa, factor Va, Ca$^{2+}$, on a membrane surface. It has been well established in the literature that the cofactor, factor Va, is of physiological importance, in that it increases the catalytic efficiency of prothrombin activation by the enzyme, factor Xa, by five orders of magnitude. Once enough thrombin is produced to stop bleeding when there is an injury to the vasculature, down regulation of the coagulation cascade begins. Thrombin serves two roles in hemostasis, as a coagulant (to promote clotting by aiding in the formation of the fibrin plug) and as an anti-coagulant (to start the down-regulation of the coagulation cascade by a positive feedback mechanism). The anti-coagulant function of thrombin is to activate protein C (PC), a zymogen to a vitamin K-dependent serine protease, one of the key components of the natural anti-coagulant pathway.

The protein Case complex is composed of the enzyme, activated protein C (APC) and the cofactor, protein S (PS), in the presence of a membrane surface. This complex is imperative to the anticoagulant mechanism of the blood coagulation cascade. The cofactor function of protein S are weak in purified systems, only enhancing the APC inactivation of factor Va by a factor of two (I). Once formed, protein Case can inactivate the cofactors to the intrinsic and prothrombinase complexes, factor VIIIa and factor Va, respectively. APC cleaves three peptide bonds in the A1-A2 domains of factor Va, at Arg$^{306}$, Arg$^{506}$, and Arg$^{679}$ and it cleaves factor VIIIa at two peptide bonds, Arg$^{336}$ and Arg$^{562}$. However, APC cleavage does not appear to be essential to inactivation because
factor VIIIa inactivation occurs spontaneously following the dissociation of the A2 domain (2-4).

Extensive studies have been performed to determine the molecular mechanism of APC inactivation of factor Va in the prothrombinase complex. It has been demonstrated that not all factor Va inactivating cleavages are equal. The first cleavage at Arg$^{506}$ is kinetically favored and results in a molecule with decreased cofactor activity (5-7). It is also known that cleavage at Arg$^{506}$ in factor Va is necessary for cleavage at Arg$^{306}$. It is cleavage at Arg$^{306}$ that leads to full loss of cofactor function and the dissociation of the A2 domain from the rest of the molecule (8, 9). The last inactivation cleavage at Arg$^{679}$ has not been extensively characterized. In addition, APC can also cleave the pro-cofactor in the presence of a membrane surface at residues Arg$^{306}$, Arg$^{506}$, Arg$^{679}$, and Lys$^{994}$. It is the first cleavage at Arg$^{306}$ that is the inactivating cleavage (10).

It is known that complete inactivation of factor Va by APC occurs in the presence of a membrane surface and requires all three cleavages at Arg$^{306}$, Arg$^{506}$, and Arg$^{679}$ (5, 11). However, factor Va can be cleaved in the absence of a membrane surface only at residues Arg$^{506}$ and Arg$^{679}$. It has been demonstrated that a factor Va molecule that has been cleaved at these two positions maintains ~80% of its cofactor activity in a prothrombinase assay under saturating conditions of factor Xa. On the other hand, when tested in a clotting assay, the same molecule retains only ~40% of cofactor activity. It has also been shown that factor Va cleaved at positions Arg$^{506}$ and Arg$^{679}$ has a reduced affinity for both factor Xa and prothrombin (12-14). Conversely, when a membrane surface is present, cleavage at Arg$^{306}$ is possible, and this cleavage is coupled to the complete loss of cofactor activity no matter what assay is used.
The importance of the first inactivation cleavage of factor Va is clear in patients with APC resistance that have a molecular defect that has been linked to a single point mutation of Arg$^{506}$ to Gln (designated factor V$^{\text{LEIDEN}}$ (5). The factor V$^{\text{LEIDEN}}$ phenotype is present in 5% of the western countries (15) and is a major risk factor for venous thrombosis in patients with this mutation (16). Because factor V$^{\text{LEIDEN}}$ does not have the Arg$^{506}$ cleavage site, APC inactivation is delayed. This delay in inactivation is due to slower cleavage at Arg$^{306}$, indicating that the cleavage at Arg$^{506}$ is mandatory for successful inactivation of the cofactor (17). These data suggest that both cleavages at Arg$^{306}$ and Arg$^{506}$ are essential for full inactivation of factor Va.

In addition, two new mutations in the factor V gene were recently reported, with mutations Arg$^{306}$ to Gly (factor V Hong Kong) and Arg$^{306}$ to Thr (factor V Cambridge) (18, 19). Factor V Cambridge was discovered in a patient that had thrombosis with unexplained resistance to APC. The factor V Hong Kong mutation was found in two Chinese patients with venous thrombosis. It was suspected that a mutation at Arg$^{306}$ would cause a severe thrombotic state, since it is cleavage at Arg$^{306}$ in factor Va by APC that causes complete inactivation of the cofactor. However, neither factor V Cambridge nor factor V Hong Kong appear to be related to patients with an increased risk for thrombosis (20, 21). Further evidence is needed to further elucidate the mechanism of these mutations in factor V.

