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Modeling the Human Prothrombinase Complex Components

Tivadar Orban

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MODELING THE HUMAN PROTHROMBINASE COMPLEX COMPONENTS

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Submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY
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at the
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May, 2008
to my dear son Alex
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Tivadar Orban, March 2008
Thrombin generation is the culminating stage of the blood coagulation process. Thrombin is obtained from prothrombin (the substrate) in a reaction catalyzed by the prothrombinase complex (the enzyme). The prothrombinase complex is composed of factor Xa (the enzyme), factor Va (the cofactor) associated in the presence of calcium ions on a negatively charged cell membrane. Factor Xa, alone, can activate prothrombin to thrombin; however, the rate of conversion is not physiologically relevant for survival. Incorporation of factor Va into prothrombinase accelerates the rate of prothrombinase activity by 300,000-fold, and provides the physiological pathway of thrombin generation. The long-term goal of the current proposal is to provide the necessary support for the advancing of studies to design potential drug candidates that may be used to avoid development of deep venous thrombosis in high-risk patients. The short-term goals of the present proposal are to (1) to propose a model of a mixed asymmetric phospholipid bilayer, (2) expand the incomplete model of human coagulation factor Va and study its interaction with the phospholipid bilayer, (3) to create a homology model of prothrombin (4) to study the dynamics of interaction between prothrombin and the phospholipid bilayer.
CITATIONS

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ABBREVIATIONS

323EY324 – a peptide with the sequence EY (part of factor Va)

330EV331 - a peptide with the sequence EV (part of factor Va)

332IWDYA336 - a peptide with the sequence IWDYA (part of factor Va)

334DY335 - acid residues D334 and Tyr335 from factor Va primary sequence

ABE-I – anion binding exosite I of thrombin

ABE-II - anion binding exosite I of thrombin

APC - Activated Protein C

Bsu361 - restriction enzyme

D5Q1,2 – a peptide with the DYDYQ sequence and having the Tyr residues are sulfated

DOPC - dioleoyl-sn-glycero-3-phosphatidylcholine

DPPC - dipalmitoyl-sn-glycero-3-phosphatidylcholine

DYDYQ - a pentapeptide with an identical sequence from factor Va's Asp695 to Gln699 region

EGF1 - epidermal growth factor 1

EGF2 - epidermal growth factor 2

EMBOSS - European Molecular Biology Open Software Suite

factor Va KF forward primer - 5’-C ATT TGG AAG TTT GCA CCT G-3’

factor Va KF forward reverse primer 5’-C AGG TGC AAA CTT CCA AAT G-3’

factor Va3/5/6 – a factor Va molecule cleaved at all three APC cleavage sites. i.e., Arg306, Arg506, and Arg 679

factor Va3/6 – a factor Va molecule cleaved at Arg306 and Arg679.

factor Va679 – a factor Va molecule cleaved atArg306 and Arg506
factor VaAA - a factor Va molecule with amino acid residues 334 and 335 changed to Ala
factor VaAA - forward primer 5’-GAG GAA GTC ATT TGG GCC GCC GCA CCT GTA ATA- 3’
factor VaAA - forward reverse primer 5’-TAT TAC AGG TGC GGC GGC CCA AAT GAC TTC CTC-3’
factor VaControl – the corresponding notation for wild type factor Va, i.e., no amino acid changes
factor VaKF - a factor Va molecule with amino acid residues 334 and 335 changed to Lys and Phe, respectively
factor VaLEIDEN - a factor Va molecule having the Arg506 changed to Gln.
factor VKF/FF - D334→K/Y335→F and E323→F/Y324→F
factor VKF/MI (D334→K/Y335→F and E330→M/V331→I)
GLA - gamma carboxyglutamic acid
HFV#17- antibody against factor Va
HFV#9 - antibody against factor Va
Hir54-65 – hirudin peptide
Hir54-65 (SO3-) – sulfated hirudin peptide
IC50 – half maximal inhibitory concentration
MD - molecular dynamics
MSD - mean square displacement
MzIIa-desF1 – a meizothrombin molecule lacking its fragment 1 region
N42R - a peptide sharing sequence identity with amino acid region
NMR - nuclear magnetic resonance
NPT - a simulation with constant number of atoms, pressure, and temperature
NVT - a simulation with constant number of atoms, volume, and temperature
P15H – control peptide
PAR-1 - protein-activated receptor 1
PCR - polymerase chain reaction
pf1 - prothrombin fragment 1
pf1Sol – simulation of pf1 in solution
pf1Sol/Ca – simulation of pf1 in solution and calcium ions
pf1Sol/Ca/Lipid – simulation of pf1 in solution, calcium and phospholipids
pf1Sol/Na – simulation of pf1 where the calcium ions of the GLA domain were replaced with sodium ions
pGEM-T - plasmind used to clone factor Va
POPC - 1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphatidylcholine
POPC:POPS - an asymmetric/mixed lipid bilayer composed of POPC only in the inner leaflet and POPC, POPS in the outer leaflet
POPE - 1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphatidylethanolamine
POPS - 1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphatidylserine
PR-MD - position restraint molecular dynamic simulation
PS - phosphatidylserine
RMSD - root mean square displacement
rMZ-II – recombinant meizothrombin molecule
Scd - deuterium order parameter
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SPC - single point charge

TIP4P - four points water model

VKD - vitamin K dependent

Xcm1 - restriction enzyme
CHAPTER I

Introduction to hemostasis

1.1 Introduction to hemostasis

Blood coagulation is the natural response the body renders following physical or chemical injury. The generally employed naming of the process, blood coagulation cascade, should not be regarded as a cascade of events, but rather as a highly interconnected network that has several up and down-regulator nodes, see figure 1.1. The process of blood clotting, clot dissolution, and repairing of the injured tissue is called homeostasis. Following vascular rupture, normal homeostasis involves a series of four processes described below.

Vascular constriction that is mainly important to arrest bleeding that occurs in large arteries. In this case, vascular constriction represents an important process because solely the fibrin plug formation and the platelet adhesion will not suffice.
Figure 1.1 The blood coagulation event.

The figure shows the up (procoagulant in green) and down-regulation (anticoagulant in red) processes that finely regulate the enforcement of the loose platelet plug through the local generation of the fibrin mesh.
The loose plug is formed following platelet activation and aggregation. Activation of platelets proceeds through binding of collagen to its receptor on platelets, the $\alpha_2\beta_1$ integrin [1, 2]. Thrombin generated on the platelets followed by binding to its receptor, protease activated receptor-1 (PAR-1), represents another important event in platelet activation. On activated platelets the $\alpha_{IIb}\beta_3$ integrin represents a receptor for fibrinogen, von Willebrand factor, thrombospondin, fibronectin, and vitronectin [3]. Binding of fibrinogen $\alpha_{IIb}\beta_3$ integrin promotes platelet aggregation during the loose plug formation. Propagation of platelet activation is achieved through shape change, flip-flop of the inner leaflet resulting in phosphatidylserine and P-selectin exposure, and the release of platelet granular constituents.

Loose plug enforcement is a requirement for a stable blood clot formation. The aggregated platelet plug is unstable and it will break away in a couple of hours resulting in late bleeding. Strengthening of the loose platelet plug by the polymerizable fibrin begins with the sequential activation of the coagulation factors. Figure 1.1 shows the sequential activation of the enzymes required to produce the insoluble fibrin plug. The conversion of fibrinogen to fibrin starts with the exposure of factor VIIa, present in blood, to its transmembrane protein receptor tissue factor. The complex between tissue factor and factor VIIa is the enzyme that catalyzes the conversion of factor X and IX to factor Xa and IXa, respectively, by limited proteolysis. Factor Xa (the enzyme) associates with factor Va (the cofactor) on a cell surface in the presence of the $Ca^{2+}$ ions to form the prothrombinase complex. The prothrombinase complex is the enzyme that catalyzes the conversion of prothrombin (factor II) to thrombin (factor IIa). The formed thrombin further activates more factor V to factor Va playing an important role in its generation.
and its own up-regulation event.

Dissolution of the fibrin plug is governed by the activation of plasmin. Plasminogen is the inactive form of plasmin and gets incorporated into the fibrin mesh. By this procedure the dissolution of the fibrin clot is ensured to progress from the inside out.

1.2 The prothrombinase complex.

1.2.1 Factor V/Va structure.

Factor V is the precursor of factor Va and circulates in blood as a high molecular weight inactive procofactor ($M_r$ 330,000) [4]. To date, factor Va does not have a complete crystal structure. Structural data available for factor Va can be summarized as follows: a two-dimensional projection map obtained using electron microscopy [5], a homology model that uses ceruloplasmin as a template (consisting of 994 residues from Ala1 - Cys656 and Ser1546 - Met1883), that is part of the A domains [6], a homology model of the C1 and C2 domains using galactose oxidase binding domain as a template [7], an X-ray structure of the C2 domain [8], a homology model that lacks only 46 amino acids (Arg664 - Arg709) from the C-terminal [9], and finally, the Activated Protein C (APC) inactivated bovine factor Va obtained by X-ray diffraction. The later crystal structure reported a different spatial arrangement for the C1 and C2 domains, and challenged the long-standing belief that the heavy and light chains are held together by the Ca$^{2+}$ ions [10].

1.2.2 Activation of factor Va.

While proteases such as factor Xa [11, 12], snake venom enzyme (RVV-V activator) [13], and plasmin [14] (also inactivates factor Va) have the capability to
activate factor V, the physiologically relevant enzyme is $\alpha$-thrombin. $\alpha$-Thrombin activates factor V through the removal of the B region following limited proteolysis at Arg709, Arg1018, and Arg1545. Factor Va is composed of a heavy chain (Ala1 - Arg709), and a light chain (Ser1546 - Tyr2196) [15]. The heavy and the light chain are non-covalently associated in the presence of divalent metal ions.

1.2.3 Inactivation of factor Va.

Activated Protein C (APC) will inactivate both factor Va (see figure 2) and factor VIIIa which results in a decreased $\alpha$-thrombin production. While under physiologic conditions factor VIIIa will be inactivated spontaneously in the absence of APC, through the dissociation of the heavy and light chain, inactivation of factor Va is APC dependent.

1.2.4 Coagulation Factor X/Xa.

Factor X/Xa structure. Factor X (a vitamin K dependent (VKD) plasma zymogen) is the precursor of factor Xa and circulates in blood as a glycoprotein (Mr 59,000). Factor X is composed of two chains: the heavy chain containing 306 amino acids (Ile195 - Lys448) and a light chain containing 139 amino acids (Ala1 - Arg139) [16]. The heavy and light chains are held together by a disulfide bond [17]. The zymogen gets activated to its functional form; factor Xa, through the removal of the activation peptide that connects Arg139 (the C-terminal end of the light chain) with the N-terminal Ile195 of the heavy chain. The Ala1-Gla39 region is $\gamma$-carboxyglutamic acid (GLA)-rich domain and contains 11 GLA residues. This region is followed by a hydrophobic region (Phe40-Lys45) and two epidermal growth factor like domains: EGF1 (Asp46-Phe84) and EGF2 (Thr85-Gly128) [16]. The heavy chain of factor Xa contains the protease domain of 254 amino
acids (residues Ile195-Lys448) and features the catalytic triad residues of His236 (His57\textsuperscript{\#}), Asp282 (Asp102\textsuperscript{\#}), and Ser379 (Ser195\textsuperscript{\#})

Factor Xa alone has the capability to distinguish and process (through limited proteolytic cleavages) several substrates such as: factor X \[18\], prothrombin, factor V \[11, 12\], factor VII \[19\], factor VIII \[20\], and factor IX \[21\]. As a consequence of these relatively wide spectra of recognized substrates, factor Xa alone has relatively small substrate specificity. Its full enzymatic activity and specificity is achieved only after its incorporation in the prothrombinase complex together with factor Va.

1.2.5 Interaction of factor Va with factor Xa.

Studies have shown that factor Va interacts with factor Xa through both its heavy and light chain. While the exact location of the amino acid residues involved in the interaction with factor Xa from the light chain remain to be identified several such amino acid regions from the heavy chain were reported \[22-25\]. Amino acid residues Arg347, Arg306, and Lys414 (165, 125, and 230 in chymotrypsin numbering, respectively) from the factor Xa sequence were also reported to be important in the interaction with factor Va \[26\]. Another cluster of amino acids from the factor Xa sequence was proposed to contain a factor Va binding site at 404-418 (231 - 244 in chymotrypsin numbering) \[27\].

\footnote{chymotrypsin numbering}
Activation of factor V by thrombin is the result of three sequential cleavages at Arg709, Arg1018, and Arg1545.

Figure 1.3 Inactivation of Factor Va.

Inactivation of factor Va by APC is the result of three sequential cleavages at Arg306, Arg506, and Arg679.

1.2.6 Interaction of factor Va with prothrombin.

It has been proposed that the C-terminal of the factor Va heavy chain, i.e. amino acid residues Asp683 - Arg709, contains an interactive site for prothrombin [28]. This region, with a large number of acidic amino acid residues, also contains hirudin like motifs. Specifically, Tyr696 and Tyr698 are two of the tyrosine residues that were proposed to be potential sulfation sites [29, 30]. This post-translational modification occurs in the trans-Golgi network [31] and is required for the cofactor function and also in the interaction with prothrombin/thrombin. Studies using overlapping peptides that encompass the Asp683 - Arg709 region showed that the peptide that shares sequence similarity with Asp695 - Gln699 (DYDYQ) inhibits prothrombinase function. [32]. The quadruple mutant factor Va Asp695,697 → Lys and Tyr696,698 → Lys was found to form a prothrombinase complex that had lowered enzymatic efficiency. This strongly suggests that a prothrombin binding site exists in the Asp695 - Gln699 region of factor Va. A group of positively charged residues from thrombin’s anion binding proexosite-I was found to be involved in the factor Va - prothrombin interaction [33, 34]. Several mutations in this region were also found to impede binding of factor Va to prothrombin [35].

1.2.7 Interaction of factor Xa with prothrombin.

Interaction sites between factor Xa and prothrombin were reported to involve Lys96 [36]. In addition, synthetic peptide studies showed that amino acids residues 557 - 571 in prothrombin and 415 - 429 in factor Xa orchestrate the interaction between factor Xa and prothrombin in the prothrombinase complex [27].

1.2.8 Interaction of factor Va with phospholipid vesicles.
It is believed that factor Va interacts with phospholipid vesicles through its light chain. The interactions are believed to be of electrostatic nature in the early phase of the binding whereas hydrophobic interactions have a role in keeping factor Va trapped in the hydrophobic core of the lipid bilayer.

Several interaction residues were characterized to date such as: the central region of the A3 domain of bovine factor V (amino acid residues 1667-1765 [37]), the amino acid residues located in the C1 domain (Tyr1956 and Leu1957) [38] and C2 domain (Trp2063 and Trp2064) [39].

1.2.9 Interaction of factor Xa with phospholipid vesicles.

It has been proposed using short chain soluble phospholipids that a lipid binding site on factor Xa requires both the γ-carboxyglutamic acid rich region and the EGF region of factor Xa [40].

1.2.10 Interaction of prothrombin with phospholipid vesicles.

Prothrombin binds to the negatively charged phospholipid surface through its ω-loop rich in γ-carboxyglutamic acids (Ala1 - Thr45). Specifically, it has been demonstrated that Trp4 is inserted 5 to 7 Å into the hydrophobic region of the lipid bilayer [41]. Interaction of prothrombin with phospholipids was also shown to induce conformational changes in the molecule.
Figure 1.4 Conversion of prothrombin to thrombin.

Activation of prothrombin can follow two pathways. Pathway I is factor Va independent whereas pathway II is factor Va dependent.

Bukys et. al. (2006) J. Biol. Chem. 39194-39204
1.2.11 The prothrombinase complex.

The protease in the prothrombinase complex is factor Xa. While factor Xa alone can catalyze the conversion of prothrombin to thrombin, the rate is physiologically insignificant (see table I). Considering the rate of factor Xa conversion of prothrombin to thrombin the unit, the lipid bound factor Xa in the presence of Ca\(^{2+}\) is 30 times faster. In the presence of factor Va and Ca\(^{2+}\) the rate of prothrombin activation increases 300 times. Finally, the lipid bound factor Xa - factor Va assembly in the presence of the Ca\(^{2+}\) ions produces the prothrombinase complex that functions with a rate of five orders of magnitude faster than factor Xa alone. Inclusion of factor Va in the prothrombinase complex has the effect in the increase of the catalytic efficiency of the enzyme.

<table>
<thead>
<tr>
<th>Component</th>
<th>Prothrombin activation rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor Xa</td>
<td>1</td>
</tr>
<tr>
<td>Ca(^{2+}), Factor Xa, PCPS</td>
<td>30</td>
</tr>
<tr>
<td>Ca(^{2+}), Factor Xa, Factor Va</td>
<td>300</td>
</tr>
<tr>
<td>Ca(^{2+}), Factor Xa, Factor Va, PCPS</td>
<td>300,000</td>
</tr>
</tbody>
</table>

Table I. Prothrombin activation rates.

The fact that the enzymatic process takes place on the membrane surface has the outcome that the Michaelis-Menten constant of the reaction is lowered through the increase of the substrate, i.e., prothrombin concentration at the site of reaction. Two reaction pathways were identified for the conversion of prothrombin to thrombin (see figure 1.4) [42]. Pathway I (the prothrombin 2 pathway) is favored when prothrombin is converted to thrombin in the absence of factor Va. In the absence of factor Va
prothrombin is cleaved at Arg271 which results in the formation of fragment 1•2 and prethrombin 2. In the next step Arg320 is cleaved resulting in the accumulation of mature α-thrombin. On the other hand in the presence of factor Va activation of prothrombin to thrombin follows pathway II (the meizothrombin pathway). In this case the cleavage at Arg320 occurs first which results in the formation of meizothrombin. Generation of the mature α-thrombin is followed after the cleavage at Arg271.

While the prothrombinase complex has been extensively characterized kinetically, little is known about its structure. This is not the case for other complexes involved in the coagulation process such as the factor VIIa-tissue factor-factor Xa complex [43, 44], factor VII-tissue factor-factor IX complex [45], and the factor VIIIa-factor IXa [46] complex whose three-dimensional models were reported.

1.3 Homology modeling.

Homology modeling represents an attractive alternative method to propose three-dimensional models of proteins that share sequence homology with proteins that have a known structure, i.e., resolved by either X-ray crystallography or nuclear magnetic resonance.

The methodology to be used in the creation of the homology models is an automated approach to solve spatial restraints [47]. Shortly, the modeling procedure requires as an input parameter the primary sequence alignment of the target protein (the one that needs to be modeled) with the template protein (the one with the known three-dimensional structure). The algorithm produces the three-dimensional model of the target protein containing all heavy atoms, i.e., no hydrogen atoms.
Two high quality protein modeling programs shall be used: SwissPdb Viewer [48] and Modeller [47]. The alignments of the primary sequence between the target and the final model shall be performed using the EMBOSS software suite [49].

1.4 Molecular dynamics (MD) simulation.

Molecular dynamics is a valuable tool to study the dynamics of a system that is the position of every atom as a function of time by implementing an algorithm that solves in an iterative fashion Newton’s classical equation of motion shown below:

\[ F_i = m_i a_i = m_i \frac{d^2 r_i}{dt^2} = -\frac{\partial V}{\partial r_i} \]

Further, the force that acts on each atom can be calculated as the negative derivative of the potential energy with respect to the location of each atom with the potential energy of a system and the coordinates of a starting structure, e.g., a crystal structure, and a set of velocities, one can calculate the force that acts on a specific atom followed by the computation of the new position. Repeating this will generate the change of coordinates as a function of time.

A simple diagram to illustrate the essence of the molecular dynamics procedure is show bellow:

\[ \text{state 1} \rightarrow \text{state 2} \]
\[ r_i^1 \rightarrow r_i^{1.1} \rightarrow r_i^{1.2} \ldots \ldots \rightarrow r_i^2 \]
\[ t \rightarrow t + \Delta t \rightarrow 2(t + \Delta t) \]

1.4.1 High temperature driven conformational search.

The only difference between high temperature driven conformational search and production MD is the temperature that is set to 400 K or higher. This allows for the
molecule in study to receive enough energy to overcome the energy barrier between a local and the global minimum.