The present study was undertaken to elucidate the molecular mechanism of factor V/Va inactivation by APC in the presence of a membrane surface. Through recombinant protein technology, factor V molecules were created with the inactivating cleavage sites of factor Va, or combination of cleavage sites, mutated to determine which products of
the individual inactivating cleavage sites of factor Va by APC will produce intermediates
that have different effects on factor Va cofactor assembly and function.
4.3 Experimental Procedures

4.3.1 Materials and Reagents

Diisopropyl-fluorophosphate (DFP), O-phenylenediamine (OPD)-dihydrochloride, N-[2-Hydroxyethyl] piperazine-N’-2-ethanesulfonic acid (Hepes), Trizma (Tris base), and Coomassie Blue R-250, were purchase from Sigma (St. Louis, MO). Factor V-deficient plasma is from Research Protein Inc. (Essex Junction, VT). Secondary anti-mouse and anti-sheep IgG coupled to peroxidase were purchased from Southern Biotechnology Associates Inc. (Birmingham, AL). L-α-phosphatidylserine (PS) and L-α-phosphatidylcholine (PC) were from Avanti Polar Lipids (Alabaster, AL). Chemiluminescent reagent ECL+ and Heparin –Sepharose were from AmershamPharmacia Biotech Inc. (Piscataway, NJ). Normal reference plasma and chromogenic substrate Spectrozyme-TH were from American Diagnostica Inc. (Greenwich, CT). Thromboplastin reagent was purchased from Organon Teknika Corp. (Durham, NC). Dansylarginine-N- (3-ethyl-1, 5-pentanediyl) amide (DAPA), human factor Xa, human thrombin, and human prothrombin were purchased from Haematologic Technologies Inc. (Essex Junction, VT). Human activated protein C and factor Xa were purchased from Enzyme Research Laboratories (South Bend, IN). Factor V cDNA is from American Type Tissue Collection (ATCC# 40515 pMT2-V, Manassas, VA). All restriction enzymes were from New England Biolabs (Beverly, MA). All molecular biology and tissue culture reagents, specific primers, and medium were from Gibco, Invitrogen Corp. (Grand Island, NY) or as indicated. Human factor V monoclonal antibodies (αHFV_{HC}#17 and αHFV_{LC}#9) and monoclonal antibody αHFV#1 coupled to
Sepharose were provided by Dr. Kenneth G. Mann (Department of Biochemistry, University of Vermont, Burlington, VT).

4.3.2 Construction of Recombinant Factor V Molecules

Recombinant factor V molecules, Factor V^{306}, factor V^{506}, factor V^{679}, factor V^{306/506}, factor V^{306/679}, factor V^{506/679}, and factor V^{306/506/679} were constructed using Stratagene’s QuikChange® XL Site-Directed Mutagenesis Kit. The factor V^{306} mutation was constructed with the following primers; 5’ GC CCA AAG AAA AAA ACC CAG AAT CTT AAG 3’ (forward) and 5’ CTT AAG ATT CTG GGT TTT TTT CTT TGG GC 3’ (reverse). The factor V^{506} mutation was constructed with the following primers; 5’ GC AGA TCC CTG GAC CAG CAA GGA ATA CAG AGG GCA GC 3’ (forward) and 5’ CG TGC CCT CTG TAT TCC TTG CTG GTC CAG GGA TCT GC 3’ (reverse). The factor V^{679} mutation was constructed with the following primers; 5’ CT ACA GTC ATG GCT ACA CAG AAA ATG CAT GAT CGT TTA GAA CC 3’ (forward) and 5’ GG TTC TAA ACG ATC ATG CAT TTT CTG TGT AGC CAT GAC TGT AG 3’ (reverse). The bold, underlined bases indicated the mutated base pairs to insert the R → Q mutation. The double and triple mutants were constructed as follows; Arg^{306/506} with Arg^{506} as the template using the Arg^{306} primers, Arg^{306/679} with Arg^{679} as the template with Arg^{306} primers, Arg^{506/679} with Arg^{679} as the template with Arg^{506} primers, and Arg^{306/506/679} with Arg^{506/679} as the template with Arg^{306} primers. The mutations were confirmed by DNA Sequencing (DNA Analysis Facility, Cleveland State University). Figure 4.1 shows a schematic of the recombinant factor V molecules.
### Figure 4.1 Schematic of Recombinant Factor V Molecules

Scheme of mutant recombinant factor V molecules constructed with mutations at Arg\(^{306}\), Arg\(^{506}\), and Arg\(^{679}\).

<table>
<thead>
<tr>
<th>Mutant Factor</th>
<th>Mutation Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor V(^{WT})</td>
<td>No Mutation</td>
</tr>
<tr>
<td>Factor V(^{306})</td>
<td>R(^{306}) → Q</td>
</tr>
<tr>
<td>Factor V(^{506})</td>
<td>R(^{506}) → Q</td>
</tr>
<tr>
<td>Factor V(^{679})</td>
<td>R(^{679}) → Q</td>
</tr>
<tr>
<td>Factor V(^{306/506})</td>
<td>R(^{306}) → Q, R(^{506}) → Q</td>
</tr>
<tr>
<td>Factor V(^{506/679})</td>
<td>R(^{506}) → Q, R(^{679}) → Q</td>
</tr>
<tr>
<td>Factor V(^{306/679})</td>
<td>R(^{306}) → Q, R(^{679}) → Q</td>
</tr>
<tr>
<td>Factor V(^{306/506/679})</td>
<td>R(^{306}) → Q, R(^{506}) → Q, R(^{679}) → Q</td>
</tr>
</tbody>
</table>
4.3.3 Transient Transfection of Recombinant Factor V Molecules.

The COS-7L cell line was be maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and the antibiotics streptomycin (100µg/ml) and penicillin (100IU/ml) in an atmosphere of 5% CO₂, 95% air, and 37°C. The purified wild type, factor V³⁰⁶, factor V⁵⁰⁶, factor V⁶⁷⁹, factor V³⁰⁶/⁵⁰⁶, factor V³⁰⁶/⁶⁷⁹, factor V⁵⁰⁶/⁶⁷⁹, and factor V³⁰⁶/⁵⁰⁶/⁶⁷⁹ plasmids were transfected into the COS-7L cells with fugene 6 (Roche Diagnostics) according to the manufacturer’s instructions. In short, 4µg of DNA, 300µL of serum free medium, and 27µL of fugene 6 per cell culture plate was pre-incubated for 15-45 minutes at room temperature. The mixture was then added drop-wise to the culture plate. After 48 hours of incubation, the cells were be washed twice with PBS buffer and 6ml of VP-SPM medium supplemented with 4mM L-glutamine was added. Following 24 hours the media was harvested and fresh VP-SPM media was added. Harvesting of protein was repeated for 3-4 consecutive days and the harvest medium was stored at -80°C. The harvest medium was concentrated using a Cole Parmer, Masterflex L/S with MW=30,000 Vivaflow 50 membrane to a volume of 5-15mLs. Then, 2mM DFP was added and the protein allowed will on ice for half an hour before purifying the recombinant protein.