1.4.2 Trajectory analysis.

The most highly desired thing in molecular dynamics simulations is a long simulation time. Checking equilibration of a system is required before one attempts to draw conclusions from a simulation. Some of the parameters that shall be checked are: temperature should oscillate around the desired temperature, the system should have constant pressure, for an NPT simulation, in the designated time range, the root mean square displacement of the atoms positions regarding the initial structure should contain periodic patterns, most of the time a plateau. If the system did not reach to an equilibrium simulation time shall be extended appropriately. From the multitude of analysis that can be performed on a simulation trajectory, documented in detail elsewhere [50], some are routinely performed such as: distance monitoring, secondary structure preservation, energy, pressure, temperature variation in function of simulation time, and root mean square displacement. Analyses of the simulation trajectory for proteins shall check: persistence of secondary structure, and heavy atoms, i.e., Ca$^{2+}$ and Cu$^{2+}$, coordination, and if RMSD has reached a plateau.

For the lipid bilayer several properties have to be checked such as: if the bilayer remains stable and intact, the average packing area per lipid for the gel phase, a component specific characteristic (simulation temperature set to 300 K), and the mean bilayer thickness.

1.4.3 Free energy perturbations.

The fundamental thermodynamic state function for Gibbs free energy, $G$,
considering an NPT ensemble, can be written as follows:

\[
\frac{\partial G(\lambda)}{\partial \lambda} = -k_BT \left[ \frac{\partial \Delta(\lambda)}{\partial \lambda} \right] = \left< \frac{\partial H(\lambda)}{\partial \lambda} \right> \lambda
\]

Integration of equation above between the two states described by the Hamiltonians (H) of these states at \( \lambda = 0 \) and \( \lambda = 1 \) can be used to determine the Gibbs free energy, that is:

\[
\Delta G = G(\lambda = 1) - G(\lambda = 0) = \int_0^1 \left[ \frac{\partial G(\lambda)}{\partial \lambda} \right] d\lambda = \int_0^1 \left< \frac{\partial H(\lambda)}{\partial \lambda} \right> \lambda d\lambda
\]

The thermodynamics integration procedure makes use of the thermodynamic cycle shown in figure 1.5. Let us first consider the simple case when one needs to calculate the difference in the free energy of binding between two inhibitors \( I_1 \) and a \( I_2 \). The thermodynamic cycle is shown in figure 1.5 panel A. The first step is to grow the perturbation, step \( I_1 \rightarrow I_2 \). For the sake of simplicity and as a preamble to test aim 2, hypothesis b, the structural difference between \( I_1 \) and \( I_2 \) is that \( I_2 \) contains one sulfated tyrosine residue. This is illustrated in figure 1.5 panel B, where two tyrosine residues are shown. The left side tyrosine is not sulfated but shows (using a yellow boundary) the future \( SO_3^- \) group to be grown. The place of the tyrosine residue (figure 1.5, panel B) is now taken by the sulfated tyrosine. The grey boundary shows the grown atoms, i.e., three oxygen atoms and one sulphur atom. The interaction parameters for the sulfated tyrosine residue are increased from \( \lambda = 0 \) (without \( SO_3^- \)) to \( \lambda = 1 \) (\( SO_3^- \) being present - that is the sulfated tyrosine residue). Thus, one has to run at least twenty simulations with ranging from 0 to 1 and using a \( \lambda_{\text{step}} = 0.05 \). Next, if the resultant values of \( \left< \frac{\partial H(\lambda)}{\partial \lambda} \right> \lambda \) can be plotted versus, the area under the graph, calculated using the trapezoid rule represents
the difference in free energy of binding between the states I1 → I2 (i.e., ΔG₄). Solvation (i.e., going from vacuum to solution) free energies shall be calculated using the same procedure but this time the λ parameter shall be used to fade the effect produced by the entire ligand on its environment.

1.5. Hardware and Software.

Three dual Apple G5 servers are available, all running under the Mac OSX 10.3.9 operating system. The GROMACS [50-52] version to be used is 3.2.3 or newer versions, compiled using the LAM-MPI environment. Other software programs include Pymol [53], Modeller [47], SwissPDB Viewer [48].
Figure 1.5 General thermodynamic cycle and procedure for calculation of $\Delta G_3$.

Panel A shows a general thermodynamic cycle of two different inhibitors (I1 and I2). Panel B depicts how the difference in the free energy of binding between the I1 and I2 inhibitors is calculated. The “growing and ‘fading’ procedure illustrated for the perturbation of the $SO_3^-$ group of the sulfated Tyr residue as follows: atoms in the gray region are present whereas atoms in the yellow region will fade or will be grown. The amino acid in the left side of the mirrored image is Tyr. In this case the S and the O atoms (yellow area) are not present. The sulfated Tyr residue is in the left side of the mirror image. The H and the O atoms (now in the yellow area) are replaced by the S and the O atoms (gray area). Panel C shows the conformations at $\lambda = 0$ (left) and at $\lambda = 1$ (right).
1.6 References


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trp2064 in the factor Va c2 domain are required for high-affinity binding to phospholipid membranes but not for assembly of the prothrombinase complex. Biochemistry, 2004. 43: p. 4385-4393.


CHAPTER II

Modeling the asymmetric/mixed POPC:POPS lipid bilayer

2.1 Abstract

Conversion of prothrombin to thrombin represents the penultimate step in the formation of the cross-linked fibrin clot. The reaction takes place on the phospholipid surface and is catalyzed by the prothrombinase complex. It has been long recognized that exposure of the POPS molecules to the outer leaflet of the membrane represents a requirement for efficient conversion of prothrombin to thrombin. We constructed an asymmetric phospholipid surface mimetic composed of a random mixture of 1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) and 1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphatidylserine (POPS) in the outer leaflet using a 4:1 ratio. The inner leaflet was composed of only POPC molecules. We next performed 60 ns molecular dynamics (MD) simulations on the asymmetric/mixed POPC:POPS phospholipid bilayer model. Such an asymmetric/mixed POPC:POPS lipid bilayer where the POPS molecules are localized on
the outer leaflet was constructed to mimic the activated platelet surface. The POPC:POPS lipid bilayer model was than validated by comparing the results from the MD simulation with nuclear magnetic resonance studies. First, our studies present for the first time an asymmetric/mixed POPC:POPS lipid bilayer model. Second, we show that conformational changes in prothrombin fragment 1 are due to the binding of calcium and not because of the presence of the phospholipids. The interaction of factor Va with the POPC:POPS lipid bilayers was also investigated. In the case of factor Va interaction the simulation was able to reproduce the binding event - the insertion of amino acid residues form factor Va's C2 domain into the hydrophobic region of the lipid bilayer (see Chapter III). Furthermore, our simulations show that removal of the calcium coordinated by the N-terminus of pf1 induces a considerably larger displacement as compared with the displacement calculated from the simulations of pf1 with calcium, the simulations of pf1 with calcium and phospholipids, and finally the simulations with pf1 where calcium ions were replaced with sodium ions (see Chapter IV).

2.2 Introduction

Human prothrombin is a VKD protein and is the precursor form of thrombin, the protease that holds a central role in the optimal regulation of the blood coagulation event. Although factor Xa can activate prothrombin to thrombin, the physiologically required activation rate is achieved only through the assembly of the fully functional prothrombinase complex (factor Xa – the enzyme, factor Va – the cofactor associated in the presence of the activated cell surface and calcium ions) [1]. The increase in enzymatic efficiency of the prothrombinase complex is attributed to the decrease of the Km
(following the interaction with phospholipid vesicles resulting in higher local substrate concentrations) and an increase in $k_{\text{cat}}$ (solely attributed to the inclusion of factor Va in the prothrombinase complex) [2-4].

Molecular dynamics simulations were found to be particularly useful in the study of lipid bilayers and agree fairly well with NMR and electron diffraction studies. Several lipid systems were successfully characterized to date such as those composed of dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) [5], dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) [6], 1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) [7], 1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphatidylserine (POPS) [8], 1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphatidylethanolamine (POPE). Several mixed lipid bilayer systems were characterized to date such as 1-Palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PDPC:POPC), dioleoyl-phosphatidylcholine: dioleoyl-phosphatidylglycerol DOPC:DOPG and di-palmitoylphosphatidyl-serine: di-palmitoylphosphatidyl-choline DPPS:DPPC [9-11]. Recently, one study also proposed an asymmetrical model of the DPPS:DPPC lipid bilayer [12].

Several models of the enzymatic complexes that participate in the blood coagulation event were successfully created [13-17]. However, these models still lack a vital component: the negatively charged phospholipid vesicles. In the current work we propose for the first time a 60 ns equilibrated mixed 1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) and 1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphatidylserine (POPS) in a 4:1 ratio in the outer leaflet. The inner leaflet contained only POPC molecules. The mixed asymmetric POPC:POPS lipid bilayer model was then used to
characterize the dynamics of the lipid binding at the molecular level of the human prothrombin fragment 1 (pf1), factor Va, and prothrombin molecules. Although it has been reported that factor Xa-factor Va is able to catalyze conversion of prothrombin to thrombin in the presence of other phospholipid mixtures/compositions other than POPC:POPS 1:4, the majority of the kinetic assay are performed using the POPC:POPS 1:4 model. This reason together with the convenient availability of the simulation parameters for POPC and POPS were the deciding point in choosing the POPC:POPS lipid bilayer model. A recent study performed similar experiments as described in Chapters III and IV by using a phospholipid bilayer model consisting of pure DOPG molecules. It is noteworthy that, although the study used a different phospholipid model, different time scales, different force field parameters the general conclusions are similar with our conclusions regarding interaction of vitamin K dependent proteins with a lipid bilayer (see Chapter IV).

Distribution of lipids molecules in an asymmetric fashion represents a fundamental property of a cell. Phosphatidylinerine (PS) containing lipids are usually localized in the inner layer. Exposure of PS phospholipids to the outer layer of the cell membrane corresponds to cell death and removal by phagocytosis. The process of phagocytosis is carried out by macrophages that specifically recognize the exposed PS molecules on the surface of the apoptotic cell. Another important implication of the PS exposure is in hemostasis and thrombosis. These two processes are controlled by the amount of exposed PS. The POPC:POPS system was chosen because of two reasons: (1) and the most important – prothrombinase assays are carried out in the presence of
POPC:POPS and (2) the POPC and POPS phospholipids were extensively characterized at the time of the model building thus enabling us to validate our model.

The POPC:POPS lipid model described herein, represents the long time needed framework in the building of the POPC:POPS bound enzymes that orchestrate the fine-tuning of the coagulation event such as the prothrombinase complex, the factor VIIa-tissue factor-factor Xa complex, the factor VII-tissue factor-factor IX complex and the factor VIIa-factor IXa.

2.3 Methods

2.3.1 Construction of the POPC:POPS lipid bilayer.

1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) and 1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphatidylserine (POPS) (see figure 2.1 for an atomic view and table 2.1 from Appendix for topology and parameters used in the molecular dynamics simulations) at 4:1 ratio in the outer leaflet were used as the lipid bilayer constituents as described and used throughout the literature to conduct in vitro experiments [18]. The coordinates of the system containing 128 equilibrated POPC molecules were used as a starting point [7]. Water molecules present in the system were removed. To achieve a 4:1 ratio of POPC:POPS lipid composition in the outer leaflet, 16 POPC molecules were transformed to POPS molecules. The choline fragment was replaced with its serine counterpart whereas the other regions components of the lipid unit such as the carbon tails were left unchanged. The system was replicated on the X and Y axes to obtain a 6×6×9 nm simulation box using the “genbox” utility from the GROMACS package [19, 20]. The system was solvated with single point charge (SPC) water molecules [21] and neutralized with Na⁺ ions.
Figure 2.1 Structures of the POPC and POPS phospholipids.
The figure shows the cell membrane model (red concentric circles) together with a zoomed in region of the lipid bilayer showing the percentage of POPC and POPS. The common fatty acid carbon tails (palmitoyl and oleoyl) are shown without the hydrogen atoms, the phosphate, methylene, ester, and methyl groups are depicted using yellow and pink rectangle and with aqua and green discoid, respectively. The only difference between the two lipid units, the head group, is show in red (choline) and blue (serine).
The level of hydration was set to be 46 (i.e., number of water molecules per number of lipid molecules). This level of hydration was found to reproduce reasonably well POPC head group areas [22]. Replacing 16 water molecules with Na\(^+\) ions neutralized the negatively charged system containing 16 POPS molecules. Na\(^+\) ions were added using the “genion” utility from the GROMACS package [19, 20]. The minimum distance between ions was set to 6 Å. All 16 POPS molecules present in the equilibration phase of the PCPS lipid bilayer were all in the outer leaflet leading us to believe that all Na\(^+\) ions are to be placed on the outer surface as well. However, the criterion imposed on the distribution of the charged molecules placed 19\% of the number of Na\(^+\) ions in the inner leaflet (i.e., on the leaflet containing only POPC).

2.3.2 Molecular dynamics simulations setup.

Molecular dynamics simulations were carried out by running a) an energy minimization procedure followed by b) a position restrained molecular dynamics simulation and finally c) the production molecular dynamics simulation.

a) Energy minimization was performed using the steepest descent method. The neighbor list frequency update was set to 10, the initial step was set to 0.1 Å and the integration step to 2 fs. Periodic boundary conditions were used in all three dimensions. Long-range electrostatics were treated using the particle mesh Ewald summation method [23, 24]. Grid dimensions were set to 1.6 Å and the Ewald summation was performed in all three dimensions with the interpolation order set to 6. Short-range electrostatics were calculated using a 9 Å cutoff. The necessary topologies and force field parameters for POPC [7] and POPS [8] lipid units were used as previously described. The force field
used for simulations was a version of GROMOS87 [25] implemented in GROMACS as “ffgmx” [19, 20].

b). Position restraint molecular dynamics simulation was performed so all heavy atoms were constrained to oscillate around their initial position. Simulation time was set to 50 ps. The temperature was set to 300 K and was kept constant using the Berendsen temperature-coupling scheme with the coupling constant set to 0.1 ps [26]. Specific groups such as the solvent, the sodium ions, and POPC and POPS lipid units were separately coupled to the Berendsen temperature bath set to 300 K. Initial velocities were calculated from the Maxwellian distribution at 300 K. In the case of the simulation with constant number of atoms, pressure, and temperature (NPT ensemble) the systems were simulated with a reference constant pressure of 1 bar. The constant pressure was controlled using the Parrinello-Rahman pressure coupling scheme with the compressibility set to $4.5 \times 10^{-5}$ bar$^{-1}$ and the time constant for coupling set to 1 ps [27].

c). Production molecular dynamics simulations. In the production run all heavy atoms were allowed to move freely. The integration step was set to 2 fs. Parameters for the simulations were used as described in sections a and b.

Equilibration of the POPS:POPC lipid bilayer. A 2 ns simulation was then performed using constant number of atoms, volume, and temperature (NVT ensemble). The NVT simulation was performed to relax the phospholipid surface from nonphysical contacts created after the change of POPC to POPS and the addition of the water cap as suggested [28]. Following the NVT simulation we performed a 60 ns simulation with constant number of atoms, pressure, and temperature. The simulation time for the equilibration of the PCPS lipid bilayer was set to 60 ns.
2.3.3 Radial distribution functions.

Radial distribution function is used to detect interactions between neighboring atoms/groups. The radial distribution function \( g(r) \) was calculated using the formula:

\[
g(r)_{A-B} = \frac{V \sum_{i \in A} \sum_{j \in B} P(r)}{4\pi r^2}
\]

where \( V \) is the volume and \( P(r) \) is the probability to find a B atom/group at a distance \( r \) from an A atom/group. The radial distribution function of several lipid groups with \( \text{Na}^+ \), \( \text{Ca}^{2+} \) and the hydrogen atoms of the amide moiety of POPC:POPS were calculated using the “\( g_{\text{rdf}} \)” tool from the GROMACS package [19, 20]. Values were averaged over the last 20 ns simulation time. The coordination number between selected groups was calculated by integrating the area under the first peak of the radial distribution function. A snapshot at the end of the 60 ns simulation time that shows the outer layer POPC:POPS mixture was calculated using the Voronoi tessellation method [29]. The points for the Voronoi diagrams represent the center of mass of each lipid molecule projected on the XY plane of the phospholipid bilayer system.

2.3.4 Electrostatic potential across the lipid bilayer.

The electrostatic potential \( \psi \) across the lipid bilayer was computed by evaluating the double integral of the averaged charge density \( \rho(z) \) over the last 20 ns of the total 60 ns simulation time using the formula:

\[
\psi(z) - \psi(-\infty) = -\frac{1}{\varepsilon_0} \int_{-\infty}^{z} \int_{z''}^{z'} \rho(z'')dz''dz'
\]

where \( z=-\infty \) is set to the center of the water phase and \( \varepsilon \) represents the permittivity.

2.3.5 Order parameters for the palmitoyl and oleoyl tails.
Order parameters are calculated to show the order of the fatty acyl chains. An order parameter of 1 shows maximum rigidity, whereas an order parameter of −0.5 shows maximum flexibility around the considered axis (the Z-axis in our case). Average orientation of the palmitoyl and oleoyl carbon tails was assessed by the calculation of the SCD order parameter. The calculations performed using the formula:

$$S_{CD} = \left\langle \frac{3}{2} \cdot \cos^2(\theta) - \frac{1}{2} \right\rangle$$

where <⋯> represents the mean value averaged over the last 20 ns of the simulation of the term between the brackets. θ represents the angle between the Z-axis of the simulation box and the vector from the (n-1)<sup>th</sup> carbon atom to the (n+1)<sup>th</sup> carbon atom (n ranges from 2 to 15 for palmitoyl tail and from 2 to 17 for the oleoyl tail [30]). Calculations were performed using the “g_order” tool from the Gromacs package [19, 20].

2.3.6 Electron density across the bilayer normal.

Electron density profiles of various groups were calculated using the “g_density” tool from the GROMACS package [19, 20]. The electron density was calculated as a scaling of the number of electrons for each group and “ffgmx” force field. Calculations were averaged over the last 20 ns simulation time.

2.3.7 Diffusion coefficients.

The lateral diffusion coefficient, D, of the lipids, pf1 was calculated using the mean square displacement (MSD) of the lipid molecules versus time using the Einstein relation described below:

$$D = \lim_{t \to \infty} \frac{\left\langle [r(t + t_0) - r(t_0)]^2 \right\rangle}{4t}$$
where $r$ represents the vector from the center of mass of each lipid molecule in the XY membrane dimension of the membrane plane. The averaging was performed starting at time $t_0$ and the averaged quantity is described in the equation by the brackets.

2.4 Results and discussion

2.4.1 Equilibration of the POPC:POPS lipid bilayer.

One physical property to check for a lipid bilayer equilibration is the variation of the area per lipid as a function of the simulation time (see figure 2.2). In a pure lipid bilayer system calculation of this parameter is trivial and is obtained by dividing the surface area of one leaflet with the number of phospholipids in that leaflet. In the case a lipid bilayer composed of mixed POPC and POPS molecules this method is unable to distinguish between the contributions of each phospholipid type to the total surface area. Nevertheless, the absence of drifting in the total surface area value as a function of the simulation time does indicate establishment of equilibrium. Figure 2.3 shows the result of the Voronoi tessellation diagrams for both POPS and POPC. After 60 ns of the simulation time several POPS molecules form patches on the outer leaflet.

2.4.2 Radial distribution functions.

Radial distribution functions were calculated for the amide group of the POPS head group (see figure 2.4). The radial distribution functions were functions of several groups of the POPS molecule such as: the ester carbonyl oxygen atoms (in red), the phosphate oxygen atoms (in blue), the carboxylate oxygen atoms (in green), and groups of the POPS lipid units such as the ester carbonyl oxygen atoms (in pink), the phosphate oxygen atoms (in aqua). Radial distribution function with the SPC oxygen atoms is
shown in black. The minima at 2.3 - 2.4 Å represent the hydrogen bond radius found between the hydrogen atoms of the POPS amide group and the oxygen atoms of groups such as the carbonyl, the carboxyl, the ester, and the water. The coordination number between the ester moieties of the POPS molecules and Na$^+$ ions was found to be 2.42 whereas the coordination number between the carboxyl moiety and Na$^+$ ions was found to be 0.24. The insertion of the Na$^+$ ions into the ester region is followed by a desolvation of the phospholipid head group.