4.3.4 Purification of Recombinant Factor V Molecules

The concentrated recombinant protein was centrifuged at 5,000 rpm for 5 minutes to remove any cellular debris. The protein was purified on a 2ml column of monoclonal antibody αhFV#1 coupled to sepharose. The column was first equilibrated with TBS plus 5mM Ca²⁺, pH 7.40 (TBS, Ca²⁺) (all buffers were filtered before use). The recombinant protein medium was added to the column and 0.5 ml fractions were collected. The
column was washed with 12 ml of TBS, Ca$^{2+}$ and eluted with 20 mM Tris Base, 2 M NH$_3$Cl, pH 7.40. The absorbance of the collected fractions was recorded at 280 nm on a HITACHI U-2000 spectrophotometer and clotting activity monitored cofactor activity. Fractions containing activity were pooled and dialyzed against TBS, Ca$^{2+}$, pH 7.40 for 2 hours at 4$^0$C. The purified protein was stored at -80$^0$C in small aliquots to avoid repeated freeze thaw cycles. The activity and the integrity of the recombinant factor V molecules were confirmed by clotting assays using factor V deficient plasma and western blotting with monoclonal and polyclonal antibodies.

4.3.5 APC Inactivation of Recombinant Factor V/Va Molecules

The recombinant factor V molecules were inactivated with APC at a 1:50 molar ratio in the presence of 20µM PCPS. The factor V species were first activated with thrombin to a final concentration of 1nM for 10 minutes in a 37°C water bath (designated factor Va). Immediately following this incubation, the PCPS was added and allowed to incubate at room temperature for 5 minutes. Then, APC was added and incubated in a 37°C water bath for 30 minutes (designated factor Va$_i$). The procofactor factor V molecules were also inactivated with APC and PCPS without the initial thrombin activation (designated factor V$_i$).

4.3.6 Determination of Factor Va Clotting Activity of the Recombinant Molecules

The cofactor activity of the recombinant molecules was measured in a clotting assay using factor V-deficient plasma following activation of the cofactor molecules by thrombin (1 nM, 10 min, 37°C). The clotting activities were also determined following inactivation of factor V/Va species with APC and PCPS. The values were measured on a ST art 4 Analyzer Coagulation Instrument (Diagnostica Stago, Parisippany, NJ) and the
values were standardized to the percentage of control. A linear semi-log graph was created using known concentrations of plasma factor Va as a control and the specific activity of each recombinant factor Va molecule was calculated (units/mg).

4.3.7 Gel Electrophoresis and Western Blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses were carried out using 5-15% gradient gels or 9.5% gels following reduction with 2% β-mercaptoethanol according to the methods of Laemmli (1970). The protein was transferred to polyvinylidene difluoride (PVDF) membranes following the described method of Towbin et al. (1979). Factor Va heavy chain and heavy chain inactivation fragments were probed with the appropriate antibody and visualized with chemiluminescence.

4.3.8 Factor Va Titrations

The ability of the recombinant factor V molecules to assemble in the prothrombinase complex and bind to the enzyme was measured in a discontinuous assay described in detail elsewhere. In short, recombinant factor V molecules were activated with thrombin (1 nM, 10 min at 37⁰C) followed by inactivation with APC/PCPS, or inactivation alone. Reaction mixtures contained PCPS vesicles (20µM), DAPA (3µM), factor Xa (varying concentration), and recombinant factor Va, factor Vaᵢ, and factor Vᵢ species in Reaction Buffer (varying concentration) HEPES, 0.15M NaCl, 50nM CaCl₂, 0.01% Tween-20, pH 7.40). For the practical calculation of the Kᵤ between the factor Va, factor Vaᵢ, and factor Vᵢ molecules and factor Xa, assays were performed in the presence of limiting factor Xa concentration (15pM) and varying concentrations of the recombinant factor Va, factor Vaᵢ, and factor Vᵢ species (25pM to 5nM). A zero point was taken and the reaction
was started upon the addition of 1.4µM prothrombin. At the following time points 10, 20, 30, and 60 seconds, aliquots of the reaction mixture were removed and diluted in 2 volumes quench solution (20nM HEPES, 0.15M NaCl, 50nM EDTA, 0.1% PEG 8000, pH 7.40) in a 96-well sample plate. The rate of thrombin generation was measured using a chromogenic substrate, Spectrozyme TH (0.4nM), which probes for thrombin generation. The initial rate of thrombin generation was analyzed with Prizm (GraphPad) software.

4.3.9 Prothrombin Titrations

Next, the capability of the recombinant factor Va, factor Va<sub>i</sub>, and factor V<sub>i</sub> molecules to assemble into the prothrombinase complex, bind to the substrate prothrombin, and the rate of catalytic efficiency was measured. For the determination of the kinetic constants of prothrombinase assembly, K<sub>m</sub> and k<sub>cat</sub>, experiments were executed with a limiting amount of factor Xa (5pM) in the presence of a fixed amount of the various recombinant factor Va, factor Va<sub>i</sub>, and factor V<sub>i</sub> molecules (10nM), PCPS vesicles (20µM), and DAPA (3µM) in Reaction Buffer. A zero point was taken and the reaction was started with varying amounts of the substrate prothrombin (25nM to 4µM). Aliquots were removed at the time points 20, 40, 60, and 120 seconds and the reaction was stopped in 2 volumes Quench Buffer. The rate of thrombin generation was measured using a chromogenic substrate, Spectrozyme TH (0.4nM), which probes for thrombin generation. The initial rate of thrombin generation was analyzed with Prizm (GraphPad) software.
4.4 Results

4.4.1 Transient Expression of Recombinant Factor V Molecules

In order to examine the molecular mechanism of the consequences of the individual inactivating cleavages of factor V/Va by APC in the presence of a membrane surface, recombinant factor V molecules were constructed with R → Q mutation at the APC cleavage sites; factor V^{306} (R306Q), factor V^{506} (R506Q), factor V^{679} (R679Q), factor V^{306/506} (R306Q and R506Q), factor V^{306/679} (R306Q and R679Q), factor V^{506/679} (R506Q and R679Q), and factor V^{306/506/679} (R306Q, R506Q, and R679Q). Upon transient transfection and purification of the recombinant factor V molecules, we discovered the recombinant proteins containing the mutation at amino acid residue 679 did not express as well as the other recombinant molecules. In fact, recombinant molecules with residue 679 mutated expressed an average of 50 times lower than the other molecules as seen in Table 4.1. Factor V^{WT}, factor V^{306}, factor V^{506}, and factor V^{306/506} expressed a total of 35 to 75µg per 10 cell culture plates transiently transfected. On the other hand, recombinant factor V^{679}, factor V^{306/679}, factor V^{506/679}, and factor V^{306/506/679} did not express over a total of one µg of protein, even when up to 40 cell culture plates were transfected. For the practical purposes of this investigation we, therefore, only used recombinant factor V molecules factor V^{WT}, factor V^{306}, factor V^{506}, and factor V^{306/506}.

4.4.2 Western Blot Analysis of APC Inactivation of Recombinant Factor V/Va Molecules

To investigate the APC inactivation fragments of the recombinant factor V/Va molecules, Western Blot analysis was utilized with a monoclonal antibody specific to the heavy chain of factor Va and the results are shown in Figure 4.2. All recombinant factor
<table>
<thead>
<tr>
<th>Recombinant Factor V Species</th>
<th>Average Total Recombinant Protein Produced/Transfection (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor $V^{WT}$</td>
<td>34.85</td>
</tr>
<tr>
<td>Factor $V^{306}$</td>
<td>40.06</td>
</tr>
<tr>
<td>Factor $V^{506}$</td>
<td>37.28</td>
</tr>
<tr>
<td>Factor $V^{306/506}$</td>
<td>74.40</td>
</tr>
<tr>
<td>Factor $V^{679}$ (any factor V with 679 mutation)</td>
<td>0.875</td>
</tr>
</tbody>
</table>

**Table 4.1 Expression of Recombinant Factor V Molecules.** Average total protein transiently expressed in COS7 cells for each of the mutant recombinant factor V molecules.
molecules activated with thrombin display complete activation and the appearance of the heavy chain of the cofactor, Mr 105,000 (factor Va\textsuperscript{WT} in lane 1, factor Va\textsuperscript{306} in lane 4, factor Va\textsuperscript{506} in lane 7, and factor Va\textsuperscript{306/506} in lane 10). Immediately following activation, the factor Va molecules were inactivated with APC. Factor Va\textsuperscript{WT} displayed the expected fragments of APC inactivation of the cofactor, accumulation of the Mr 30,000 fragment (lane 2), and inactivation of the wild type procofactor also shows the Mr 30,000 fragment (lane 3). Factor Va\textsuperscript{306}, which is unable to be cleaved at Arg\textsuperscript{306}, does not get completely inactivated and there is no appearance of the Mr 30,000 fragment; following APC incubation both factor Va\textsuperscript{i306} and factor V\textsuperscript{i306} display the Mr, 75,000 fragment indicating only the initial first cleavage at Arg\textsuperscript{506} (lanes 5 and 6). Conversely, when the Arg\textsuperscript{506} APC cleavage site is missing in factor Va\textsuperscript{506} we see only the appearance of the Mr, 45,000 fragment indicating that factor Va\textsuperscript{506} and factor V\textsuperscript{506} were only cleaved at Arg\textsuperscript{306}. Because cleavage at Arg\textsuperscript{506} occurs first and is necessary for effective cleavage at Arg\textsuperscript{306}, factor Va\textsuperscript{i506} and factor V\textsuperscript{i506} were not fully cleaved at Arg\textsuperscript{306} (lanes 8 and 9, respectively). On the other hand, the factor Va\textsuperscript{306/506} and factor V\textsuperscript{306/506} molecules are unable to be inactivated by APC, we do not see any of the APC inactivation fragments (lanes 11 and 12, respectively). These data demonstrate that our recombinant factor V mutants display the expected fragments of APC inactivation and can be used to further elucidate the molecular mechanism of factor V/Va APC inactivation.