2.4.3 Electrostatic potential across the lipid bilayer.

Figure 2.5 shows the potential across the box length on the Z-axis (i.e., perpendicular to the bilayer). Even though the total charge of the POPC:POPS mixture in water was neutralized with Na$^+$ ions, it is evident that there still exists a potential gradient due to the POPS molecules from the outer leaflet (i.e., ~ 200 mV). Because the system was neutralized with sodium ions it is questionable that only the presence of POPS on the outer leaflet would result in a difference in potential. This issue was recently addressed by a study performed by Gurtovenko et al. [31]. In this work the authors describe a lipid bilayer composed of POPC:POPE phospholipids but in the absence of ions. The major finding of the study is that the membrane is able to produce a nonzero potential across the normal to the bilayer and that the intrinsic membrane potential is due to the asymmetric nature of the lipid bilayer. They also found that the potential calculated (100 ± 24 mV) is in good agreement with the experimental results.
Figure 2.2 Equilibration of the head group area.
The area (nm$^2$) is represented as a function of the last 10 ns of the total 60 ns simulation.
Figure 2.3 The Voronoi tessellation diagrams.
The diagrams show the outer and inner leaflets and are color-coded as follows: red for POPS and green for POPC. Blue lines show the boundaries of each polygon obtained after the Voronoi tessellation.
Figure 2.4 Interatomic radial distribution functions.
g(r) as a function of distance (nm) is shown for the oxygen atoms with the hydrogen atoms of the amine group of the POPS lipid units. The radial distribution of ester carbonyl oxygen atoms of POPS is shown in red, the phosphate oxygen atoms of POPS is shown in blue, the carboxylate oxygen atoms of POPS are shown in green, the ester carbonyl oxygen atoms of POPC is shown in pink, the phosphate oxygen atoms of POPC is shown in aqua, and the SPC oxygen atoms in yellow.
Figure 2.5 The potential across the Z dimension of the system.
The potential is calculated as a function of the box length. The center of the lipid bilayer is at 4.5 nm (bilayer thickness is 6 nm).
2.4.4 Order parameters for the palmitoyl and oleoyl fatty acyl tails.

Figure 2.6 shows the deuterium order parameters for the oleoyl (panel A) and palmitoyl (panel B) fatty acyl chains. The results from the POPC:POPS lipid bilayer system (in red, +) are compared with results from a molecular dynamics simulation of a pure POPS lipid bilayer (in green, ×) and from NMR experiments performed on pure POPC bilayer (in blue, Θ). The small value of the oleoyl tail between the 9th and 10th carbon atom is due to the presence of the double bond and shows a less ordered chain. Deuterium order parameters of the two acyl chains of the mixed asymmetric POPC:POPS lipid bilayer agree fairly well with previous NMR results for pure POPC bilayer and with the order parameters obtained from MD simulations of the pure POPS lipid bilayer [8, 22, 32, 33]. The increase in the deuterium order parameter, especially for the beginning of the palmitoyl fatty acyl chain, as compared with the NMR results of the pure POPC bilayer can be attributed to the restricted motion of the head group. The restricted movement in turn is linked to the insertion of the Na\(^+\) ions in the ester region.

2.4.5 Electron density profile across the Z-axis of the bilayer.

Electron density profiles are shown in figure 4 (panels A and B). The electron density (e/nm\(^3\)) is shown as a function of bilayer normal (nm) with the center of the bilayer at 3 nm. Na\(^+\) ions were found to penetrate the lipid bilayer into the ester group region of POPS molecules (figure 4 panel B, red and yellow lines). This result coincides with previous findings obtained from MD simulations of pure POPS lipid bilayer in the presence of Na\(^+\) ions [8].

2.4.6 Diffusion coefficients.

Diffusion coefficients were calculated using the Einstein equation described in section
2.3.7. The diffusion coefficient of the lipid molecules present in the POPC:POPS lipid bilayer was found to be $1 \times 10^{-8}$ (cm$^2$/s). The value agrees well with the experimentally determined diffusion coefficient range, $0.306 \times 10^{-8} - 2.34 \times 10^{-8}$ (cm$^2$/s), of the mixed phospholipids [34].
Figure 2.6. A Deuterium order parameters ($S_{CD}$) for the oleoyl chain.
The deuterium order parameters are shown as a function of carbon atom numbers of the fatty acyl chains disregarding the fact that the fatty acyl chain belongs to the POPC or POPS lipid unit. The POPC:POPS asymmetric model is shown in green (5). Averaged values obtained from the 10 ns MD simulation are shown in red lines (+) whereas discrete values from NMR experiments [32, 33] are shown with blue lines (*).
Figure 2.6.B Deuterium order parameters ($S_{CD}$) for the palmitoyl chain.
The deuterium order parameters are shown as a function of carbon atom numbers of the fatty acyl chains disregarding the fact that the fatty acyl chain belongs to the POPC or POPS lipid unit. The POPC:POPS asymmetric model is shown in green (5). Averaged values obtained from the 10 ns MD simulation are shown in red lines (+) whereas discrete values from NMR experiments [32, 33] are shown with blue lines (*).
Figure 2.7.A Electron density profiles across the Z-axis of the bilayer (9 nm).
The center of the bilayer corresponds to 4.5 nm in the figure. The bilayer thickness is 6 nm. The electron density profile (e/nm$^3$) of several groups: POPC lipid unit in red; the POPS in green, the solvent molecules in blue, the phosphate oxygen of the POPC molecule in pink, the ester oxygen of POPC in aqua and both POPC and POPS lipid units in yellow.
Figure 2.7.B Shows the electron density profiles for different atoms and ion types. Na\(^+\) ions are shown in red, the phosphorus atom of the POPC in green, the phosphorus atom of the POPS in blue, the carboxyl oxygen of POPS in pink, the phosphate oxygen of the POPS molecule in aqua, the ester oxygen of POPS in yellow, the POPC methyl in black, and the POPS methyl orange.
2.5 References


33. Seelig, J. and N. Waespe-Sarcevic, Molecular order in cis and trans unsaturated
CHAPTER III

Modeling of human coagulation factor Va

3.1 Abstract

Factor Va is the critical cofactor for prothrombinase assembly required for timely and efficient prothrombin activation. In the absence of a complete crystal structure for the cofactor, Pellequer et al. [(2000) Thromb. Haemostasis 84, 849-857] proposed an incomplete homology model of factor Va (it lacks 46 amino acids from the carboxyl terminus of the heavy chain), which is a static model in a vacuum. A recently published X-ray structure of Activated Protein C (APC) inactivated bovine factor Va, (without the A2 domain) suggests a completely new arrangement of the C1 and C2 domains as compared with the previously published structure of the recombinant C1 and C2 domains. Our aims were (a) to exchange the C1 and C2 domains of the homology model with the modified bovine C1 and C2 domains using the X-ray structure as a template, (b)
to determine by computation the three-dimensional model for the carboxyl terminal peptide of the factor Va heavy chain (Ser664-Arg709) and incorporate it into the incomplete model, (c) to obtain a complete model of the cofactor folded in solution that might account for its physiological functions and interactions with other components of prothrombinase, and (d) to use the model in order to understand the mechanism of factor Va inactivation by APC. In the first step a sequence alignment of the human and bovine C1 and C2 domains was performed followed by amino acid changes in the three-dimensional structure where the sequences were not identical. The new model of the C1 and C2 domains was then attached to the homology model. The analysis of the MD simulation data revealed that several domains of the cofactor were significantly displaced during simulation. Using our completed model of human factor Va, we are also demonstrating for the first time that cleavage of membrane-bound normal factor Va as well as membrane-bound factor Va\textsuperscript{LEIDEN} by APC at Arg306 is required for the dissociation of the A2 domain from the rest of the molecule. Thus, differences in the inactivation rates of the two cofactor molecules are due to differences in the rate of cleavage at Arg306. Based on the studies of cleaved factor Va at only Arg306 and Arg506 we also conclude that the presence of cleavage at Arg679 is required for the dissociation of the fragment Gly507-Arg679 from factor Va molecule. The Gly507-Arg679 sequence is encapsulated under amino acid group Lys680-Arg709, hence release of the A2 domain from factor Va is obstructed. The data demonstrate that our model represents the foundation for the establishment of a complete prothrombinase complex model, which might be successful in describing accurately the ternary protein-protein interaction and thus accounts for experimental observations.
3.2 Introduction

Factor Va is the cofactor required for prothrombinase complex assembly and function [1]. Following vascular injury, the active cofactor combines with factor Xa, on a membrane surface in the presence of calcium ions [2], and the complex will readily activate prothrombin to thrombin [3]. Thrombin, which has multiple functions, rapidly activates platelets, converts fibrinogen to fibrin, and activates factor XIII. Cross-linked fibrin and activated platelets will produce the insoluble plug, which is necessary to stop blood leaking outside the vasculature [4]. Factor Xa alone converts prothrombin to thrombin with a rate of activation which is five orders of magnitude lower than the rate of the reaction catalyzed by factor Xa bound to factor Va on a negatively charged membrane surface in the presence of calcium ions [3].

The increase in the catalytic efficiency of prothrombinase when compared to the activation of prothrombin by factor Xa alone appears to result from a decrease in the $K_M$ representing higher local substrate concentrations and an increase in the $k_{cat}$ of the enzyme corresponding to more efficient catalysis because of the altered pathway for prothrombin cleavage and activation [5-7]. Thus, incorporation of factor Va into prothrombinase and its interaction with the components of the ternary complex is a requirement for normal hemostasis.

Factor V circulates in plasma as an inactive procofactor with high molecular weight (Mr 300,000). The procofactor is activated to factor Va by thrombin (2). Factor Va is composed of a heavy chain (Mr 105,000, Ala1 - Arg709), containing the A1 and
A2 domains [8] and a light chain (Mr 74,000, Ser1546 - Tyr2196), containing the A3, C1, and C2 domains non-covalently associated in the presence of divalent metal ions. While both chains of factor Va are required for the interaction with factor Xa, only the heavy chain of the cofactor binds prothrombin [9-12]. Cleavage of factor Va by activated protein C (APC) at Arg506/Arg679 results in decreased affinity of the molecule for factor Xa and the elimination of its interaction with prothrombin [10, 13, 14]. Subsequent cleavage of the membrane-bound cofactor at Arg306, completely abolishes the ability of the cofactor to interact with factor Xa because of dissociation of the A2 domain of the cofactor, resulting in efficient down-regulation of prothrombinase activity [13, 15].

To date, factor Va does not have a complete crystal structure. Structural data available for factor Va can be summarized as follows: a two-dimensional projection map obtained using electron microscopy [16], a homology model that uses ceruloplasmin as a template (consisting of 994 residues from Ala1 – Cys656 and Ser1546 – Met1883), that is part of the A domains [17], a homology model of the C1 and C2 domains using galactose oxidase binding domain as a template [18], an X-ray structure of the C2 domain [19], a homology model that lacks only 46 amino acids (Ser664 – Arg709) from the C-terminal [20], and finally, the Activated Protein C (APC) inactivated bovine factor Va obtained by X-ray diffraction. The later crystal structure reported a different topology for the C1 and C2 domains, and challenged the long-standing belief that the heavy and light chains are held together by the Ca\(^{2+}\) ions [21].

The model described by Pellequer et al. [20], lacks 46 amino acid residues from the carboxyl-terminal of the heavy chain (amino acid residues Ser664-Arg709). Furthermore, the model does not account for the presence of the solvent. This model will
be referred in the text as the “original homology model” since it is used in part as a foundation to propose a completed model for the cofactor.

Studies have shown that factor Va interacts with factor Xa through both its heavy and light chain. While the exact location of the amino acid residues involved in the interaction with factor Xa from the light chain remain to be identified several such amino acid regions from the heavy chain were reported [22-25]. Amino acid residues Arg37, Arg38, and Lys39 (165, 125, and 230 in chymotrypsin numbering, respectively) from the factor Xa sequence were found to be important in the interaction with factor Va [26]. Another cluster of amino acids from the factor Xa sequence was proposed to contain a factor Va binding site 404-418 (231 - 244 in chymotrypsin numbering) [27].

The factor Xa binding site (amino acid residues Glu323 - Val331) on factor Va [24, 25, 28] was buried in the original homology model under residues Tyr371 - His379 of the heavy chain. The prothrombin-binding site (amino acid residues Asp695 - Gln699) [29] could not be analyzed since it was located on the carboxyl-terminal region of the heavy chain of factor Va that was missing from the original model. This missing region contains important moieties required for the normal physiological activity of factor Va such as a cleavage site for APC (Arg679) [12] and the interaction site for prothrombin [29, 30].

The present study was undertaken to provide a complete model of factor Va that can be used for the investigation and understanding of its physiological functions. After the remodeling of the C1 and C2 domains of factor V and inserting the folded 46 amino acid sequence, the protein was allowed to adjust to the new structural environment by performing molecular dynamics (MD) simulations. We focused our investigation on the
factor Xa binding site on factor Va heavy chain, amino acid residues Glu323 and Val331, [12, 24] and the recently reported prothrombin binding site on the carboxyl-terminal portion of the heavy chain of the cofactor [29, 30]. We have also used our model to understand the mechanism for APC inactivation of the cofactor.

3.3 Methods

The recently reported X-ray structure of the bovine factor Va, [21] describes a cofactor with a rather different arrangement of the C1 and C2 domains compared with the earlier models [16-20] (see figure 3.3 panels B-C). In the X-ray structure of bovine factor Va, the C1 and C2 domains have their axes of the beta barrels in parallel orientation, whereas in the earlier models the axes of the beta barrels have a nearly coaxial orientation. In the first step we remodeled the C1 and C2 domains in the original homology model by replacing them with the C1 and C2 domains found in the bovine factor Va, crystal structure. Sequence alignment performed with the European Molecular Biology Open Software Suite (EMBOSS) server [31] showed that there is a 85.5 % identity and 93.1% similarity between the sequence of the C1 and C2 domains of human and bovine factor V. Only 46 amino acids were found to be different between the two species within the C1 and C2 domains. There are no gaps between the two sequences in this region. Therefore, in the first step we changed 46 amino acids from the bovine factor V C1 and C2 sequence into the corresponding amino acids in the human factor V C1 and C2 sequence (see Appendix table 3.1). The second step in our study was to extend the model of human coagulation factor Va proposed by Pellequer et al., (derived from human ceruloplasmin) [20], by adding the missing 46 amino acids at the carboxyl-terminal end
of the heavy chain (Ser664 - Arg709) and perform MD simulations on the completed model. Since we could not find a homologous peptide fragment for the carboxyl-terminal 46 amino acid sequence (based on its primary structure), we used computer simulation techniques to obtain a folded three dimensional model for this sequence [8]. Because of the low homology with other known X-ray/NMR structures no specific fold could be attributed to the 46 amino acid peptide.

**Preparing the MD simulations.** The modeling of the complete factor Va structure required several MD simulations (performed with GROMACS [32, 33] program). For all systems subjected to simulations we used the same parameters. These systems are:

(i) high temperature driven simulation of the incomplete factor Va model,
(ii) folding of the 46 amino acid peptide, and
(iii) the relaxation of the new model. The number of particles, the groups chosen for temperature and pressure coupling, the simulation time, and temperature were chosen according to the needs of each simulation. Parameters that differ from one simulation to another are described in the following sections.

For the MD simulations the protein was placed in a water box by setting the distance between the box walls and protein/peptide to 5 Å. Initial velocities for atoms were taken from the Maxwellian distribution at 300 K. For solvent we used the transferable intermolecular potential with four points water model (TIP4P) [34]. OPLS-AA parameters set was used to describe the interactions (potential and forces) for all atoms of the system [35]. Ions (Cl⁻ or Na⁺) were added when the system was positively or negatively charged, to keep the system neutral. Ions were added using the program
“genion” (a GROMACS [32, 33] tool) by replacing water molecules with the corresponding ions. The positioning of the ions was performed by using an electrostatic potential criterion with a 0.9 Å Coulomb cut-off and setting the minimum distance between ions to 0.6 Å. The LINCS algorithm was used for bond constraints [36].

Energy minimizations were carried out with the steepest-descent method with the initial step set to 0.1 Å. The neighbor list updated frequency was set to 10 and we used the “grid” option for neighbor search. Periodic boundary conditions were employed in all three dimensions. The cutoff distance for the short-range neighbor list was set to 9 Å. Long range electrostatic interactions were treated using the particle mesh Ewald summation method [37, 38]. Ewald summation was performed in all three dimensions. The fast Fourier transform grid dimension was set to 1.6 Å and the interpolation order was set to 4. During energy minimization bonds were not constrained.

Position restraint molecular dynamic simulation (PR-MD) was performed using the “md” integrator with all bonds constrained and all heavy atoms from the protein restrained to move around their initial position with a harmonic oscillator function. The integration step was set to 2 fs and the total PR-MD simulation was 20 ps long. Long-range electrostatic interactions were calculated as in the energy minimization step. The system was treated as an NPT ensemble (constant number of atoms, constant pressure and constant temperature). The reference temperature was set to 300 K for those simulations that did not employ a high temperature driven conformational search. The simulation was carried out at constant pressure using Parrinello-Rahman [39] isotropic pressure coupling with the compressibility set to $4.5 \times 10^{-3}$ bar$^{-1}$ and a reference pressure of 1 bar.
Parameters used in the molecular dynamics simulation were the same as described at PR-MD step, except that the heavy atoms (all atoms except the hydrogen atoms) were allowed to move freely.

3.3.1 Modeling the C1 and C2 domains of factor Va.

The bovine and human primary structures of the C1 and C2 domains were first aligned using the “matcher” program [40, 41] provided by the EMBOSS online serve [31]. Deep View Swiss-PdbViewer [42] was then used to change the amino acids from the bovine sequence that were not identical with the human factor V C1 and C2 domain sequence. Forty-six amino acids from the C1 and C2 domain were manually changed using the “mutate” tool from Deep View Swiss-PdbViewer [42]. The best rotamer for each amino acid, which is the form of the amino acid with the lowest score, was chosen each time (data not shown).

The modeled C1 and C2 domains were then inserted in the original homology model by fitting the Ca^{2+} atoms of two amino acid groups. The first group is formed by amino acids Arg1877, Asp1878, and Cys1879 and is part of the original homology model, whereas Arg1864, Glu1865, and Cys1866 make a second group, which is part of the bovine factor Va\textsubscript{i} structure. These groups were fitted with the “fit” tool from Deep View Swiss-PdbViewer. The \( \phi \) and \( \varphi \) angles of Cys1879 were changed according to the values found in the bovine factor Vai crystal structure. The steric strain on Glu1608 (due to the insertion of C1 and C2 domains), was removed by using a new rotamer generated by Deep View Swiss-PdbViewer, which has the lowest score (-2) of a set of eight generated rotamers.

3.3.2 Modeling of the calcium and copper binding sites.
The original homology model contains two calcium ions and one copper ion. The crystal structure of bovine factor Va, shows the copper ion in the vicinity of amino acid residues His1802, His1804 and Asp1844. We have included in our model one copper and two calcium according to the literature \[21, 43, 44\]. Residues Asp111, Asp112 together with the oxygen from the carbonyl group of Lys93 and Glu108 were identified to coordinate a calcium ion on factor Va in the crystal structure of bovine factor Va. These data confirm the existence of the earlier described calcium binding site in the region Glu96 - Asp111 of the cofactor \[43\].

Previous findings have also described two calcium binding sites with lower affinity \[44\]. Therefore one of the calcium ions that was not found in the bovine factor Va crystal structure, but was modeled in the original homology model, i.e., in the vicinity of amino acid residues Asp1579, Glu1576, Glu1572, and Glu1583 was retained in the present study. The other calcium ion was placed in the proximity of Asp111, Asp112, Lys93, and Glu108 whereas the copper ion was positioned nearby His1802, His1804, and Asp1844. Distances from the ions to the corresponding amino acids were adjusted based on the bovine factor Va crystal structure geometry.

3.3.3 Modeling of the 46 amino acid residues from the C-terminal of factor Va.

The last residue, Asp663, from the heavy chain of the original homology model of factor Va \[20\], protrudes outward from the protein surface (e.g. Asp663 is 20.4 Å away from the closest solvent accessible residue, Val654, which is part of an outer loop). We thus decided to fold the peptide in a separate simulation before attaching it to factor Va. The starting model of the peptide had three straight-line backbone domains, separated by two kinks. These kinks are due to Pro671, Pro672, and Pro688. The final three
A three-dimensional model of the peptide was obtained following three simulations:

(i) 14 ns simulation at 300 K.
(ii) 1 ns high temperature conformational search simulation at 400 K; and
(iii) 2 ns simulation at 300 K for equilibration.

All other simulation parameters used are described in the “Preparation of the MD simulations” section. Because the total charge of the system was -7, seven water molecules were replaced with seven Na$^+$ ions. The system was coupled to the Berendsen thermostat (46) using a reference temperature of 300 K for the first and third simulation and 400 K for the high temperature conformational search. The root mean square displacement (RMSD) of the C$^\alpha$ atoms was plotted for the final simulation to ascertain equilibration of the system. The starting coil model of the peptide (based on its primary structure [8]) was built using the Pymol program [45].

After a 1 ns simulation (temperature driven conformational search) in water we performed 2 ns simulation to equilibrate the system. The final snapshot, obtained from a cluster of the longest life conformers, was saved and after minimization was inserted in the incomplete factor Va model. The general fold of the peptide is shown in figure 3.3 panel A. The minimized three-dimensional model of the peptide was inserted in the original homology model, containing the newly modeled C1 and C2 domains.

Extending the factor Va heavy chain required two steps:

(1) making the peptide bond between Asp663 and Ser664 (Ser1 in the folded peptide), and

(2) performing MD simulation of the extended factor Va molecule in solution.

The peptide bond, connecting the carboxyl-terminal residue of the incomplete
model, Asp663, with the amino-terminal residue of the folded peptide, (Ser664 in factor Va), was made with Deep View Swiss-PdbViewer program [42]. The $\phi$ and $\varphi$ angles of residue Asp663 were adjusted in such a manner that the $\phi$ and $\varphi$ angles of the inserted 46 amino acid residues are positioned in the allowed regions of the Ramachandran plot [46].

To ascertain peptide equilibration, the root mean square displacement (RMSD) of $C^\alpha$ of the peptide was plotted as a function of time. The hydrogen bond network formed following peptide folding was also verified (see Appendix, table 3.2). The final simulation removes the steric strain and allows the peptide to adjust to the factor Va three-dimensional model.

3.3.4 Amino acid substitutions in the heavy chain of factor Va.

The newly developed factor Va model was used to study the effect of amino acid substitutions in the heavy chain on the activity of prothrombinase. We constructed a factor Va molecule with amino acid residues 334 and 335 changed to Lys and Phe, respectively, (factor VaKF). These mutations were undertaken to assess the effect of these mutations on the structure of factor Va on the vicinity of the factor Xa binding site.

It has been demonstrated that amino acids E323, Y324 and E330, V331 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 [25]. It has been also demonstrated that amino acid region 332-336 contains residues found to be involved in the function of factor Va. Using overlapping peptides (see figure 3.1.A) from the 332-336 region we have identified amino acid residues 334DY335 as important contributors for factor Va cofactor activity [47]. The peptides
were assayed for their ability to inhibit prothrombinase activity in an assay that uses purified reagents and a fluorescent thrombin inhibitor (figure 3.1.B). The data demonstrate that AP5 inhibits prothrombinase with an IC$_{50}$ of ~11 µM (figure 3.1.B, filled diamonds) while the control peptide (P15H) had no effect on prothrombinase activity under the conditions employed (figure 3.1.B, closed squares) even at concentrations as high as 500 µM (not shown). Complete inhibition of prothrombinase by AP5 occurred at 200 µM peptide (not shown). Site-directed mutagenesis was used to study the effect of these amino acids on the catalytic efficiency of prothrombinase. The recombinant factor V molecules with the mutations D334 → K and Y335 → F (factor VKF) were constructed using the primers: 5'-C ATT TGG AAA GTT GCA CCT G-3' (forward) and 5'-C AGG TGC AAA CTT CCA AAT G-3' (reverse) (the mutated bases are indicated using underlined letters) in a two-stage PCR method and characterized as previously described [25]. Factor V$^{AA}$ 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 GCC GCCCCA AAT GAC TCC CTC-3' (reverse). The resulting amplicon was sub cloned into pGEM-T and the mutations were confirmed by DNA sequencing (DNA Analysis Facility, Cleveland State University). The pGEM-T plasmid was digested with restriction enzymes; Bsu361 and Xcm1, to remove the factor V insert and the insert containing the mutation was ligated into pMT2-FV at the same restriction sites.
Figure 3.1.A Factor Va heavy chain peptides.

On the top the sequence of N42R is illustrated (part of the box). The bold underlined sequence (323-335, 13 amino acids) is the regulatory amino acid sequence of factor Va heavy chain. Overlapping peptides from the central portion of factor Va heavy chain (AP3-AP7) are also shown. The role of the underlined amino acid region included in the box (i.e. Arg334-Tyr335) and common to IWDYA, AP5 and AP6, is under investigation in the present work. Bold underlined are the amino acid residues mutated in this study [47].

Figure 3.1.B Inhibitory potential of factor Va heavy chain peptides.
Inhibition of prothrombinase activity. Increasing concentrations of AP5, AP6, P15H, I5A, and AP5_{DY→KF} were preincubated with factor Xa and assayed for prothrombinase activity as described in the “Experimental Procedures” section. P15H (filled squares) represents the control peptide containing amino acids 337-351 of human factor Va heavy chain (see Fig.2 for details). AP5 (filled diamonds) represents amino acid region 327-336 of factor Va. AP5_{DY→KF} (open triangles) represents amino acid residues 327-336 with residues 334-335 mutated from DY to KF. IWDYA (open squares) is amino acid residues 332-336 of human factor Va heavy chain. AP6 is shown by filled circles. 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[47].

[Barhoover et al. 2008 – in press]
The remaining recombinant plasmids were constructed using Stratagene’s QuikChange® XL Site-Directed Mutagenesis Kit according to the manufacturer’s instructions. The mutations were confirmed by DNA sequencing (in the DNA Analysis Facility, at Cleveland State University). Kinetic studies showed that while factor VaKF had a $K_D$ for factor Xa similar to the $K_D$ observed for wild type factor Va, the clotting activity of the mutant molecule was significantly impaired and the $k_{cat}$ of prothrombinase assembled with factor VaKF was notably reduced. The second order rate constant of prothrombinase assembled with purified recombinant factor VaKF, for prothrombin activation, was 6-fold lower than the second order rate constant for the same reaction catalyzed by prothrombinase assembled with purified wild type factor Va. We have also created two quadruple mutants, factor VKF/FF (D334K/Y335F and E323F/Y324F) and factor VKF/MI (D334K/Y335F and E330M/V331I). Prothrombinase assembled with factor VaKF/FF showed a 47-fold decrease in the second order rate constant for prothrombin activation while under similar experimental conditions prothrombinase assembled with factor VaKF/MI had a 400-fold decrease in the second order rate constant. Time courses studying prothrombin activation by gel electrophoresis [47] (figure 3.2) demonstrated that prothrombinase reconstituted 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. Briefly, amino acid residues Asp334 and Tyr335 were substituted with lysine and phenylalanine, respectively in order to generate a structural model of factor VaKF. The amino acid change was performed using the “mutate” tool from the SwissPDBViewer package [42].
Figure 3.2 Prothrombin activation by prothrombinase assembled with recombinant factor Va molecules.

Plasma-derived factor Va, factor VaWT (corresponding to factor VaControl in the theoretical study), factor VaKF, and factor VaAA were incubated with prothrombin, PCPS vesicles, and DAPA at a final concentration of 10 nM. Factor Xa was added to start the reaction to a final concentration of 1nM. Aliquots were withdrawn at selected time intervals. M represents the lane with the molecular weight markers (from top to bottom): Mr 98,000, Mr 64,000, Mr 50,000; Mr 36,000. Lanes 1-19, represent samples from the reaction mixture before (0 min) the addition of factor Xa and 20 sec, 40 sec, 60 sec, 80 sec, 100 sec, 120 sec, 140 sec, 160 sec, 180 sec, 200 sec, 220 sec, 240 sec, 5 min, 6 min, 10 min, 20 min, 30 min, and 60 min respectively following the addition of factor Xa. Panel A, factor Xa with plasma-derived factor Va; panel B prothrombinase assembled with wild type factor Va; panel C, prothrombinase assembled with factor VaKF, panel D prothrombinase assembled with factor VaAA. The legends to the right of the gels indicate the prothrombin activation fragments: II (prothrombin), F1•2-A (Fragment 1•2-A chain), F1•2 (fragment 1•2), and B (B chain of thrombin). Experiments were performed with at least four separate preparations of purified proteins and one representative gel is shown. [47]

The factor Va wild type structure was left unchanged.

The molecular dynamics simulation of each factor Va model consisted in a three-step setup:

(a) the solvation of the protein followed by energy minimization,
(b) position restraint molecular dynamics simulation, and
(c) production run molecular dynamics simulation. Simulations were run and analyzed using the GROMACS package version 3.2.1 [32, 33].

Energy minimization. The protein submitted for simulation was first placed in a box so that the periodic images of the protein were at 10 Å of each other. This resulted in both cases in a simulation box with the dimensions 11 × 13 × 10 (nm) and 46832 water molecules. The positively charged systems were neutralized by the addition of Na⁺ ions to the system by replacing water molecules. Replacement of the water molecules was performed based on an electrostatic potential criterion with a 0.9 Å Coulomb cutoff. The minimum distance between the added sodium ions was set to a minimum of 6 Å. Neutralization of charges with the Na⁺ ions was performed using the “genion” utility from the GROMACS package [32, 33]. The minimization procedure was performed using the steepest descent algorithm with an initial step size of 0.1 Å. Periodic boundary conditions were employed in all three directions. The system was solvated using the SPC water model (37). The force filed used to describe the interaction parameters was set to GROMOS-87 implemented in GROMACS as “ffgmx” [32, 33]. Molecular topologies for both wild type factor Va and factor VaKF were generated using the atom types described in the “ffgmx” force field. Bonds were constrained using the LINCS algorithm (39). The neighbor list for short-range interactions was set to 9 Å cutoff. Long-range electrostatic
interactions were evaluated with the particle mesh Ewald summation method performed in all three dimensions and using a fourth order Fourier interpolation on a 1.6 Å spaced grid (40, 41).

*Position restraint molecular dynamics.* Following the minimization procedure both systems (i.e., solvated wild type factor Va and solvated factor VaKF) were subjected to 20 ps molecular dynamics simulation with the restriction that only water molecules were allowed to move freely whereas the protein was restrained. This allowed water molecules to fill in the interstices that were previously unoccupied. Several groups such as the protein, the solvent, and ions were separately coupled to temperature baths. The simulation temperature was set to 300 K and was maintained using the Berendsen temperature coupling with the coupling parameter set to 0.1 ps. Initial velocities were based on the Maxwellian distribution at 300 K. The constant reference pressure of 1 bar was maintained throughout the simulation using the Parrinello-Rahman isotropic pressure coupling scheme with the compressibility set to $4.5 \times 10^{-5}$ bar$^{-1}$ [39].

The systems were considered having constant number of particles, constant temperature and pressure. All other parameters were kept the same as in the “Energy minimization” protocol.

*Production run molecular dynamics.* Simulations were run until the systems reached equilibrium. Equilibration of each system was confirmed when the root mean square displacement of the protein reached a plateau and the drift was less than 1 Å during a whole nanosecond simulation time. The integration step was set to 2 fs. All heavy atoms were allowed to move freely. All other parameters were kept the same as in the position restraint simulation. The last nanosecond of the equilibrated trajectories were
analyzed using tools included in the GROMACS package \[32, 33\].

**3.3.5 Activated protein C mediated cleavages on human coagulation factor Va.**

The factor Va model obtained following 1.4 ns simulation was used to test the behavior of the factor Va-derived fragments following APC cleavage (13, 15). We prepared a cleaved factor Va molecule at Arg306, Arg506, and Arg679, a molecule that mimics APC-induced inactivation of the cofactor on the membrane surface (factor Va3/5/6).

A second factor Va molecule was cleaved at Arg506 and Arg679. This sequence of cleavages mimics the APC cleavages of the cofactor in the absence of a membrane surface (factor Va5/6). A third factor Va molecule was cleaved at Arg306 and Arg679 mimicking the APC cleavage of membrane-bound factor Va (factor Va3/6). To study the fragment dissociation pattern of the factor Va molecule in the absence of cleavage at Arg679, a fourth factor Va molecule was cleaved at Arg306 and Arg506 (factor Va679).

Finally the fifth control simulation involved a factor Va molecule cleaved only at Arg306. Simulations parameters were the same as previously described for the intact model of factor Va. All simulations were performed for 2 ns. Distances between the mass centers of the generated fragments following cleavage of the heavy chain and the light chain were calculated using the “g_dist” utility package from GROMACS \[32, 33\].

Factor VaKF and factor VaAA were modeled using the recent factor Va model (pdb code 1y61) \[48\]. Briefly, amino acid residues Asp334 and Tyr335 were substituted with lysine and phenylalanine (factor VaKF) or with alanine (factor VaAA). The amino acid change was performed using the “mutate” tool from the SwissPDBViewer package \[42\]. The factor Va wild type was left unchanged.

**3.3.6 Building the factor Va - POPC:POPS system.**
Construction of the factor Va - POPC:POPS systems. The coordinates of the lipid bilayer model composed of 1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) and 1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphatidylserine (POPS) (4:1 ratio in the outer leaflet) was taken from the equilibrated 60 ns long simulations as described (see Chapter II). The coordinates for the factor Va molecule were taken from the previously equilibrated factor Va homology model (Chapter III, sections 3.3.1-3.3.3) [48]. In the previous study of the factor Va C2 domain interaction with neutral phospholipids the authors inserted the C2 domain into the phospholipid bilayer. In our studies we wanted to check first if factor Va is able to insert into the POPC:POPS phospholipid bilayer during the simulation. This approach together with the control simulation where we considered the POPC lipid bilayer only would be useful to draw conclusions about the importance of the POPS molecules in the interaction. The factor Va molecule was placed in the vicinity of the POPS containing leaflet but making sure that there are no interactions. The factor Va molecule was positioned so the C2 domain was in the close proximity of the POPS rich phospholipid leaflet. This positioning ensured that amino acid residues Trp2063, Trp2064, Tyr1956, Leu1957 previously found to be involved in membrane binding [49, 50], were placed at a distance of ~ 10 Å from the outer leaflet plane. Amino acid residue Cys2113 found to interact with soluble PS molecules [51] in the initial configuration was at 15 Å to the closest lipid molecule.

Molecular dynamics simulations setup. The system composed of factor Va and the POPC:POPS lipid bilayer factor Va was solvated using the single point charge (SPC) water model [52]. The solvated system was kept neutral by replacing some water molecules with sodium ions. The simulation protocol was started with an energy
minimization of the solvated system using the steepest descent method. Periodic boundary conditions were employed in all three dimensions. Periodic boundary conditions are employed in molecular dynamic simulations of proteins to effectively simulate an infinitely tiled system. This procedure ensures that at the boundaries of the simulation box instead of vacuum there is another image of the simulation box. Long-range electrostatics were evaluated using the particle mesh Ewald summation method [37, 38]. Grid dimensions were set to 1.6 Å and the Ewald summation was performed in all three dimensions with the interpolation order set to 6. Short-range electrostatics was calculated using a 9 Å cutoff. During the 50 ps simulation that followed the energy minimization all heavy atoms from the system were constrained to oscillate around their initial position. This short simulation enabled the rearrangement of solvent molecules around the protein. The temperature was set to 300 K and was kept constant using the Berendsen temperature-coupling scheme with the coupling constant set to 0.1 ps [53]. The solvent, factor Va, the calcium and sodium ions, the POPC, and the POPS lipid units were separately coupled to the Berendsen temperature bath set to 300 K. Initial velocities were calculated based on the Maxwellian distribution at 300 K. The system was simulated as an NPT ensemble (i.e., constant number of atoms, pressure, and temperature) using a reference constant pressure of 1 bar. The constant pressure was controlled using the Parrinello-Rahman pressure coupling scheme with the compressibility set to $4.5 \times 10^{-5}$ bar$^{-1}$ and the time constant for coupling set to 1 ps [39]. In the production run (simulation time set to 9 ns) all heavy atoms were allowed to move freely. RMSD of $\alpha$C of factor Va was calculated using the “g_rms” program and mean RMSD value per residue was calculated using the “g_rmsf” program from the
GROMACS package [32, 33].

3.4 Results and discussion

3.4.1 Validation of the factor Va model.

(1) Superposition of the new model with the model obtained from bovine factor Va<sub>i</sub> crystal structure. Following 1.4 ns simulation, the equilibration of the system was verified by plotting the change in temperature, potential energy, and RMSD of C<sup>α</sup> of the factor Va molecule versus time (see figure AIII.1 in Appendix). Further analysis of the trajectory of the complete factor Va model was made using the equilibrated portion of the simulation (i.e., from 0.8 ns to 1.4 ns). The DSSP [54] program was used to assign the secondary structure.

The recently solved crystal structure of bovine factor Va<sub>i</sub> provides a structure for a significant part of the A1, A3, C1 and C2 domains of the bovine cofactor [21]. However, the crystal structure lacks the entire A2 domain and several segments throughout the entire molecule. In contrast, we are providing a three-dimensional model encompassing the entire structure of the factor Va molecule. To validate the positions of our model containing unconserved sequences (i.e., several segments throughout the A1 and A3 domains and the entire A2 domain), we superimposed all homologous regions present in both the crystal structure and our model.

Seven out of nine RMSD values were found to be in the range 1.6 - 2.5 Å. The other two regions corresponding to a total number of 88 and 422 amino acids residues have an RMSD of 4.77 and 4.64 Å respectively (figures A3.2g and A3.2i in Appendix). The large RMSD observed between the crystal structure of bovine factor Va<sub>i</sub> and the model of human factor Va presented herein within regions Asn1670 - Asp1757 and
Lys1773 - Tyr2196 can be explained by the fact that in the crystal structure of bovine factor Va, the amino and carboxyl-termini of the segments move freely, while the corresponding amino acids in the model of human factor Va are part of the continuous polypeptide chain. It is hence expected that the segments resulting from the cleaved factor Va X-ray structure adopt sometime different orientations of the termini as compared with our model where the orientation of the side chains/backbone has a more restricted flexibility (see figures A3.2g and A3.2i in Appendix). The region His228-Thr267 has a relatively high RMSD 2.52 Å (for such a short sequence as presented in panel c) between the X-ray structure and our model. Although in the previous cases such as A3.2g compared with A3.2i the high RMSD could be attributed to the flexible C and N-terminal ends in this case the difference arises from the middle of the sequence with residues His252, His253 having the major contribution. This sequence has both the C and the N-terminus buried in the core of factor Va but the middle of the loop is exposed to solvent. It is well known that the structure in the inside of a globular protein tends to be less affected by the overall dynamics of the protein. On the other hand, the loops exposed to the solvent are known to be quite flexible. This flexibility of such a loop could have an effect on the protein’s function although in factor Va such an effect was not reported for this region.
Figure 3.3 Model of the complete human factor Va molecule.
Panel A shows the three dimensional fold of the 46 amino acid peptide. The N-terminus is shows at Ser664 whereas the C-terminus corresponds to Arg709. Panel B and C show the folds of the C1 and C2 domains as described in the Pellequer homology model [20] and the factor Va X-ray structure [21], respectively. Trp2063-Trp2064 (human numbering) and the bovine factor Va correspondents are shown as red sticks. Panel D. The extremities of the factor Xa binding site are shown in pink (residues Glu323, Tyr324, Glu330, and Val331) with the rest shown in blue (residues Phe325-Glu329). Red spheres represent the prothrombin binding site on factor Va (residues Asp695-Gln699). APC cleavage sites Arg306, Arg506, and Arg679 are shown in magenta, cyan, and lime, respectively. Yellow spheres show the rest of the 46 newly added amino acids (Ser664-Arg679, Lys680-Ala694, and Asn700-Arg709). The position of the C1 and C2 domains is also depicted to show the relative position of the important moieties of the heavy chain of the cofactor from the membrane surface.