4.4.3 Clotting Activity of Recombinant Factor V/Va Molecules Following APC Inactivation in the Presence of a Membrane Surface

Next, we analyzed the factor V recombinant molecules with the APC inactivating cleavage sites mutated for their clotting activity. Clotting times were recorded before the
Figure 4.2 Western Blot Analysis of Recombinant Factor V Molecules with an Antibody Specific to the Heavy Chain of Factor Va. Recombinant Factor V molecules were subjected to activation by thrombin and inactivation by APC in the presence of a membrane surface. The molecules were incubated with APC and PCPS with or without activation with thrombin. The lane assignments are as follows; Lane 1 factor Va<sup>WT</sup>, Lane 2 factor Va<sub>i</sub><sup>WT</sup>, Lane 3 factor V<sub>i</sub><sup>WT</sup>, Lane 4 factor Va<sup>306</sup>, Lane 5 factor Va<sub>i</sub><sup>306</sup>, Lane 6 factor V<sub>i</sub><sup>306</sup>, Lane 7 factor Va<sup>506</sup>, Lane 8 factor Va<sub>i</sub><sup>506</sup>, Lane 9 factor V<sub>i</sub><sup>506</sup>, Lane 10 factor Va<sup>306/506</sup>, Lane 11 factor Va<sub>i</sub><sup>306/506</sup>, and Lane 12 factor V<sub>i</sub><sup>306/506</sup>.
addition of a membrane surface and APC and following 15 and 30 minutes of APC incubation and the results are shown in Figure 4.3. All factor V and factor Va species are able to produce a clot at the expected times, 20-25 seconds for the factor V species and 16-18 seconds for the factor Va species. Following 15 minutes of APC inactivation in the presence of a membrane surface, factor $\text{Va}_i^{\text{WT}}$ and factor $\text{V}_i^{\text{WT}}$ have clotting times increased to 60 and 80 seconds, respectively, and following 30 minutes of inactivation the wild type factor $\text{Va}_i$ and factor $\text{V}_i$ have further increased clotting times of 90 and 100 seconds, respectively. This demonstrates that the wild type molecule is fully inactivated, even after a 15 minute incubation with APC and PCPS and that the procofactor and activated cofactor behave similarly during inactivation. Factor $\text{V}_i^{306}$ and factor $\text{Va}_i^{306}$ behave like the wild type molecules, the molecules are completely inactivated following APC inactivation in the presence of a membrane surface having clotting times over 75 seconds. On the other hand, when factor $\text{V}^{306}$ is activated with thrombin before inactivation, the factor $\text{Va}_i^{306}$ molecule does not get completely activated and still retains a clotting time of approximately 45 seconds even after 30 minutes of incubation with APC and PCPS. Suggesting that the mechanism of APC inactivation of factor V/Va with the Arg$^{306}$ cleavage site mutated is different between the procofactor and active cofactor. Conversely, factor $\text{V}^{506}$ and factor $\text{Va}^{506}$ have comparable clotting times when exposed to APC and a membrane surface. These recombinant molecules retain partial activity even after a 30 minute incubation. Since factor $\text{Va}^{306/506}$ does not contain the APC inactivating cleavage sites at Arg$^{306}$ and Arg$^{506}$ it is not susceptible to APC inactivation as evidenced by unchanging clotting times. On the contrary, similar to factor $\text{V}_i^{306}$, factor $\text{V}_i^{306/506}$ does not behave like the activated version of the mutant. Its clotting
Figure 4.3 Time Course of Clotting Times. Clotting times were measured as described in ‘Experimental Procedures’. The recombinant factor V/Va molecules were analyzed prior to APC inactivation in the presence of a membrane surface and following 15 and 30 minute incubation times.
times are increased to 30 seconds following incubation with APC. These data demonstrate that upon thrombin activation of factor $V^{306}$ and removal of the B chain of the procofactor, it causes APC inactivation to proceed at a different rate.

4.4.4 Factor Va/Va\textit{i} Titrations

In order to test the ability of the recombinant factor Va and factor Va\textit{i} to assemble in the prothrombinase complex, effectively bind the enzyme, and activate prothrombin an assay using purified reagents and a chromogenic substrate was used. The results with the recombinant factor V molecules activated were as expected. Figure 4.4 and Table 4.2 demonstrate that factor Va\textit{WT} (filled squares), factor Va\textit{306} (filled inverted triangles), factor Va\textit{506} (filled triangles), and factor Va\textit{306/506} (filled circles) have similar $k_d$ values, indicating the mutations do not affect the interaction between factor Va and factor Xa.

We also analyzed the effect of APC inactivation of the recombinant cofactors. Inactivation of the wild type cofactor does not have any effect on the affinity with the enzyme (open squares). On the other hand, the mutated cofactors have a decreased affinity for factor Xa. Factor Va\textit{i}\textit{306} has a 9-fold increase in the $k_d$ (open inverted triangles), factor Va\textit{i}\textit{506} has a 8-fold increase in the $k_d$ (open triangles), and factor Va\textit{i}\textit{306/506} has a 5-fold increase in the $k_d$ (open circles). These data demonstrate that partial inactivation of the factor Va when its APC inactivation sites are mutated results in a molecule that has a decreased affinity for the enzyme in the prothrombinase complex.

4.4.5 Prothrombin Titrations with Limiting Concentrations of Factor Xa

Next the ability of the recombinant factor Va molecules to assemble in the prothrombinase complex and activate prothrombin using kinetic experiments designed to determine the $K_m$ and $k_{cat}$ values for prothrombinase function. These experiments were
Figure 4.4 Factor Va Titrations. Thrombin generation experiments were carried out as described under “Experimental Procedures”. Prothrombinase complex assembled with varying concentrations (30 pM to 5 nM) of purified recombinant factor Va\textsuperscript{WT} (closed squares), factor Va\textsubscript{i,WT} (open squares), factor Va\textsuperscript{306} (closed inverted triangles), factor Va\textsubscript{i,306} (open inverted triangles), factor Va\textsuperscript{506} (closed triangles), factor Va\textsubscript{i,506} (open triangles), factor Va\textsuperscript{306/506} (closed circles), and factor Va\textsubscript{i,306/506} (open circles). The solid lines represent a nonlinear regression fit of the data using Prizm GraphPad software for a one binding site model. Titrations were performed in triplicate with at least three different preparations of purified protein.
performed in the presence of limiting amounts of factor Xa (5-10pM) and the concentration of the cofactor was kept constant (10nM) while the substrate concentration was varied (25nM to 4µM). The results are shown in Figure 4.4 and Table 4.2 and demonstrate that factor Va WT (filled squares), factor Va 306 (filled inverted triangles), factor Va 506 (filled triangles), and factor Va 306:506 (filled circles) have similar K_m values indicated similar affinities for the substrate, prothrombin. Factor Va_i 506 (open triangles) and factor Va_i 306:506 (open circles) also have K_m values close to the wild type factor Va. These results suggest cleavage that the first cleavage at Arg 506 does not have an effect on binding the substrate. However, when the cleavage site at Arg 306 is not present in factor Va_i 306 (open inverted triangles) and it can only be cleaved at Arg 506, it causes a slight decrease in the affinity for the substrate. Likewise, factor Va_i 306:506 (open circles) also has an increased K_m value.