It is noteworthy that while the alignment of residues Asn1760 - Met2182 (from bovine factor Va) with the corresponding region from the model of human factor Va (Lys1773 - Tyr2196), results in a large Cα RMSD, the alignment of two large segments within these regions, representing the entire C1 and C2 domains as well as residues Lys1773 - Gly2032 taken separately, have RMSD values of 1.79 Å and 1.56 Å, respectively. In conclusion, the comparison between the model of human factor Va and the crystal structure of bovine factor Va provides a valuable confirmation for our model.

(2) Superposition of the new model with the model obtained from the crystal structure of ceruloplasmin. Polypeptide segments available from the ceruloplasmin crystal structure (pdb code 1KCW), were also superimposed with the corresponding fragments from the model of factor Va. Cα RMSD values between the model and four fragments from ceruloplasmin, i.e., Lys1 - Cys338, Ile347 - Pro474, Val483 - Pro884, and Arg892 - Asn1040 were found to be 1.58 Å, 1.32 Å, 1.72 Å, and 1.83 Å, respectively (not shown). These data together with the data presented above validate our model and provide for the first time a working model of a complete human factor Va molecule in solution. The model has been deposited in the Protein Data Bank, pdb code 1Y61.

*Factor Xa binding sites on factor Va.* Figure 3.3 panel D shows the complete factor Va molecule following 1.4 ns simulation with the C1 and C2 membrane-binding domains together with a portion of the factor Xa binding site of the cofactor located on the A2 domain. Amino acid residues Glu323, Tyr324, Glu330, and Val331 [24, 25] (shown in pink) represent the extremities of the factor Xa binding site on factor Va [24], whereas amino acid residues Asp695 - Gln699 (in red) illustrate a prothrombin interactive site on the cofactor [24].
Figure 3.4 Structural details of factor Xa and the prothrombin interactive site on factor Va.
Gray spheres (residues Tyr371-His379) represent residues that block solvent accessibility of the factor Xa binding site; pink spheres (Glu330-Val331 and Glu323-Tyr324) and red spheres (Phe325-Glu329) show portions from factor Xa binding site. The prothrombin binding site on factor Va (amino acid residues Asp695-Gln699) is shown in green while amino acid residue Ser692 is shown in violet. Amino acid residues Met410 and Lys408 are shown in blue; the rest of the newly added 46 amino acid peptide (Ser664- Glu691, Asp693-Ala694, and Asn700-Arg709) is shown in yellow.

Amino acid residues Tyr371 - His379 (in grey) correspond to amino acids that cover the factor Xa binding site. Amino acid residues Ser664 - Ala694 and Asn700 - Arg709 (in yellow) represent the rest of the newly added 46 amino acid residues carboxyl-terminal peptide.

We next studied the change in the conformation of amino acid residues Tyr371 - His379, which partially cover the factor Xa binding site on factor Va, following the simulation. The RMSD of these amino acids was calculated with respect to amino acid residues Met410 and Lys408. Similar measurements were performed with amino acid stretch Glu323 - Val331. We found that the displacement of amino acid residues Glu372 - Lys378 (~ 9 Å) from their initial position was twice as big as that observed for displacement of amino acid residues Glu323 - Val331 (~ 4 Å) when compared to Lys308. Tyr371 was found to participate in a hydrogen bond (Tyr371 donor atom OH - hydrogen atom HH - acceptor Glu329 atom OE2 using atom nomenclature as described [55]) that lasts 800 ps out of 1.4 ns simulation.

That is statistically enough for hydrogen bond persistence [56]. Lys378 has two hydrogen bonds before simulation with Glu372, but only one hydrogen bond is preserved during simulation. It appears that the existence of these hydrogen bonds is at the origin of the small RMSD observed for amino acid residues Tyr371 and His379 during the 1.4 ns simulation. On the other hand, amino acid sequence Glu372 - Lys378 is part of a loop and is not involved in hydrogen bonding (which would restrict its movement with respect to amino acid residues Glu323 to Val331).

It has been hypothesized [24] that for optimal expression of factor Va cofactor activity within the prothrombinase complex, amino acid residues Tyr371 - His379 could
hinder the solvent accessibility of Tyr324 - Phe325, which are involved in binding factor Xa (i.e., Glu323 - Val331). Perhaps factor Va exposes residues Tyr324 - Phe325 only when bound to a procoagulant cell surface. This assumption [24] has been made based on studies of the incomplete homology model. Further, the amino acid sequence missing in the original model (Ser664 - Arg709) is found to be located at ~22 Å to the amino acid region Glu323 - Val331 (distance measured between the C\(^\alpha\) atoms of Tyr698 and Val331).

After 1.4 ns simulation, the effect of the newly added amino acid residues on amino acid segment Tyr371 - His379 led to an increased exposure of amino acids Tyr324 and Phe325 (see Appendix figure A3.3, panel B); however, despite this significant change during simulation amino acid residues Tyr324 and Phe325 are still not solvent accessible (the solvent accessible surface area (SASA) does not increase after simulation). Figure 3.4 shows details of factor Xa binding site on factor Va after 1.4 ns simulation. Even though amino acid residues Tyr371 - His379 (see Appendix figure A3.3, gray), are shifted by 8.0 Å after simulation, Met410 and Lys408 (see Appendix figure 3.3, dark blue) are still partially blocking access to amino acid segment Glu323 - Val331. As recently demonstrated, amino acids Glu323, Tyr324, Glu330 and Val331 are essential in binding factor Xa to factor Va [25].

It has been shown experimentally [25] that amino acid residues Glu330 and Val331 are more important for binding of factor Xa than amino acid residues Glu323 and Tyr324. Since amino acids Glu330 and Val331 are exposed to the solvent they could be responsible for the weak interaction observed between factor Va and factor Xa in solution (i.e., the binding site of factor Xa on factor Va in not totally solvent accessible when the
proteins are not bound to a procoagulant cell membrane surface. Amino acid residues Glu467, Ala511, Arg652, and His1683, previously shown to represent a surface for factor Xa binding, remained solvent exposed after simulations (23). However, it was found that SASA of Glu467 slightly decreased during simulations, mainly due to the new hydrogen bond formation with Lys607. During simulations, Arg652 formed hydrogen bonds with Thr571 and Asp653, while His1683 formed hydrogen bonds with Glu1560.

3.4.2 Calcium and Copper binding sites on factor Va.

Ion binding sites are shown in figure 3.5 together with the distances prior and after simulation. Before simulation, the distance from the carboxy oxygens of Glu1859 to the copper ion were found to be in the range of 4 - 5.5 Å (figure 3.5 panel A). Following simulation, the same distance decreased to 2.12 - 2.20 Å (see figure 3.5). The modeled copper ion, using factor Va, as a template is not at the interface of A1 and A3 as previously suggested [20], hence it may not be directly involved in the association of the heavy and light chains. As proposed [21] the copper ion may have a role in providing additional stabilization of the heavy and light chains. The closest amino acid residue from the heavy chain is found at a distance of 5 Å from the copper ion (Gln87). This model assumes that the heavy and light chains are held together primarily by means of hydrogen bonds between the A1 and A3 domains as suggested [21]. The distance between the calcium ion to the neighboring residues shows no significant change (see figure 3.5 panel C before simulations and figure 3.5 panel D after simulations). Amino acids Asp1579 and Glu1583 are found to be slightly closer to the other calcium ion after simulation (2.28 Å and 2.31 Å respectively, figure 3.5 panel F) when compared with the same arrangement before simulation (3.78 Å and 2.82 Å respectively, figure 3.5 panel E).
Figure 3.5 Detail views of the Ca$^{2+}$ and Cu$^{2+}$ ions.
Amino acid residues involved in binding of the Cu$^{2+}$ and Ca$^{2+}$ ions are shown with sticks. Panels A and B show the Cu$^{2+}$ ion coordination before and after the simulation, respectively. Panels C, D and E, F show the two Ca$^{2+}$ ions before and after simulations. Distances, in angstroms, are shown from the Cu$^{2+}$ and Ca$^{2+}$ ions to the nearest amino acid residues.

3.4.3 Prothrombin binding site on factor Va.

In the newly extended factor Va model, the prothrombin binding site (residues Asp695 - Gln699 (24)) is in the proximity of factor Xa binding sites that is 22 Å to residues Glu323 - Val331 (22), and 12 - 36 Å to residues Glu467, Ala511, Arg652, and His1683 (23). The RMSD from the initial model calculated for all five residues, Asp695 - Gln699, shows that Asp695 and Tyr696 have the largest RMSD value (8 Å and 6 Å, respectively), while Asp697, Tyr698, and Gln699 have smaller, but similar values compared to each other (~2 Å). After simulations, the phenyl moiety of amino acid residues Tyr696 and Tyr698 are facing towards the outside the protein, whereas Gln699, even though it is partially solvent accessible, is found in a pocket close to Arg709.

3.4.4 Amino acid changes in the factor Va molecule.

We next used the model of the factor Va molecule as a control system to understand the structural consequences of the mutations at positions D334 and Y335 [48]. The MD simulation of factor VaControl reached equilibrium after 1 ns and was extended for another nanosecond for analysis. The simulation of factor VaKF reached equilibrium after 2.5 ns and the simulation was extended to 3.5 ns for analysis of the last equilibrated nanosecond. The simulation of factor VaAA reached equilibrium at 2 ns and the last 500 ps were used for analysis. Checking several simulation parameters such as the potential energy, pressure, and temperature also assessed equilibration for all three systems. These parameters did not fluctuate significantly during the last 1 ns of each simulation (not shown). Figure 3.6 panel A shows the final snapshot of factor VaControl with the amino acid residues D334 and Y335 shown as blue sticks. The corresponding amino acids in factor VaKF (K334 and F335, figure 3.6 panel B) and in factor VaAA (A334 and A335,
The $S528 \rightarrow F538$ α-helix. Analyses of the simulations of factor VaControl, factor VaKF, and factor VaAA reveal a difference in the α-helix motif encompassing the region $S528 \rightarrow F538$. While in factor VaControl the α-helix is preserved throughout the simulation (figure 3.6.A, red ribbon), the specific fold of the α-helix is disrupted in the factor VaKF and factor VaAA molecules (figure 3.6. panels B-C in red ribbon). It is interesting to note that the α-helix gets disordered starting at S528 in the case of factor VaKF, whereas in the case of factor VaAA the disordering starts at the F538-end of the α-helix. The disordered $S528 \rightarrow F538$ α-helix shown at the final snapshots in the case of factor VaKF arises from the fact that Y530 looses its capability to form the necessary hydrogen-bonding network with the main chain of amino acid D533. This bonding is required for the stability of the $S528 \rightarrow F538$ α-helix. The same bond between Y530 and D533 was found to be persistent in both factor VaControl and factor VaAA. Removal of the bond between Y530 and amino acids D533 in the case of factor VaKF is a consequence of the formation of a new hydrogen bond between Y530 and R413. The hydrogen-bonding with amino acid E330 that is part of a neighboring β-sheet that contains the 334 and 335 sequence, is preserved. In the case of the factor VaAA molecule disruption of the $S528 \rightarrow F538$ α-helix can be attributed to the removal of the hydrogen bond between the main chain atoms of F538 and I535. This bond was found to be persistent in both factor VaControl and factor VaKF. The removal of this hydrogen bond in the factor VaAA molecule is due to the formation of a new hydrogen bond between F538 with H362.

The $R321 \rightarrow V331/K365 \rightarrow T369$ β-sheet. The anti-parallel β-sheet contained in the
region R321→V331 (first strand) and K365→T369 (second strand) is preserved in the case of factor VaControl during the simulation. On the other hand, in the case of the factor VaAA and factor VaKF molecules, the β-sheets are extended at the V331 and K365 ends by three amino acids. Elongation of the R321→V331/K365→T369 β-sheet to R321→A334/H362→T369 in the factor VaAA molecule positioned H362 in a conformation that ensured hydrogen bonding with F538 thus directly affecting the stability of the S528→F538 α-helix as described above.

To assess the structural perturbations created by the mutations in the functional domains of the molecule, we next assessed the distances between the centers of masses between two groups, one representing amino acids 334→335 and the other corresponding to the factor Xa binding site on factor Va (amino acid residues 323→331). The average distance between the centers of the masses between these two amino acid groups was 21 Å in factor VaControl and factor VaAA, and 19 Å in factor VaKF (data not shown). However, the distances between the Ca atoms between amino acids 334→335 and the extremities of the factor Xa binding site taken separately, one by one, did not significantly changed. Similarly, distance analysis between the center of masses of amino acid group D695→Q699 that are important for expression of cofactor activity and D334→Y335 showed an approximate distance of 16.1 Å in factor VaControl. A similar distance analysis in factor VaKF yielded a distance of 13.6 Å whereas in VaAA the distance was 17.5 Å. These data demonstrate a rearrangement of amino acids belonging to both sites following amino acid substitution D334→K and Y335→F for factor VaKF and D334→A and Y335→A for factor VaAA. Thus, the structural changes observed are specifically due to the mutations at amino acids 334 and 335.
Collectively, through a systematic approach, using both kinetic studies with synthetic peptides and recombinant proteins, our data demonstrate that amino acid residues D334 and Y335 are crucial for optimum rearrangement 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 [29].

The mechanism of inhibition of prothrombinase function by AP5, AP5DY→KF, and IWDYA, 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 332IWDYA336 might contribute to the activity of prothrombinase [57].

The present data show that the 332IWDYA336 motif shared by AP5 and AP6 contains the amino acids accountable for the inhibitory effect of the peptides. This is indicative by the similar $K_i$ values of peptides AP6 and 332IWDYA336. AP5 has an 8-fold lower $K_i$ value than AP6 and I5A, because it also contains amino acids Glu330 and Val331, which have been previously shown to be involved in factor Xa binding (24).

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 332IWDYA336, 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.
Site directed mutagenesis was 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 prothrombinase produces an enzyme with altered $K_M$ and $k_{cat}$. In addition, the study of prothrombin cleavage by prothrombinase by gel electrophoresis assembled with factor VaKF demonstrated an 8-fold lower rate of prothrombin cleavage than prothrombinase assembled with the wild type cofactor molecule.
Figure 3.6 Detailed view of the factor VaControl molecule in the near vicinity of amino acid residues 334-335.

A portion of the prothrombin binding site containing amino acid residues D695 to Q699 is shown as magenta spheres. The α-helix contained in S528→F538 is represented in red. The β-sheet contained in the R321→V331/K365→T369 sequence is shown in yellow. The loop contained in the 400→420 sequence is shown in cyan ribbons. Amino acid residues F538 and H362 are shown as green sticks; S412 is shown as cyan sticks; N534 and K364 are shown as lime sticks. Amino acid residues Y530 and E330 are shown as olive sticks and E323 is shown as orange sticks. The final snapshot at 2ns of factor VaControl is shown in Panel A with amino acid residues D334 and Y335 represented as blue sticks. Panel B shows the final snapshot at 3.5 ns of factor VaKF with the mutated amino acid residues K334 and Phe335 in blue sticks and R413 and S412 as cyan sticks. Panel B Panel C Snapshot at 2ns of factor VaAA with the mutated amino acid residues A334 and A335 in blue.

Since the $K_D$ of factor VaKF is similar to the $K_D$ of the wild type molecule, these data like the kinetic data strongly suggest that the inability of prothrombinase assembled with factor VaKF 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 $k_{cat}$) and because of impaired prothrombinase-substrate interaction (increase in the $K_m$).

Prothrombinase is composed of factor Va and factor Xa assembled on a membrane surface in the presence of divalent metal ions. We can thus hypothesize 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 in the regulatory subunit may influence (modify) the stability of the catalytic site of the catalytic subunit.

Thus, the consequences of mutations in factor Va affecting factor Xa catalytic efficiency can be measured relative to the change in transition-state stabilization free energy of the enzyme as previously established. The kinetic data showed that the DY $\rightarrow$ KF mutation in the regulatory subunit of prothrombinase results in a significant decrease of the catalytic efficiency of the enzyme which in turn is translated by a positive value of $\Delta G_{WT} \rightarrow KF$.

The results obtained from the MD simulations of wild type factor Va and factor VaKF were able to offer an explanation for both the similar $K_D$ values found for the interaction of wild type factor Va and factor VaKF with factor Xa as well as for the impaired clotting and cofactor activity of factor VaKF. The modeling data demonstrate
that while the average distance of the center of the mass between amino acid groups 334-335 and 323-331 in wild type is 21 Å, the same distance in factor VaKF is 19 Å. On the other hand, the distances between the Cα of amino acids 334-323, 334-324, 335-323, and 335-324 analyzed separately do not change significantly when comparing wild type factor Va with the mutant molecule.

Thus, there are no changes in the overall conformation of the 323-331 amino acid segment because of the mutations. The combined data rather suggest that the difference in the distance of the centers of mass between the two amino acid segments observed is due to the presence of different amino acid side chains that interact with each other.

In addition, the significant decrease in the second order rate constants of prothrombinase assembled with factor VaKF/FF and factor VaKF/MI together with the positive values of $\Delta \Delta G_{\text{int}}$ implies that combination of the mutations in factor Va have a cumulative detrimental effect on prothrombin catalysis by factor Xa with destabilization of the transition-state complex resulting in a slower rate of cleavage. These results provide evidence that in addition to amino acid residues Glu323, Tyr324, Glu330, and Val331, representing an interactive site of factor Xa for factor Va, residues Asp334 and Tyr335 of factor Va are also indispensable for optimum rates of activation of prothrombin by factor Xa as a member of prothrombinase.

Factor VaKF showed an important conformational change in the region Ser528-Phe538, resulting in a distorted $\alpha$-helix (see figure 3.6). The distorted $\alpha$-helix contained in the 528 to 538 amino acid sequence may have an influence on the overall decreased catalytic efficiency observed in the kinetic studies as a result of the drastic changes in the secondary structure elements. Although the primary sequence of the disrupted $\alpha$-helix
lies in the continuation of what was found to contain a factor Xa and protein S binding site [31] (amino acid residues 493-506), these amino acids are located on the opposite side of the molecule with the connecting peptide (amino acid residues 507 to 527) buried inside the factor Va molecule.

At this point it is important to underline the complementarity of all the data provided herein. Our data show that in the presence of the mutation there is a relocation of the COOH-terminal region of factor VaKF as compared with the wild type cofactor molecule. However, while the MD simulations are able to detect considerable structural changes at the carboxyl-terminus of factor VaKF, these calculations are unable to distinguish whether the changes will enhance or decrease the catalytic efficiency of the prothrombinase complex assembled with the mutated cofactor molecule. On the other hand, the kinetic and thermodynamic data demonstrate a detrimental effect of the mutations on cofactor activity without providing a structural explanation.

Together the data suggest that the rearrangement at the COOH-terminus of factor VaKF is detrimental to the overall catalytic efficiency of factor Xa as part of prothrombinase. Therefore, the MD simulations are very useful in providing the theoretical explanation for the 6-fold decrease in the second order rate constant of prothrombinase assembled with factor VaKF. Thus, in addition to all the original findings presented herein, our data demonstrate that the recently published model of factor Va can be extremely useful in explaining the behavior of mutated factor Va molecules. 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 demonstrate the cofactor requirement for the efficient rearrangement of enzyme (factor Xa) and substrate
(prothrombin) within prothrombinase required for efficient catalysis. Thus, our data strongly suggest that factor Va directs (regulates) catalysis of prothrombin by factor Xa within prothrombinase at two spatially distinct sites.

3.4.5 Dissociation of the A2 domain from APC-cleaved factor Va.

All three cleavage sites of APC on the factor Va model are solvent accessible (see figure 3.3, Arg306 in magenta, Arg506 in cyan, and Arg679 in lime). Previous data using bovine factor Va and immunoprecipitation techniques have suggested that factor Va inactivation is consequence of both cleavage and dissociation of the A2 domain from the rest of the molecule [13].

However, the latter data were unable to distinguish which of the two events was the major contributing factor to the inactivation process. Similar biochemical data are not available for the human molecule. Moreover, by using an inter-domain engineered disulfide bond recombinant factor Va molecule it was suggested that cleavage at Arg506 and Arg306 are critical for inactivation [58]. This latter study used a recombinant human factor Va molecule with a disulfide bond between Cys609 and Cys1691. Finally, it has been shown that inactivation of factor Va is much slower than inactivation of normal plasma factor Va [59-62]
Figure 3.7 RMSD of fragments generated after cleavage at Arg306, Arg506 and Arg679 (T =300 K).
Cα RMSD (nm) from initial model at t=0 ns is shown versus the simulation time (ps). The fragments resulted after cleavage at Arg 306, 506 and 679 are shown as follows: Ala1 - Arg306 in red, Asn307 - Arg506 in blue, and Gly507 - Arg679 in green.
Figure 3.8 RMSD of fragments generated after cleavage at Arg506 and Arg679 (T=300K). Cα RMSD (nm) from initial model at t=0 ns of fragments Ala1-Arg506 (red line) and Gly507-Arg679 (green line).
Figure 3.9 RMSD of fragments generated after cleavage at Arg306 and Arg679 (T=300K).

Ca RMSD (nm) from initial model at t=0 ns of fragments Ala1 - Arg306 (red line), Asn307 - Arg709 (green line).
Figure 3.10 RMSD of fragments generated after cleavage at Arg306, Arg506 (T = 600 K).
Cα RMSD (nm) from initial model at t=0 ns of fragments Ala1 - Arg306 (red line), Asn307 - Arg506 (blue line), and Gly507 - Arg709 (green line)
The question that remains unanswered is: is factor Va3/6 inactivation slower because of slower rate of cleavage at Arg306 (because of absence of cleavage at Arg506) or because of slower rate of dissociation of the A2 domain from the rest of the molecule?

To ascertain the role of cleavages at Arg306 and Arg506 by APC for human factor Va inactivation, a 2 ns simulation of factor Va fragments derived from membrane-dependent inactivation of factor Va by APC was performed using the factor Va model developed herein (factor Va3/5/6, figure 3.7). The data reveal that the two A2 domain-derived fragments (Asn307 - Arg506 and Gly507 - Arg679) have persistently larger RMSD values than the A1 domain (amino acid residues Ala1 - Arg306, figure 3.7). The number of hydrogen bonds between the A2 domain-derived fragments and the light chain was also found to decrease.

Distance analyses, between the A2 domain-derived fragments and a contact region from the light chain, residues Val1736 - Leu1836, shows that the distance between fragments composed of amino acids Asn307 - Arg506 and Gly507 - Arg679 increases by 1.8 Å and 2.2 Å respectively following 300 ps and remains constant following a 2 ns simulation (data not shown).

In contrast, the distance of the A1 domain of factor Va (Ala1 - Arg306) from the same contact point of the light chain remains approximately the same following a 2 ns simulation (data not shown). It thus appears that while the A1 domain remains connected to the light chain by non-covalent interaction, the distance between A2 domain fragments and the rest of the molecule increases.

These data strongly suggest that the A2 domain of the cofactor dissociates from the rest of the molecule following APC cleavage.
The simulations of factor Va5/6 (simulation of factor Va cleaved by APC in the absence of a membrane surface at Arg506 and Arg679) show that the two resulting fragments, i.e., Ala1 - Arg506, Gly507 - Arg679, have the same RMSD values (figure 3.8, red and green). Distance analysis of these two fragments shows that the distance between these segments and residues Val1736 - Leu1836 of the light chain, does not significantly increase during the 2 ns simulation. Additionally, the hydrogen-bonding network between these fragments and the light chain is also preserved.

Finally, a similar analysis of factor Va3/6 (simulation of APC-cleaved membrane bound factor Va3/6, figure 3.9, red and green) reveals that cleavage at Arg306 even in the absence of cleavage at Arg506 results in the release of the A2 domain of the cofactor in a time frame similar to that observed for plasma factor Va3/5/6 (650 ps). These data are original and provide compelling evidence suggesting that inactivation of factor Va occurs because of dissociation of the A2 domain of factor Va and loss of a portion of the factor Xa binding domain.

Overall the data imply that cleavage of the cofactor at Arg506 is not required for bisection of the A2 domain in two fragments. Cleavage of factor Va at Arg506 is rather required to facilitate cleavage at Arg306. Thus, slower inactivation of factor Va by APC occurs because of delayed cleavage at Arg306, which in turn is required for dissociation of the A2 domain from the rest of the molecule. Altogether these data demonstrate that factor Va inactivation occurs in a similar manner as factor VIIIa inactivation following dissociation of the enzyme binding-site from the rest of the cofactor molecule. However, while inactivation of the latter occurs spontaneously at pH 7.4, factor Va inactivation is induced following cleavage by APC at Arg306.
3.4.6 Interaction of factor Va with the lipid bilayer.

*Interaction of factor Va with the lipid bilayer.* Figure 3.11.A shows the final snapshot of the factor Va molecule (heavy chain (1-709) in red ribbon, light chain 1546-2196 in blue ribbon) bound to the lipid bilayer. Amino acid regions: Asn1913-Asn1918, Gly1902-Trp1904, Lys2114-Met2120, Lys2087-Lys2092, and Thr2179-Ser2183 from factor Va, found to interact at the end of the simulation with lipid head groups, are shown as spheres. At the end of the simulation the distance from Cys2113 to the closest lipid molecule was found to be ~ 7Å (distance at t = 0 was set to 15 Å). Cys2113 was found to interact with a soluble phosphatidylserine molecule thus suggesting a possible involvement in the lipid bilayer binding [51]. A recent factor V mutant molecule having Arg2074 changed to Cys was found to have reduced. The authors concluded that mutated Arg2074→Cys will form a disulfide bridge with amino acid residue Cys2113 which would in turn affect the function of prothrombinase because of the impaired lipid binding [63].

*Solvent accessible surface of regions from factor Va found to interact with the POPC:POPS lipid bilayer.* Several regions from factor Va’s C2 domain were found to interact with the phospholipid bilayer during the simulation. Solvent accessible surface areas of these regions (shown in figure 3.11 as spheres) were calculated. Solvent accessible surface area (SASA) [64] of these amino acid regions generally increased as compared with the starting configuration t = 0 (see table II).

Figure 3.12 shows the root mean square displacement, RMSD (nm), of the αC of the factor Va molecule as a function of the simulation time (ns) (panel A). The RMSD of αC atoms reaches a plateau at 0.52 nm at 6.5 ns. After 6.5 ns the RMSD has fluctuations...
<table>
<thead>
<tr>
<th>Factor Va region</th>
<th>C1 domain</th>
<th>C2 domain</th>
<th>Initial [Å²]</th>
<th>Final [Å²]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly1902-Trp1904</td>
<td>+</td>
<td>-</td>
<td>278</td>
<td>302</td>
</tr>
<tr>
<td>Asn1913-Asn1918</td>
<td>+</td>
<td>-</td>
<td>401</td>
<td>369</td>
</tr>
<tr>
<td>Lys2087-Lys2092</td>
<td>-</td>
<td>+</td>
<td>428</td>
<td>372</td>
</tr>
<tr>
<td>Lys2114-Met2120</td>
<td>-</td>
<td>+</td>
<td>358</td>
<td>367</td>
</tr>
<tr>
<td>Thr2179-Ser2183</td>
<td>-</td>
<td>+</td>
<td>333</td>
<td>334</td>
</tr>
</tbody>
</table>

**Table II.** Solvent accessible surface area of the lipid bilayer interacting regions from factor Va.
of ± 0.02 nm. The distance between the center of mass of the factor Va molecule and the center of mass of the lipid bilayer was monitored during the simulation (panel B). The plot of the distance as a function of the simulation time has a distinctive profile: during the first ns of the total simulation time the distance decreases by 47% of the total decrease at the end of the simulation. This region is marked as region I in the figure. The next 5 ns (marked as region II) also show a decrease in the monitored distance, however at a slower pace when compared to region I. During the 5 ns simulation the distance decrease represents 11% of the total decrease at the end of the simulation. Region III (between 6 and 7 ns) resembles with the profile from region I and represent the second highest drop in distance accounting for the remaining 42% of the total decrease in distance between the centers of masses of factor Va and the lipid bilayer. After 7 ns (region IV) the distance reaches a plateau and for the next 2 ns the fluctuations are in the range of ± 0.1 nm. These data, the RMSD plot and the center of mass distance profile, demonstrate that the system reaches at least a local equilibrium after 6.5 ns of the simulation time.

Panel C shows the RMSD as a function of factor Va residue number. Several residue groups were found to have increased RMSD values as compared with the starting structure. The region encompassed between amino acid residues 660-680 increased more than twice as compared with the initial snapshot of the molecule. The amino acid region containing Arg506 (504 –507) was found to have RMSD values of 0.25 nm and represents one of the highest among all residues. This region contains an important APC cleavage site on factor Va and the high mobility of this loop could be a requirement for efficient cleavage.

3.4.7 Factor Xa binding site on factor Va in factor Va – POPC:POPS.
Interaction sites on factor Va with factor Xa were identified in several regions: 311-325 [23], 323-331 [24], 493-506 [22]. Solvent exposed area surface of amino acid residues 323-331 remained the same throughout the simulation, i.e., an average value of 13.3 nm$^2$.

### 3.4.8 Prothrombin binding site on factor Va in factor Va – POPC:POPS.

The prothrombin binding site located at position 695-699 [29] is contained in the C-terminus of the heavy chain region where RMSD values range from 0.1 to 0.25 nm. The amino acid region 695-699 has the smallest RMSD ~ 0.1 nm of all residues from the C-terminus.

This study describes for the first time a model of factor Va in the presence of phospholipids composed of POPC and POPS lipids in explicit atomistic detail. It also follows the dynamics of binding of factor Va to the asymmetric phospholipid mixture. Interaction of factor Va with phospholipids was described as a two-step process: (a) adsorption and (b) penetration (37). In our studies we found that the profile of interaction from the molecular dynamics simulations shows a fast adsorption profile during the first ns of the simulation time. This is followed by a 5 ns period where the speed of factor Va insertion drops four times. After 6 ns the factor Va insertion increases again and the behavior of the system resembles the first ns. The insertion continues for another ns and after 7 ns the monitored distance reaches a plateau (figure 3.12.B, region IV). Analysis of the mass density profiles (see figure 3.11 panel C) of several groups such as the methyl groups, ester groups of the fatty acyl chains, the phosphorus atom of the head groups, and amino acid residues Trp2063/Trp2064 revealed that factor Va inserted slightly deeper than the phosphorus atoms and in the vicinity of the ester group and the hydrophobic and
hydrophilic interface (figure 3.11 panel C). This positioning of the Trp residues is in agreement with the NMR studies of Trp analogues that study the insertion level into phospholipid membranes [65]. This study showed that Trp analogues were found to be most probable at the interface of the lipid/water interface of the bilayer. This is most probably due to their dual properties: the possibility to be involved in hydrophobic interaction due to the indole ring and the possibility to participate in hydrogen bonding with groups from the hydrophilic phase of the lipid bilayer such as carbonyl groups and/or water molecules. Another study showed that Trp residues are preferentially found at the membrane interface [66]. Another study found that the indole moiety is located at ~9 Å placing the backbone atoms at the membrane interface [67]. At the end of the simulation time the factor Va molecule was not inserted in the lipid bilayer at the extent suggested in other studies [68, 69] but rather was found at an equilibrium state after the adsorption process. The backbone atoms of amino acid residue Trp2068 is located ~11.4 Å from the peptide bond between Trp2063-Trp2064 [21]. This distance remains in the same range (i.e., 12.4 Å) in the presence of the phospholipid bilayer and has no interaction with phospholipids as suggested [19, 67]. A region, Tyr1956-Leu1957, located in the C1 domain of factor Va was also found to interact with the phospholipids at the end of the simulation as suggested [50]. At the end of the simulation, the ratio between factor Va and lipid molecules was found to be 1:28 a ratio comparable with the one suggested (i.e. 25 to 30 lipid molecules to 1 factor Va molecule) [70]. Even though the ratio (between factor Va and the phospholipids) is similar with the experimentally determined value, a visual inspection shows that this ratio will be unlikely to change significantly even in the case of a deeper insertion of factor Va.
Although the insertion of factor Va deep into the hydrophobic core is not visible during this simulation time frame, amino acid residue Arg506 was found to be positioned ~75 Å from the lipid plane. This distance agrees well with the distance obtained using FRET [71] measurements recorded for Arg506 and the lipid bilayer plane. The farthest region from the phospholipid membrane was found to be located at ~93 Å as suggested from FRET measurement [72] (a minimum of 90 Å). The distance between Arg306 and the phospholipid surface (i.e., ~84 Å) is in good agreement with earlier fluorescence resonance energy transfer studies based on a fluorescein dye in the active site of APC and octadecylrhodamine dye from the bilayer surface (~84-86 Å) [71, 73].

The small variations in the solvent accessible surface area of the Xa binding site together with the small RMSD of residues 320-335 (i.e., 0.7 Å) does not indicated a direct effect of the phospholipids on the factor Xa interacting region. The RMSD of the prothrombin binding site (i.e. 695-699) does show an increase as compared with the initial configuration (i.e., RMSD ~1.1 Å). However, it is interesting to note that region 695-699 has the smallest RMSD of the last ~100 amino acids from of the C-terminus where other RMSD values are in the 2.5 Å range (see figure 3.12, panel C). This implies that interaction of factor Va with the phospholipid surface does not result in a rearrangement of the prothrombin binding site.

3.4.9 Activated protein C cleavage sites.

Arg506 represents a cleavage site on factor Va by Activated Protein C. In factor Va bound to POPC:POPS Arg506 was found at ~75 Å from the outer layer of the lipid bilayer defined by the lipid head groups.
This study describes the binding of factor Va to the asymmetric phospholipid mixture composed of POPC and POPS lipids. Interaction of factor Va with phospholipids was described as a two step process: (a) adsorption and (b) penetration [74]. The profile of interaction from the molecular dynamics simulations shows a fast adsorption profile during the first ns of the simulation time. At this time, the factor Va molecule is still in solution. This is followed by a 5 ns period where the speed of factor Va insertion drops four times. During the 6 ns the factor Va molecule is slowly inserting. At this time the factor Va molecule is in the lipid head group region – the hydrophilic region of the bilayer. After region II the insertion rate increases although the effective distance is not large (i.e. 5 Å considering the centers of masses). Region III resembles to the first ns. The insertion continues for another ns and after 7 ns the monitored distance reaches a plateau (figure 3.12.B, region IV). At this time the factor Va molecule reached the hydrophobic layer of the lipid bilayer. It could be that hydrophobic forces further drive deeper insertion of the factor Va molecule. Analysis of the mass density profiles (figure 3.11 panel C) of several groups such as the methyl groups, ester groups of the fatty acyl chains, the phosphorus atom of the head groups, and amino acid residues Trp2063/Trp2064 revealed that factor Va inserted slightly deeper than the phosphorus atoms and in the near vicinity of the ester group. At the end of the simulation time the factor Va molecule was not inserted in the lipid bilayer at the extent suggested in other studies. After 9 ns of simulation the factor Va molecule only equilibrated after the adsorption process.

Although the insertion of factor Va beyond the boundary of the hydrophobic core is not visible during this simulation time frame amino acid residue Arg506 was found to
be positioned ~75 Å from the lipid plane. This distance agrees well with the distance obtained using FRET [71] measurements (~84 Å).
Figure 3.11 Final snapshot of factor Va inserted into the POPC:POPS lipid bilayer.

Panel A shows the final snapshot of factor Va inserted into the POPC:POPS lipid bilayer. The heavy chain (1-709) is shown in red ribbon, the light chain (1546-2196) is shown in blue ribbons, POPS molecules are shown as cyan stick structures, POPC molecules are shown yellow sticks, phosphorus atoms from the lipid head groups are shown in magenta. Amino acid regions from factor Va found to interact with the lipid bilayer are shown in spheres: Asn1913-Asn1918 in red, Gly1902-Trp1904 in orange, Lys2114-Met2120 in green, Lys2087-Lys2092 in magenta, Thr2179-Ser2183 in yellow. Panel B shows Trp2063 and Trp2064 in blue sticks, factor Va is depicted with slate cartoons and lines, phospholipids in the near vicinity of Trp2063 and Trp2064 are shown having the fatty acyl chains as green sticks. Panel C shows the mass density profiles of POPC methyl groups (—), Trp2063, Trp2064 (- - -), Phosphorus atoms (…), POPC oleoyl double bond (---)
Figure 3.12 RMSD (nm) of the Cα of the factor Va molecule as a function of the simulation time (ps).

Panel A, RMSD (nm as a function of residue identifier (factor Va numbering 1-709 heavy chain and 1546-2196 for the light chain)) – panel B, distance (nm) between the center of mass of the factor Va molecule and the center of mass the lipid bilayer as a function of the simulation time (ps) The cartoons under the plot present a general view of the insertion of factor Va deep into the boundary of the hydrophobic core of the bilayer. Solvent is shown in blue, the interface between solvent and hydrophobic layer is shown in green, and the hydrophobic region is shown in yellow. The position of Trp2063, Trp2064 is shown as a red sphere. Panel C shows the RMSD of each residue from the last 2 ns of the simulation time. The RMSD values were compared with the snapshot after the second insertion (i.e., after 7 ns)
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CHAPTER IV

Modeling of human prothrombin and its components

4.1 Abstract

Conversion of prothrombin to thrombin represents the penultimate step in the formation of the cross-linked fibrin clot. The reaction takes place on the phospholipid surface and is catalyzed by the prothrombinase complex. We constructed an asymmetric phospholipid surface mimetic composed of a random mixture of 1-palmitoyl, 2-oleoyl-\textit{sn}-glycero-3-phosphatidylcholine (POPC) and 1-palmitoyl, 2-oleoyl-\textit{sn}-glycero-3-phosphatidylserine (POPS) in the outer leaflet using a 4:1 ratio. The inner leaflet was composed of only POPC molecules. We next performed 60 ns molecular dynamics (MD) simulations on the asymmetric/mixed POPC:POPS phospholipid bilayer model. The POPC : POPS lipid bilayer model was than validated by comparing the results from the MD simulation with nuclear magnetic resonance studies (See Chapter II for specific details). Analysis of the MD simulations of prothrombin fragment 1 (pf1) bound to the equilibrated POPC : POPS lipid bilayer showed that several $\gamma$-carboxyglutamic residues
of pf1 interacted through polar contacts with phospholipid molecules. Our studies show that conformational changes in prothrombin fragment 1 are due to the binding of calcium and not because of the presence of the phospholipids. Furthermore, our simulations show that removal of the calcium coordinated by the N-terminus of pf1 induce a considerably larger displacement as compared with the displacement calculated from the simulations of pf1 with calcium, the simulations of pf1 with calcium and phospholipids, and finally the simulations with pf1 where calcium ions were replaced with sodium ions. The interaction of factor Va with the POPC : POPS lipid bilayers was also investigated. In the case of factor Va interaction the simulation was able to reproduce the binding event - the insertion of amino acid residues form factor Va's C2 domain into the hydrophobic region of the lipid bilayer. We also propose a model for the prethrombin 1 molecule - a thrombin precursor. In the second part of the study the interaction of prothrombin with POPC:POPS vesicles was also studied. This newly developed prothrombin “bent” model associated with the POPC:POPS lipid bilayer is able to provide the necessary framework for the construction of the whole prothrombinase complex (factor Va, factor Xa, and phospholipids) and its substrate (prothrombin).