The catalytic efficiencies were also calculated from the prothrombin titrations with limiting concentrations of factor Xa and the results are shown in Figure 4.5. The results show that all the recombinant factor Va/Va_i species with the exception of factor Va_i WT and factor Va_i 506 have similar k_cat values compared to the wild type cofactor in the presence of APC under the experimental conditions used. Factor Va_i WT has a dramatically decreased catalytic efficiency as to be expected, with a 40-fold reduction in the k_cat, while factor Va_i 506 only had a 2-fold reduction in the catalytic efficiency of prothrombin activation.

4.4.6 Prothrombin Titrations with Limiting Concentrations of Factor Va/Va_i

Next, the ability of limiting concentrations of the recombinant factor V/Va molecules before and after APC inactivation in the presence of excess amounts of the enzyme,
Prothrombin Titrations With Limiting Concentrations of Factor Xa

![Graph showing prothrombin titrations with limiting concentrations of Factor Xa.](image)

**Figure 4.5 Prothrombin Titrations with Limiting Concentration of Factor Xa.** Thrombin generation experiments were carried out as described under “Experimental Procedures” by varying the substrate concentration (25 nM to 4 µM) with 5 pM of factor Xa saturated with the factor Va species. Prothrombinase complex assembled with recombinant purified wild type factor Va (filled squares), factor Va\textsubscript{WT} (open squares), factor Va\textsubscript{306} (closed inverted triangles), factor Va\textsubscript{306} (open inverted triangles), factor Va\textsubscript{506} (closed triangles), factor Va\textsubscript{506} (open triangles), factor Va\textsubscript{306:506} (closed circles), and factor Va\textsubscript{306:506} (open circles). The data shown are the average of three different titrations performed in triplicate with at least three different preparations of purified proteins.
Prothrombin Titrations with Limiting Concentrations of Factor V Species

Figure 4.6 Prothrombin Titrations with Limiting Concentration of Factor Va. Thrombin generation experiments were carried out as described under “Experimental Procedures” by varying the substrate concentration (25 nM to 4 µM) with 10 nM of factor Xa saturated with the factor Va species. Prothrombinase complex assembled with recombinant purified wild type factor Va (filled squares), factor Va\textsubscript{WT} (open squares), factor Va\textsuperscript{306} (closed inverted triangles), factor Va\textsubscript{306} (open inverted triangles), factor Va\textsuperscript{506} (closed triangles), factor Va\textsubscript{506} (open triangles), factor Va\textsubscript{306\,506} (closed circles), factor Va\textsubscript{306\,506} (open circles), and factor Xa alone (closed diamonds). The data shown are the average of three different titrations performed in triplicate with at least three different preparations of purified proteins.
Table 4.2 Kinetic Constants of Recombinant Factor Va Species Before and After APC Inactivation. The dissociation constants of recombinant factor Va species for plasma derived factor Xa were calculated at limiting concentrations of the enzyme as described in “Experimental Procedures”. The $K_m$ constants were determined at described in “Experimental Procedures” using limiting amounts of the enzyme and varying concentrations of substrate.

<table>
<thead>
<tr>
<th>Recombinant Factor V Species</th>
<th>$K_d$ (nM)</th>
<th>$K_m$ (µM) with Limiting Factor Xa</th>
<th>$K_m$ (µM) with Limiting Factor Va</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor Va$_{WT}$</td>
<td>0.221</td>
<td>0.131</td>
<td>0.674</td>
</tr>
<tr>
<td>Factor Va$<em>{i}$$</em>{WT}$</td>
<td>0.143</td>
<td>0.535</td>
<td>0.506</td>
</tr>
<tr>
<td>Factor Va$_{306}$</td>
<td>0.301</td>
<td>1.13</td>
<td>0.606</td>
</tr>
<tr>
<td>Factor Va$<em>{i}$$</em>{306}$</td>
<td>1.24</td>
<td>0.175</td>
<td>0.497</td>
</tr>
<tr>
<td>Factor Va$_{506}$</td>
<td>0.121</td>
<td>0.262</td>
<td>0.414</td>
</tr>
<tr>
<td>Factor Va$<em>{i}$$</em>{506}$</td>
<td>1.09</td>
<td>0.189</td>
<td>0.975</td>
</tr>
<tr>
<td>Factor Va$_{306}/506$</td>
<td>0.299</td>
<td>0.149</td>
<td>0.932</td>
</tr>
<tr>
<td>Factor Va$<em>{i}$$</em>{306}/506$</td>
<td>0.755</td>
<td>0.182</td>
<td>0.569</td>
</tr>
</tbody>
</table>
factor Xa, were studied in an assay employing purified reagents and a chromogenic substrate that probes for thrombin generation. Recent data have shown that in the presence of excess amounts of the enzyme and limiting amounts of the cofactor in the prothrombinase complex results in an enzymatic complex that cleaves prothrombin first at Arg\textsuperscript{271} and has an approximate 5-fold decrease in the affinity for the substrate (nesheim, unpublished data). Our results are consistent with these findings. Factor Va\textsuperscript{WT}, factor Va\textsubscript{306}, factor Va\textsubscript{506}, and factor Va\textsubscript{306/506} show approximately a 5-fold decrease or more in the affinity for the substrate under the experimental conditions used.
Figure 4.7 Comparison of $k_{\text{cat}}$ Values of Recombinant Factor Va Species Before and After APC Inactivation. The values of $k_{\text{cat}}$ were determined using the $k_d$’s and the quadratic equation to determine the saturation concentration of the various recombinant factor Va species.
4.5 Discussion