4.2 Introduction

Human prothrombin is a vitamin K-dependent protein and is the precursor form of thrombin, the protease that holds a central role in the optimal regulation of the blood coagulation event. Although factor Xa can activate prothrombin to thrombin, the physiologically required activation rate is achieved only through the assembly of the fully functional prothrombinase complex (factor Xa – the enzyme, factor Va –the cofactor associated in the presence of the activated cell surface and calcium ions) [1]. The increase
in enzymatic efficiency of the prothrombinase complex is attributed to the decrease of the
Km (following the interaction with phospholipid vesicles resulting in higher local
substrate concentrations) and an increase in $k_{\text{cat}}$ (solely attributed to the inclusion of
factor Va in the prothrombinase complex) [2-4].

Prothrombin (M, 72 kDa) is the precursor form of thrombin, an enzyme known to
have important roles in activation of blood coagulation factors and platelets. Prothrombin
has three structural regions: (a) fragment 1 composed of the GLA domain (residues 1-40)
and the kringle 1 domain (residues 41-155), (b) fragment 2 composed of kringle 2
domain (residues 156-271) and (c) the serine protease domain (residues 272-579).
Activation of prothrombin to thrombin is catalyzed by prothrombinase composed of
factor Xa – the enzyme, factor Va – the cofactor both associated in the presence of
negatively charged phospholipids and calcium ions). Prothrombinase converts
prothrombin to -thrombin following two sequential cleavages at Arg320 and Arg271.
This pathway represents the physiologically relevant pathway (Arg320 cleaved first,
Arg271 second) and follows through the formation of meizothrombin intermediate. In the
absence of factor Va – factor Xa will cleave Arg271 first and Arg320 second and
thrombin will form through the prothrombin 2 intermediate.

Prothrombin is part of the so-called vitamin K dependent proteins together with
factor VII, factor IX, factor X, and protein C. These zymogens are known to have
elongated structures as previously described (1-3). However, even though prothrombin
has a homologous Gla domain with these zymogens and a catalytic domain of a serine
protease that is homologous with chymotrypsin and trypsin, prothrombin possesses two
kringle domains in place of the “epidermal growth factor like” domains found in the other
zymogens. To date, there is no atomic model about the overall structure of prothrombin. Although the X-ray structures of the majority of prothrombin’s domains (such as: catalytic domain, fragment 1, and meizothrombin lacking fragment 1) are available in parts, the spatial orientation of these domains remains uncertain at best.

Prothrombin is composed of a $\gamma$-carboxyglutamic acid rich region (GLA domain), two kringle domains, and the catalytic region. The GLA domain (amino acid region 1 to 45) participates in the binding to phospholipid vesicles by bridging $\gamma$-carboxyglutamic residues with calcium and phosphatidylserine lipid head groups. Site directed mutagenesis of the $\gamma$-carboxyglutamic residues, acetylating the amino groups from the prothrombin N-terminus and removal of the calcium ions all resulted in abolished membrane binding of prothrombin fragment 1 [5-7]. Binding of calcium was also shown to induce conformational changes in pf1 [8].

Several models of the enzymatic complexes that participate in the blood coagulation event were successfully created [9-13]. However, these models still lack a vital component: the negatively charged phospholipid vesicles. In the current work we propose for the first time a 60 ns equilibrated mixed 1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) and 1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphatidylserine (POPS) in a 4:1 ratio in the outer leaflet. The inner leaflet contained only POPC molecules. The mixed asymmetric POPC:POPS lipid bilayer model was then used to characterize the dynamics of the lipid binding at the molecular level of the human prothrombin fragment 1 (pf1) molecule.

Pf1 is a highly homologous region among species, except zebrafish and hagfish. In the absence of a crystal structure of the entire human prothrombin molecule, we
created a homology model of human pf1 encompassing amino acid residues 1-155. The pure homology modeling of the complete pf1 and its dynamics in solution is not a novelty in the field [14, 15] and specific interactions between pf1 and the lipid bilayer were previously proposed [16]. However, our work offers for the first time a view of the dynamics of the interaction between pf1 and the POPC:POPS mixed lipid bilayer using molecular dynamics simulations on 20 ns time scale. Furthermore, the POPC:POPS lipid model described herein, represents the long time needed framework in the building of the POPC:POPS bound enzymes that orchestrate the fine-tuning of the coagulation event such as the prothrombinase complex, the factor VIIa-tissue factor-factor Xa complex, the factor VII-tissue factor-factor IX complex and the factor VIIIa-factor IXa.

4.3 Methods

4.3.1 Modeling of human pf1.

Human pf1 homology modeling was performed using the Modeller package [17]. Alignments between the target pf1 sequence [18] and templates (see Appendix figure A4.1) were performed with the “matcher” tool from the EMBOSS server [19]. The modeling of human pf1 was performed using three template structures determined by X-ray crystallography pdb codes 1NL1 [20], 2PF1 [21]. Because the two templates share sequence identity, enabled a modeling procedure where the N terminus of pf1 was modeled using the 1NL1 structure whereas the C terminus region was modeled based on the three dimensional fold of the 2PF1 structure. First the sequence of human pf1 was aligned with the amino acid sequence found in 1NL1. The aligned sequences were found to be 83.0 % identical and 91.2 % similar with one gap corresponding to Gly4 of the bovine pf1. In the first step of the homology modeling procedure, the water molecules
found in the 1NL1 structure were removed together with the N-acetyl-D-glucosamine, the calcium ions and the carboxyls from the γ-carboxyglutamic residues. This simplified model was used as a template for amino acid 1-55 of human pf1 and represented the input parameter form the Modeller package version 8.2. [17]. Using superposition with the X-ray structure (1NL1) the γ-carboxylated glutamic residues and the calcium ions were added to the model. One extra calcium ion was added as proposed in an earlier human pf1 homology model and as described in the strontium bound prothrombin fragment 1 [14, 22]. The model produced by Modeller [17] was first validated using the PROSAII program [23]. The z-score corresponding to pf1 was compared with the z-score of the templates (i.e., 1NL1 and 2PF2 see Appendix figure A4.2). The second validation method of the pf1 homology model was based on its stereochemistry and was performed using the PROCHECK program (resolution for the validating procedure was set to 1.5 Å) [24] (see Appendix figure A4.3).

4.3.2 MD simulation of pf1 inserted into the PCPS lipid bilayer.

Pf1 was inserted into the PCPS lipid bilayer so the region composed of amino acid residues 1-10 was positioned in the hydrophobic core of the outer leaflet. This positioning of pf1 ensured that the ω-loop of the γ-carboxyglutamic acid rich region was inserted in the lipid bilayer as previously proposed using triptophane fluorescence studies [16]. Such positioning of pf1 in the lipid bilayer is also based on several site directed mutagenesis studies of the first 20 amino acid residues that showed decreased or inexistent binding ability. Following the insertion of pf1, one POPC lipid unit was removed because of the overlap. The final box size was set to 6 × 6 × 22 nm. The system was solvated with 22266 water molecules and contained a total of 128 phospholipid
molecules. A minimization procedure was then performed where prothrombin fragment was held fixed. The free movement of the POPC and POPS lipid units achieved removal of the sterical impediments in the pf1 interacting leaflet. Production MD simulations were performed as described in the Molecular dynamics simulations setup sections a, b and c. pf1Sol/Ca/Lipid.

2.3.3 MD simulations of the pf1 in the absence of phospholipids.

Three MD simulations of pf1 in solution were used as control experiments. The simulations parameters were as described in the Molecular dynamics simulations setup sections a, b, and c. All three systems were considered as an NPT ensemble. The first control simulation consisted of the solvated pf1 in the presence of Ca$^{2+}$ ions (pf1Sol/Ca). The second simulation of pf1 was performed in the absence of Ca$^{2+}$ ions (pf1Sol). In the third simulation of pf1 the Ca$^{2+}$ ions were replaced with Na$^{+}$ ions (pf1Sol/Na). The simulation time for pf1Sol/Ca, pf1Sol, and pf1Sol/Na was set to 20 ns.

4.3.4 Modeling of human prethrombin 1 using the bovine meizothrombin des fragment 1 crystal structure as a template.

Molecular modeling was performed for amino acid residues Ser164-Glu579. Alignment of the human and bovine amino acid sequences of prothrombin, performed with the matcher tool in the EMBOSS [19] online software suite, showed 81.8 % identity and 89% similarity with a gap of two amino acids. The modeling was performed using the Modeller package version 8v1 [17]. The algorithm implemented in Modeller uses the primary sequence alignment of the target and template. The modeling procedure employed herein uses two template structures: the crystal structure of human prethrombin 2 (pdb entry 1HAG) [25], and the crystal structure of bovine meizothrombin lacking
fragment 1 (meizothrombin des-fragment 1, pdb entry 1A0H) [26]. Thus, the amino acid region of prethrombin 1 Ser164-Arg284 was modeled entirely using the three-dimensional model of the bovine meizothrombin des-fragment 1, amino acid region Thr285-Lys308 was modeled using the structures of both bovine meizothrombin des-fragment 1 and human prethrombin 2, and finally the region Asp292-Glu579 was modeled using only the human prethrombin 2 as a template. The modeling procedure ensured that the region that coincides with the catalytic domain of thrombin was modeled using the prethrombin 2 structure since cleavage at Arg320 and meizothrombin formation is accompanied by a significant structural change of the protease domain of prothrombin [27]. Next, the algorithm produced a three-dimensional model by solving the spatial restraints (see Appendix figure A4.5).

The model was validated using several methods and tests including cation-π interactions [28]. Evaluation was performed using several software packages: PROSAII [23], PROCHECK [24], and CAPTURE [28]. Energy minimization was performed using the steepest descent method. The neighbor list frequency update was set to 10, the initial step was set to 0.1Å and the integration step to 2 fs. Periodic boundary conditions were used in all three dimensions. Long-range electrostatic interactions were treated using the particle mesh Ewald summation method [29, 30]. Grid dimensions were set to 1.6 Å and the Ewald summation was performed in all three dimensions with the interpolation order set to 6. Short-range electrostatic interactions were calculated using a 10 Å cutoff. The force field used for simulations was a version of GROMOS87 [31] implemented in GROMACS as “ffgmx” [32, 33]. The system was solvated using the single point charge water molecule [34]. The minimum distance between the solute and the simulation box.
was set to 10 Å while the periodic boundary conditions were employed in all three directions. The negatively charged system was neutralized with 10 Na\(^+\) ions using the “genion” tool from GROMACS [32, 33].

**4.3.5 Modeling of human prothrombin.**

One hypothesis is that prothrombin is an elongated molecule where the F1 and catalytic domain are place on opposite sides of the F2 domain [35]. This hypothesis is supported by small angle X-ray scattering studies of prothrombin in solution (a large and a small ellipsoid with the following semiaxes: a1=b1=40 Å, c1=20 Å and a2=30 Å, b2=c2=20 Å) [36] and low-angle neutron scattering studies of prothrombin in presence of vesicles consisting of phosphatidylcholine and phosphatidylserine (the long axis of prothrombin ~90 Å) [36]. The prothrombin molecule binds to negatively charged phospholipids through the GLA residues rich domain and has its kringle and catalytic domains projected outward of the phospholipid surface [37]. This extended prothrombin molecule would seem to be easily accommodated by the factor Va-factor Xa models available to date [13, 38].

A second hypothesis is that prothrombin would adopt a more or less globular shape in the presence. This hypothesis is suggested by studies showing that the fragment 1 domain of prothrombin interacts with the catalytic domain [39-41]. Another study, that used prothrombin 2 molecule incubated with prothrombin fragment 1, found that the membrane binding capability of the complex has regained and was able to be converted to thrombin through the meizothrombin pathway [42], also promotes the idea of a globular prothrombin in presence of phospholipid vesicles. In the absence of Ca\(^{2+}\) ions a direct interaction between fragment 1 and the protease domain was demonstrated [36, 43, 43].
Several ligands bound to exosite I of meizothrombin lacking its fragment 1 but not to meizothrombin also suggests involvement of fragment 1 in the exposure of exosite I. A prothrombin molecule cleaved by thrombin to generate prethrombin 1 (a prothrombin molecule lacking its fragment 1) had a 6-fold increase in affinity for hirudin peptides suggesting a role for fragment 1 in the exposure of proexosite I on prothrombin [45, 46].

The need to study the interactions of proteins with phospholipids using atomistic simulations is clearly evident from because the phospholipids play a major role in the coagulation process. Our current study, together with a similar study by Ohkubo et al. [47], lay the framework to study the major complexes from the coagulation process: the extrinsic tenase, the intrinsic tenase, and the prothrombinase complex.

Alignment of the amino acid sequences of the human prothrombin with the amino acid sequences showed a good alignment (see Appendix figure A4.6). The alignment between the model and templates, together with the structure files of the templates were used as the input for the MODELLER v8.2 package. Human prothrombin fragment 1 (Ala1-Arg155) containing the Gla domain was modeled as previously described (see section 4.3.1). Modeling of prethrombin 1 fragment (Ser164-Gly578) was performed as previously described [42]. Before starting the modeling procedure for the entire prothrombin molecule the specific orientation the prothrombin fragments (i.e., prothrombin fragment 1 and prethrombin 1) was performed using SwissPdbViewer [48]. First, the kringle 1 domain of the prothrombin fragment 1 was superposed with the kringle 2 domain of molecule II from the crystal structure of meizothrombin-des fragment 1 [26] (see Appendix A4.7 for a graphic representation of the modeling steps). This positioning of the fragment 1 model ensured that the Cα of Arg155 is located at
~10Å from His363\(^{57}\) (part of the catalytic site) as suggested. Meizothrombin was also found to autocatalytically be able to remove its fragment 1 by cleaving after Arg155 [49]. This would position Arg155 in the near vicinity of the catalytic site residues. The active site of meizothrombin was found to be placed at ~67-70 Å from the phospholipid surface [50]. Differential scanning calorimetry showed a direct interaction between prethrombin 2 and fragment 1 [41] which is well represented in our model.

\textit{Molecular dynamics simulation of the prothrombin.} Molecular dynamics simulations were performed using the GROMACS program suite. A modified force field [51] was used where the γ-carboxyglutamic acid residues. The protein was solvated using the SPC water model and the solvated molecule was placed in a rhombic dodecahedron box. After a short energy minimization a 20 ps position restraint molecular dynamics simulation was performed. The production run molecular dynamics simulation was set to 5 ns. Electrostatics was evaluated using the particle mesh Ewald summation method – a Fourier spacing of 0.16 nm and the interpolation order of 6.

4.4 Results and discussion

4.4.1 Validation of the pf1 homology model.

Evaluation of the quality of the pf1 model was performed using the PROSAII analysis software. The plot of the pf1 model revealed that several residues were found to have positive z-scores (see Appendix figure A4.2). Although usually this means a badly modeled sequence, evaluation of the z-score profiles of the two templates revealed positive peaks in the same region as well. As a consequence the presence of positive peaks in the model is attributed to the presence of the corresponding peaks in the

\footnote{1 Chymotrypsinogen numbering}
templates. The \( \phi \) and \( \psi \) angles shown in the Ramachandran plot have values that place almost all amino acids in the allowed regions (see Appendix figure A4.3). Figure 4.1 shows the system composed of the solvated lipid bilayer with the pf1 molecule inserted in the outer leaflet.
Figure 4.1 Pf1 interactions with the phospholipid bilayer.
Panel A shows the interaction of Pf1 with the phospholipid bilayer composed of POPC and POPS. Pf1 is represented using secondary structure elements as follows amino acid residues 1-32 in magenta and amino acid residues 33-155 in red. γ-carboxyglutamic acid residues are shown with blue sticks. The POPS lipid units are shown using black lines whereas the POPC lipid units are represented with cyan lines. Panel B shows a detailed view of the Gla domain of Pf1 (amino acid residues 1-32) and the interacting phospholipid units depicted using sticks (same color coding as in panel A). Calcium ions are shown as yellow spheres. The orientation of the system used throughout the paper is as described by the X, Y, and Z axes. The figure shows the system with its periodic image in the X dimension.
4.4.2 Simulations of pf1.

Figure 4.2 panel A. shows the average of the RMSD for each amino acid residues (1-155) obtained from four different simulations pf1\textsuperscript{Sol/Ca} in blue, pf1\textsuperscript{Sol/Ca/Lipid} in red, pf1\textsuperscript{Sol} in magenta, pf1\textsuperscript{Sol/Na} in green. Molecular dynamics simulations on the 20 ns time scale of the pf1 bound to the POPC:POPS lipid bilayer showed a conformational change in pf1. This conformational change can be described as a rearrangement of three regions: region A (composed of amino acid residues 1-30), region B (composed of amino acid residues 31-69), and region C (composed of amino acid residues 70-155). Amino acid residues from pf1 were categorized in these three regions due to the overall dynamics of the molecule (i.e., large conformational changes between regions and small conformational changes within the region). Root mean square deviation (RMSD) of region A compared to the starting model was found to be 1.61 Å whereas for region C was found to be 1.91 Å. The RMSD of region B was found to be 1.99 Å. Because region B contains two $\alpha$-helix motifs the deviation is due to the amino acids found at both ends (i.e., the ones that connect region A and region C with region B). During the simulations, the initial calcium ions positions were preserved in the vicinity of the malonate-like moiety of the $\gamma$-carboxyglutamic amino acid residues. The secondary structure elements of pf1 (see figure 4.1.A depicted using ribbons) were not altered during simulations.

Root mean square displacement of all atoms as a function of the simulation time was calculated for pf1 compared with the coordinates at simulation time t=0. The graph shows a plateau of the RMSD values after 4 ns in the simulation of pf1 bound to POPC:POPS lipids in the presence of Ca$^{2+}$ ions (pf1\textsuperscript{Sol/Ca/Lipid}, see figure 4.2A.). The
negligibly small drift of these parameters during the last 10 ns shows again that equilibration of the systems is reached after the first 10 ns of the total simulation time.

The simulation performed in the absence of the POPC:POPS lipid bilayer but in the presence of Ca\(^{2+}\) (pf1\(_{\text{Sol/Ca}}\)) was found to reach equilibrium after only 4 ns of the total 20 ns simulation time. The simulation performed in the absence of the POPC:POPS lipid bilayer and with the Ca\(^{2+}\) ions replaced by Na\(^{+}\) ions (pf1\(_{\text{Sol/Na}}\)) was found to reach equilibrium after 5 ns. On the other hand, the simulation pf1Sol the RMSD values between 10 ns and 15 ns of the simulation time were found to have fluctuating values of almost 1 Å. This system was equilibrated only after 15 ns of the total 20 ns simulation time. The RMSD as a function of the residue number for the pf1\(_{\text{Sol}}\) system has the highest values as compared with the other three systems. More specifically, several residue groups such as 18-25, 1-10, and 140-150 reach RMSD values twice as much as compared with the same RMSD values for the other three systems, i.e., pf1\(_{\text{Sol/Ca}}\), pf1\(_{\text{Sol/Ca/Lipid}}\), and pf1\(_{\text{Sol/Na}}\). Another amino acid region from the pf1Sol simulation that is high is encompassed in the region 95-100. Although in these region the RMSD values are comparable with the values obtained in the pf1\(_{\text{Sol/Ca/Lipid}}\) system these values are twice as much as in the case of the other two systems pf1\(_{\text{Sol/Ca}}\) and pf1\(_{\text{Sol/Na}}\). The smallest RMSD values were found to be for the pf1\(_{\text{Sol/Ca/Lipid}}\) system except the 95-100 region described previously. The other system with small RMSD values is the pf1\(_{\text{Sol/Na}}\). Surprisingly enough this system was the first to equilibrate and among all four. Figure 4.2C shows the pf1 conformations at t=0 ns (in red) and at t=20 ns (in blue) superposed using the Gla domain. The general conformational change detected was between the previously
described regions A, B, and C was reproduced by normal mode analysis performed on the ElNémo web server [52].