Proteolytic inactivation of factor Va by APC requires a membrane surface and cleavage at both Arg$_{506}$ and Arg$_{306}$ located in the heavy chain of the active cofactor. Our data demonstrate that inactivation of the procofactor, factor V, and the active cofactor, factor Va, occur at different kinetic rates at the different inactivation cleavages. The proteolytic inactivation of factor V/Va at Arg$_{306}$ appears to occur at different rates in the presence of a membrane surface. When the cleavage site at Arg$_{306}$ is mutated to a glutamine residue it can no longer be cleaved by APC. Analysis of clotting times of factor Va$_{i}^{306}$ and factor V$_{i}^{306}$ demonstrate that the inactivation of the active molecule occurs much slower and retains clotting activity after 30 minutes of incubation with APC in the presence of a membrane surface. On the other hand, inactivation of the procofactor molecule with the mutation at Arg$_{306}$ results in a recombinant molecule with greatly decreased clotting activity in the presence of APC and a membrane surface even after only 15 minutes of incubation.

It has long been established that a single point mutation in the factor V gene which results in a Arg$_{506}$ to Gln replacement is a common risk factor for thrombosis and caused APC resistance in patients. It has been demonstrated in the literature and our present data support that cleavage at Arg$_{506}$ does not result in complete inactivation of factor Va and that Arg$_{306}$ is required for complete inactivation of the cofactor by APC. Therefore, one would presume that mutation at Arg$_{306}$ would cause a severe APC resistance leading to a thrombotic state. On the contrary, patients with this amino acid residue mutated, factor V$^{\text{CAMBRIDGE}}$ (with a Arg$_{306}$ to Thr mutation) and factor V$^{\text{HONGKONG}}$ (with a Arg$_{306}$ to Gly mutation), do not appear to be at high risk for thrombosis.
The double mutant, factor V\textsuperscript{306/506} was vastly resistant to APC inactivation with no considerable loss of cofactor activity under all conditions tested herein. Western blot analysis showed no heavy chain fragments when the cofactor and active cofactor molecules were exposed to APC in the presence of a membrane surface. This clearly demonstrates the importance of APC cleavage sites at Arg\textsuperscript{306} and Arg\textsuperscript{506} and that cleavage at Arg\textsuperscript{679} does not play a role in APC inactivation of factor Va under the experimental conditions employed. This could be because cleavage at Arg\textsuperscript{679} does not occur in the absence of cleavage at Arg\textsuperscript{306} and Arg\textsuperscript{506} or it does not contribute to inactivation.

There are conflicting results described in the literature in relation to the cleavage sites of APC catalyzed inactivation of factor Va. Kolfschoten et al. report that factor Va can be inactivated by APC in the absence of cleavage site at Arg\textsuperscript{306}, Arg\textsuperscript{506}, and Arg\textsuperscript{679} (22). This laboratory constructed recombinant factor V molecules with the cleavage sites at Arg\textsuperscript{306}, Arg\textsuperscript{506}, and Arg\textsuperscript{679} mutated using constructs with a large portion of the B domain deleted claiming the B domain of the cofactor does not play a crucial role in the inactivation of factor Va. This contradicts preliminary data from our laboratory showing the importance of the B region in factor Va inactivation (unpublished data). Their data demonstrate that a recombinant molecule with all three APC inactivation cleavage sites mutated is still able to be inactivated under their experimental conditions, while our data clearly demonstrates the importance of cleavage at Arg\textsuperscript{306} and Arg\textsuperscript{506}. This difference could be in the experimental conditions used to inactivate the cofactor with APC; our laboratory uses small concentrations of thrombin to activate the procofactor and small concentrations of APC to inactivate the active cofactor with a 30 minute incubation time,
whereas their laboratory uses large amounts of thrombin and APC in their experiments with a three hour incubation time. This difference in enzyme concentrations may explain the dramatic difference in results; spontaneous degradation of the purified recombinant cofactor cannot be ruled out due to their prolonged incubation times. Moreover, their data show a truncated heavy chain with residues 1-643 generated following activation with thrombin. This can be alternatively explained by the fact that under prolonged exposure to thrombin, factor Va can further be cleaved at Arg$^{643}$ (unpublished data, Kalafatis et al.).

Overall, our data demonstrate the importance of factor Va APC inactivation cleavage sites at Arg$^{306}$ and Arg$^{306/506}$. We show a recombinant factor Va molecule with both these cleavage sites removed is unable to be proteolyically cleaved by APC in the presence of a membrane surface following prolonged exposure.
4.6 References


5.1 Overall Conclusion

In view of the fact that heart disease is the number one killer of men and women in the United States and the current anti-coagulant medical therapies possess many serious side effects, more knowledge of the specific molecular mechanisms of the proteins involved in the blood coagulation cascade is needed. The penultimate step in the blood coagulation cascade is the activation of prothrombin by the prothrombinase complex. In order to find a better, more specific anti-coagulant drug, we have further defined the mechanism by which factor Va exerts its cofactor function and increases the catalytic efficiency of the prothrombinase complex. The advantageous molecular mechanisms of factor Va cofactor function are currently unknown. It has been suggested that the binding of factor Va to factor Xa exposes cryptic exosites on the
enzyme improving its catalytic function. One likely mechanism is that the incorporation of factor Va into the prothrombinase complex rearranges the enzyme and substrate into a position where efficient catalysis can occur. The residues involved in binding to the substrate, prothrombin, may serve as a guide for the design of a more specific anti-coagulant drug that only targets the members of the prothrombinase complex. The goal is to reduce the side effects of the current anti-coagulants on the market.

Factor Va binding sites for factor Xa have been identified on both the heavy and light chains of the cofactor (16, 32, 37, 112). While the binding sites on the light chain of factor Va remain to be identified, the binding sites on the heavy chain have been well studied. Our laboratory has demonstrated amino acid residues Glu\textsuperscript{323}, Tyr\textsuperscript{324}, Glu\textsuperscript{330}, and Val\textsuperscript{331} located on the A2 domain of the factor Va heavy chain are vital for expression of factor Va cofactor activity and contain a binding site for factor Xa (112). In addition, our laboratory has shown that mutation at one or two of the factor Xa binding sites has no major consequence on the kinetic parameters of prothrombinase assembly and function, however mutation of all four of these residues results in a recombinant molecule unable to efficiently convert prothrombin to thrombin (131).

Collectively, our data presented herein further establish the role of residues from the A2 domain of factor Va heavy chain in prothrombinase assembly and function. We have established that amino acid residues Glu\textsuperscript{334} and Tyr\textsuperscript{335} are necessary for optimal rearrangement of the enzyme and substrate for proficient prothrombin activation. Specifically, our data illustrate that substitution of amino acid residues
334-335 (factor $\text{Va}^{\text{KF}}$ and factor $\text{Va}^{\text{AA}}$) are enough to have an effect on cofactor function, nevertheless it takes mutating four residues (factor $\text{Va}^{\text{MI/KF}}$, factor $\text{Va}^{\text{MI/AA}}$, factor $\text{Va}^{\text{FF/KF}}$, and factor $\text{Va}^{\text{FF/AA}}$) to entirely eliminate factor $\text{Va}$ cofactor activity. Kinetic analysis with these molecules show decreased affinity for the enzyme, factor Xa, and dramatically reduced catalytic efficiencies.

Furthermore, our laboratory has confirmed the COOH-terminal portion of the factor $\text{Va}$ heavy chain is important for the activation of the procofactor by thrombin and its interaction with prothrombin under physiological conditions within the prothrombinase complex. Analysis of a peptide $^{695}\text{DYQ}^{699}$ binds prothrombin in a competitive manner with the prothrombinase complex (25). We examined the ability of amino acid motifs $^{334}\text{DY}^{335}$ and $^{695}\text{DY}^{698}$ to act in concert and allosterically regulate prothrombinase assembly and function. Allosteric regulation is the regulation of an enzyme by binding of a cofactor molecule, in this case factor $\text{Va}$, to the enzyme at its allosteric site, or a site other than the active site of the molecule. Our data presented currently show that recombinant factor $\text{Va}$ molecules with mutations at the aforementioned residues (factor $\text{Va}^{3K}$, factor $\text{Va}^{4A}$, and factor $\text{Va}^{6A}$) have a noteworthy consequence on cofactor activity. In particular, kinetic investigation with these molecules have a decreased catalytic efficiency. Molecular modeling data from our laboratory has also shown that residues 334-335 and 695-698 are located on the surface of the heavy chain and are in close proximity to each other. Taken together these observations support the conclusion that amino acid residues 334-335 work together with 695-698 and are indispensable to factor $\text{Va}$ cofactor function and timely activation of prothrombin by the prothrombinase complex.
Since the blood coagulation system has to maintain a delicate balance between thrombosis (blood clotting) and haemorrhage (excessive bleeding), the details of the down regulation of the coagulation cascade have been under intense investigation. Once enough thrombin is produced to form the fibrin plug at the site of vascular injury, it activated protein C to its active form, activated protein C (APC). APC subsequently goes on to inactivate factor Va by proteolytic cleavages so it can no longer participate in the prothrombinase complex, therefore ceasing thrombin production. Factor Va is cleaved by APC at arginine residues 306, 506, and 679 on the heavy chain of the active cofactor. In addition, the procofactor molecule can also be cleaved by APC at residues Arg^{306}, Arg^{506}, Arg^{679}, and Lys^{994}. Our data demonstrate a different rate of inactivation between factor V and factor Va in the presence of a membrane surface. Factor V_{i}^{306} retains clotting activity after a 30 minute incubation APC and lipids; on the other hand, factor V_{i}^{306} was rapidly inactivated and had a greatly decreased clotting activity even after a 15 minute incubation. These results are consistent with patients that have an Arg^{306} mutation, in that they do not appear to have a high risk for thrombosis. Overall the data presented suggest the significance of APC inactivating cleavages Arg^{306} and Arg^{506} on the factor Va heavy chain for complete inactivation and the down regulation of the coagulation cascade.

Overall, the ultimate goal of our research is to define the molecular mechanism of the incorporation of factor Va into the prothrombinase complex as it relates to the advantageous process of hemostasis and the detrimental phenomena of thrombosis. The research presented herein brings us one step closer to this goal, we have shown
that the expression of factor Va cofactor activity is required for the rearrangement of
the enzyme and substrate within the prothrombinase complex necessary for efficient
prothrombin catalysis. We expect this research will lead to the design of a specific
peptide or small molecule that will inhibit the procoagulant activities of thrombin
production.