4.4.3 Pf1 interaction with POPS molecules.

At the end of the 20 ns simulation several $\gamma$-carboxyglutamic acid residues of pf1 were found to form polar contacts with phospholipid molecules.
Figure 4.2. A Root mean square displacements (RMSD) of pf1 from different setups. RMSD (in nm) is shown as a function of simulation time is shown for all four systems: pf1$^{\text{Sol/Ca/Lipid}}$ in red, pf1$^{\text{Sol/Na}}$ in green, pf1$^{\text{Sol/Ca}}$ in blue, pf1$^{\text{Sol}}$ in magenta.
Figure 4.2.B RMSD of specific amino acids from different setups. Shows the averaged RMSD (from the snapshot at 10 ns) over the last 10 ns of the simulation time calculated from the initial structure as a function of the pf1 residue number. The color-coding for the four systems was kept the same as in panel A.
Figure 4.2.C Superposed conformations of pf1.
Shows the initial conformation (in red) superposed with the final snapshot at 20 ns (in blue). The conformers where superposed by aligning the Gla domains.
To assess the persistence of the contacts between the phospholipid molecules and the γ-carboxyglutamic residues during the 20 ns we monitored the distance between the interacting groups. Distances between POPS molecules and γ-carboxyglutamic residues were calculated based on their center of the mass of the amine moiety (POPS) and the carboxyl groups of the malonate like moiety (γ-carboxyglutamic amino acids). Amino acid residues Gla25 (see figure 4.3 panel A), Gla32 (panel B), and Gla30 (panel C) were found to interact directly with POPS molecules through the carboxyl and amine groups.

One interesting property of the POPC:POPS membrane is the increased interaction of the ester moieties of the POPS molecules with Na\(^+\) ions. This was first observed in a pure POPS lipid bilayer and had an effect of restricting the motion of the head groups.

The huge increase in the RMSD values for the first 20 amino acid residues from the simulation of pf1 without calcium ions reflects the drastic structural rearrangement and explains the loss of membrane binding capability of pf1 in the absence of Ca\(^{2+}\) as previously described \[7, 53\]. This result is also consistent with previous MD simulations on the bovine prothrombin fragment 1 that contained 65 residues and was found to undergo substantial unfolding in the Gla domain and a simulation of factor VIIa where the Ca\(^{2+}\) ions were displaced 17.5 Å from the corresponding Gla residues and resulted in increased dynamics of the first eleven residues \[15, 54\]. The X-ray structure of prothrombin fragment 1 solved in the absence of Ca\(^{2+}\) ions also showed a distorted structure of the first 35 amino acid residues \[55\]. These findings together with the comparable RMSD values from the simulation together with results from the simulation of pf1 in the presence of Ca\(^{2+}\) and the simulation of pf1 in the presence of both Ca\(^{2+}\) and
phospholipids demonstrate that conformational changes are due to the binding of calcium and not because of the presence of the phospholipids.

The simulations also showed two different models of the interaction between γ-carboxyglutamic amino acid residues and POPS molecules first a classic hydrogen-bonding network between the amine group of the POPS molecule and carboxyl group of the γ-carboxyglutamic amino acid residue. The second interaction mode is through chelation of calcium by both the carboxyl and phosphate groups. The later binding mode was long recognized and served as a model of interaction with negatively charged phospholipids of proteins with γ-carboxyglutamic amino acid residues.

Site-directed mutagenesis of prothrombin revealed that amino acid residues 16, 26, or 29 result in a considerable increase of the Michaelis-Menten constant (K_M) for the interaction of prothrombin with the prothrombinase complex. It has been previously shown that such changes in the K_M are due to the interaction of prothrombin with the phospholipid component of the prothrombinase complex. Based on site-directed mutagenesis studies it has been hypothesized that amino acid residues 16, 26 or 29 are required to preserve the internal structure of the Gla domain. On the other hand changes of amino acid residues 7, 14, 19, 20 or 25 resulted in a prothrombin molecule that was capable to retain partial procoagulant capability.

A recently reported crystal structure of pf1 (pdb code 1NL2) shows that the serine head group interacts with two calcium ions (Ca5 and Ca6) that are coordinated by the Gla17 and Gla21 amino acid residues [20]. Although after the simulations amino acid residues Gla17 and Gla21 do not interact with a serine head group of the lipid moiety, these residues are in the membrane plane.
Figure 4.3. A Distance between CGU25 and a POPS lipid.
Distance analysis performed on the whole length of the simulation trajectory. Distances are shown between the carboxyl moieties of amino acids 25 and the closest POPS molecule.
Figure 4.3.B Distance between CGU32 and a POPS lipid.
Shows distance analysis performed on the whole length of the simulation trajectory. Distances are shown between the carboxyl moieties of amino acids 32 and the closest POPS molecule.
Figure 4.3.C Distance between CGU30 and a POPS lipid.
Shows distance analysis performed on the whole length of the simulation trajectory. Distances are shown between the carboxyl moieties of amino acids 30 and the closest POPS molecule.
From the simulations we are unable to clearly define which Gla residue interacts with what lipid unit since the placement of the POPC and POPS units was random and the simulation time of 20 ns is too short to statistically account for the lateral diffusion of the lipid units in the bilayer’s outer leaflet. During the 20 ns simulation time the pf1 molecule was found to diffuse in the XY plane of the outer leaflet (i.e., at time = 0 ns pf1 is located in the center of the XY plane whereas at time = 20 ns pf1 was found at the boundary of the square that delimits the outer leaflet (see Appendix figure A4.4). The diffusion coefficient calculated for pf1 using the $\text{pf1}^{\text{Sol/Ca}}$ system was found to be $1.1 \times 10^{-7}$ cm$^2$/s which comparable with the experimentally determined value of the bovine prothrombin 4.6$\times 10^{-7}$ cm$^2$/s [56] and 7.3$\times 10^{-7}$ cm$^2$/s [57]. A more detailed analysis revealed that the lipid molecules found to interact with pf1 at time = 0 ns diffused in the XY plane with a similar pattern as the pf1 molecule. Ten lipid molecules maintained close contact with the pf1 molecule. At the end of the 20 ns simulation several $\gamma$-carboxyglutamic acid residues of pf1 were found to form polar contacts with phospholipid molecules.

The simulations show that after 20 ns pf1 has increased interacting surface with the lipid bilayer plane as compared with the starting model where only the Gla domain was interacting with the POPC:POPS surface.

One model of the pf1 binding to phospholipid vesicles is through the interaction of the hydrophobic residues from the N-terminal with the hydrophobic core of the carbon fatty acyl chains of the lipid units. This model successfully places the calcium ions on the same level with the lipid units head group for efficient chelation. However, this model
would suggest that pf1 would be discriminative between small and large unilamellar vesicles, which is not the case [58].

While the crystal structure of pf1 shows only seven calcium ions, it has been also proposed that binding of pf1 to the phospholipids membrane would be accompanied by ten calcium ions. It is reasonable to expect that three calcium ions would create bridges between acidic amino acid residues of pf1 and negatively charged groups of lipid units such as the carboxyl or phosphate moieties. While the Gla domains of several K-dependent proteins share a high amino acid sequence homology, their affinities for phospholipid are strikingly different suggesting that other parameters or regions are involved in the lipid-binding event [59].

Addition of the 8th calcium ion to the Gla32 has been suggested to be required for membrane binding [59]. In our simulations we found that amino acid Gla32 interacts with a POPS molecule. Site-directed mutagenesis at position 32 in factor VII showed a 13-fold increase in the membrane binding affinity compared with the wild type [60]. Although amino acid residue 32 is located far from the N-terminal region it was proposed that contains a membrane binding site of the vitamin K-dependent proteins [61].

**4.4.4 Validation of the prethrombin 1 homology model.**

Eight energetically significant cation-π interactions were found in the modeled prethrombin 1 molecule. The best alignment between prethrombin 1 model and the bovine meizothrombin des f1 structure was found to be 0.59 Å (a total of 370 amino acid residues representing ~ 93% of all residues found in the MzIIa-desF1 structure). Several loops (Asn463 to G475, Asp513 to G523, and Gly549 to Gly555) were found to have relatively large RMSD values compared with the corresponding loops in MzIIa-desF1.
This is not surprising since the same large RMSD values were found when the structures of prethrombin 2 and bovine MzIIa-desF1 were compared. All of these regions were modeled using the prethrombin 2 structure. Alignment of prethrombin 1 with prethrombin 2 resulted in a RMSD value of 0.48 Å (RMSD value of a total of 289 amino acid residues representing ~ 98% of all residues from the prethrombin 2 structure).

The z-score values (see ref. [23] for detailed description) of the prethrombin 1 homology model were found to compare well with the z-score values of the templates. The model (2IIN.pdb) was deposited in the Protein Data Bank [62].

Figure 4.5 shows the prothrombinase cleavage sites on the prethrombin 1 molecule (i.e., Arg320 in yellow and Arg271 in magenta). The factor Va dependent binding site of the prothrombinase complex is shown in blue spheres and the factor Va independent binding site of factor Xa is shown in green spheres. The distance between the C$^\alpha$ of the two cleavage sites of prothrombinase, i.e., Arg271 and Arg320, was found to be 42 Å.

The distance between the two C$^\alpha$ of amino acid residues Arg271 and Arg320 in prethrombin 1 is higher than the previously reported distance between the two prothrombinase cleavage sites. A previous model of prethrombin 1 placed the two amino acid residues at 15 Å [63]. The small distance in the model was attributed to a different arrangement of the linker loop as compared with the crystal structure of MzIIa-desF1. In the X-ray structure of MzIIa-desF1 the distance between Thr271 and Arg320 was found to be 36 Å. The smaller distance in the case of MzIIa-desF1 as compared with the prethrombin model may be attributed to the conformational changes after the cleavage at Arg320 as described [27] and references therein.
Figure 4.5 The prethrombin 1 model (Ser164 to Glu579).
Amino acid residues part of anion binding exosite I are shown in white spheres, Arg320 is shown in yellow, Arg271 is shown in magenta. Amino acid residues Gly473 to Ile487 [64] found to participate in binding of factor Va in the prothrombinase complex are shown with red spheres whereas amino acid residues Tyr557 to Gln571 proposed to interact with factor Xa in a factor Va independent manner [65] are shown in blue.
4.4.5 Validation of the prothrombin model.

The output model of prothrombin from the homology modeling procedure was subjected for analysis. The analyses, found to be satisfactory, were performed using PROSA [23], PROCHECK [24], CAPTURE [28] and included checking for overlapping atoms, cation-π interactions, and Ramachandran plots (data not shown).

4.4.6 MD simulation of prothrombin in solution.

Equilibration of the MD simulation of prothrombin in solution was attested by evaluating the RMSD of the protein backbone over the simulation time. Figure 4.6.A shows the RMSD over the 10 simulation time of the equilibrated system. Other parameters checked for the consistency of the simulation were temperature and pressure (data not shown). Throughout this thesis the equilibrium of a system is assessed if the RMSD was shown not to have a significant drift. This case represents a particular case of the general concept where one needs to see a repetitive pattern in the parameters of the system throughout the simulation. This is the case in the case of the solvated prothrombin molecule. Figure 4.6.A also shows in black lines the repetitive pattern that emerges after 10 ns of the simulation. The next approach was to see which residues contribute more to the increase in the RMSD at a given time point. The inset of figure 4.6.A shows the RMSD not as a simulation time but as a function of the residue number averaged over the simulation time. The line from the inset shows the calculated averaged value of RMSD as a function of time. It is clearly visible that the N terminal (residues 1-200) is the one with the higher RMSD during the 10 ns simulation. The simulations show that fragment 1 has a considerably increased dynamics as compared with the rest of the molecule (the catalytic region). Such movements of the fragment 1 region were also reproduced by a
simple normal mode analysis of the prothrombin molecule.

4.4.7 MD simulation of prothrombin in the presence of POPC:POPS.

Equilibration of the prothrombin-POPC:POPS system was validated using the same properties as described above (section 4.4.6). Figure 4.6.B shows the RMSD of the backbone atoms of prothrombin during the last ns from the simulation of the equilibrated system. The distance between Arg320 and Arg271 (the prothrombinase specific cleavage sites on prothrombin) was found to be 41 Å. This distance is similar with the ~ 42 Å found in the case of prethrombin 1 and is also persistent throughout the simulation. It is interesting to note that the RMSD values of the prothrombin molecule in the presence of the POPC:POPS lipid bilayer were found to have a negligible drift (i.e., a sign of the system equilibration) after 3 ns. In the case of the simulation where prothrombin was in solution in the absence of the lipid bilayer longer time (10 ns) was required for equilibration (see section 4.4.6). Reaching a rapid plateau of the RMSD values is probably due to the entrapment of prothrombin on the XY plane hence restricting the degrees of freedom as compared with the system where atoms are following the dynamics with no restriction imposed by the lipid bilayer plane. The hydrogen bonding network between the fragment 1 of prothrombin and the rest of the molecule was found to be preserved during the simulations. The calcium ions were found to be at the same level with the phospholipid head group level. This finding is similar with the simulations performed with pf1 in the previous section. A recent study that studied the same kind of interaction, i.e., VKD protein interacting with phospholipids through its GLA domain, studied factor VIIa and phospholipids and reached to the same conclusion [47]. The distance between the residues of meizothrombin/thrombin (His363, Asp419, and Ser525)
were found to be ~ 60 Å from the phospholipid surface. The distance agrees well with the 67 ± 3 Å as reported using FRET measurements of the meizothrombin active site in the presence of factor Va [50]. The insertion of the Gla domain into the phospholipid bilayer is similar with the insertion reported in case of factor VIIa interaction with phospholipids. It is noteworthy that the two studies ended up with similar distances although following different approaches. In our study using pf1 we placed the GLA domain into the phospholipid in the initial starting model. This reasoning was based on the results of Falls et al. that placed the Trp residue from the ω-loop at 7 Å deep into the phospholipid bilayer [16]. Our simulations needed 20 ns to reach equilibrium. On the other hand, Ohkubo et al. studied the interaction of factor VIIa with phospholipids using a different approach. They placed the factor VIIa molecule distant from the phospholipid bilayer (we also employed a similar approach that studied the actual binding event for factor Va, see chapter 3, section 3.4.7). In this way, the authors wanted to study the actual binding event of the factor VIIa molecule [16]. The different approach explains why the system studied by Ohkubo et al. needed a long equilibration time as compared with our simulations that do not need to go further than 20 ns to assess equilibration. The fact that the distance between the active site residues and the phospholipids (~60 Å calculated and 67 ± 3 Å from FRET measurements) clearly shows that the insertion of the ω-loop is well reproduced from the simulations.
Figure 4.7 RMSD values of Cα atoms of prothrombin.
Panel A shows RMSD of prothrombin in solution. The thick black line shows the pattern that was used to check equilibration. The arrows show the relative time points of the two conformers having considerable different RMSD values. The inset shows the RMSD as a function of the residue number and in black the averaged value of RMSD over time. Panel B shows the RMSD of prothrombin in the presence of POPC:POPS. The time frame, i.e., 3-4 ns is the interval used for analysis.
Figure 4.8. Prothrombin in the presence of POPC:POPS phospholipids.

The figure shows the final snapshot at 4 ns of the prothrombin –POPC:POPS simulation. The POPS phospholipids are shown in red lines, POPC phospholipids are shown in cyan lines. Arg271 is shown in red spheres whereas Arg320 is shown in red spheres. The rest of prothrombin is shown in green lines and the secondary structure I shown as cartoons.
4.5 References


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CHAPTER V

Characterization of peptides from factor Va sequence that inhibit prothrombinase/thrombin function

5.1 Abstract

Peptides that share sequence similarity with the primary amino acid sequence of factor Va corresponding to regions Asp665-Gln669 (DYDYQ) and the sulfated version (D5Q1 - having both tyrosine residues sulfated) were found to inhibit prothrombinase and thrombin function. In this study we characterize these two peptides using three molecules: a recombinant mezothrombin molecule with amino acid residues Arg155, Arg284, Arg271 changed to Ala (only ABE-I exposed) (rMZ-IIa), α-thrombin (both ABE-I and ABE-II available), and β-thrombin (only ABE-I available). The clotting time was increased by 8-fold when using β-thrombin. In the case of rMZ-IIa the clotting time was decreased by 4-fold as compared with α-thrombin under similar conditions. While α-thrombin alone was able to activated factor V to factor Va (through cleavages at
Arg709, Arg1018, and Arg1545), in the presence of the D5Q1,2 peptide, the activation was profoundly inhibited. β-thrombin was unable to cleave factor V at Arg1545 – a cleavage required for the light chain – and the cleavage at Arg709 was slowed down as compared with α-thrombin. Under similar conditions the membrane bound rMZ-IIa was able to cleave and activate the factor Va molecule. Again, addition of D5Q1,2 efficiently inhibited activation of factor V. These data demonstrate that the ABE-I region of α-thrombin alone can account for the interaction of factor V with α-thrombin resulting in timely and efficient activation. These findings also suggest that meizothrombin may be the physiological activator of factor V in vivo in the presence of a procoagulant membrane surface in the early stage of the coagulation event.

5.2 Introduction

Thrombin is a trypsin like serine protease that has a central role in the maintenance of normal hemostasis through regulation of both the procoagulant and anticoagulant events. Its main role as a protease is accomplished through proteolytic cleavages at specific arginine residues of its substrates and is highly dependent on interactions that involve its anion binding exosites. This requirement for thrombin to perform its function as a protease makes it more selective towards arginine residues as compared to other proteases like trypsin or chymotrypsin.

Meizothrombin is an intermediate formed during the activation of prothrombin to thrombin and is formed after the proteolytic cleavage of prothrombin at Arg320 (see Chapter I, figure 1.4).

In the presence of Na⁺ ions allosterically induced conformational changes occur
that favor the high activity (fast form) instead of low activity (slow form) [1]. Thrombin, in its fast form, performs its procoagulant role through limited proteolysis of several clotting factors such as factors XIII, VIII, V, and XI. Activation of these factors is a prerequisite for the conversion of fibrinogen to fibrin; the penultimate event catalyzed by thrombin before the formation of the insoluble fibrin plug. Thrombin, in its slow form [1], plays its anticoagulant role mediated by thrombomodulin through proteolytic cleavage of protein C. The fast and slow forms are almost equally populated (3:2 ratio) in vivo because of the physiological Na concentration that is not high enough to fully saturate thrombin's sodium binding site.

At the initial stage of the coagulation process small amounts of thrombin activate factor V and factor VIII through limited proteolysis. The activated factor V serves as a cofactor [2] for factor Xa associated in the presence of activated cell surface and Ca$^{2+}$ ions to form the prothrombinase complex [3]. The prothrombinase complex next catalyses the conversion of prothrombin to thrombin [4].

Thrombin is composed of two chains interconnected by disulfide bonds. The light chain composed of 36 amino acids residues has no specific involvement assigned in thrombin's function. The heavy chain contains two positively charged exosites, denoted as Anion Binding Exosite I and II (ABE-I, ABE-II), whose integrity is required for the expression of the enzymatic activity of thrombin. ABE-II is known as the heparin binding site whereas ABE-I has a role in binding of thrombomodulin [5], the protein-activated receptor 1 (PAR-1) a platelet transmembrane receptor [6], hirudin [7] and fibrinogen [8]. As a consequence any inhibitor that is targets ABE-I of thrombin represents an attractive anticoagulant.
It has been shown that amino acid region Asp665-Gln699 (DYDYQ) from factor V/Va is important for the procofactor activation and the cofactor function [9]. Because it was proposed that these two tyrosine residues may be sulfated a second peptide denoted D5Q1,2 was constructed so both tyrosine residues were sulfated [10, 11]. It has been also proposed that thrombin interacts with factor V through its ABE-I [12, 13].

**5.2.1 Effect of synthetic peptides on prothrombin activity during clotting.**

The effect of the peptides on clot formation was determined using prothrombin deficient plasma. In the presence of 250 µM D5Q1,2 the clotting time of prothrombin and rMZ-II increased by 3 fold as compared with the control experiment, i.e., 25 nM prothrombin and 25 nM rMZ-II (see Appendix figure 5.1). In inhibitory potential of D5Q1,2 was found to be comparable with the non-sulfated Hirudin (Hirudin\textsuperscript{54-65}) but was two fold smaller when compared with the sulfated Hirudin (Hirudin\textsuperscript{54-65SO\textsuperscript{3-}}). These results clearly show the inhibitory potential of D5Q1,2 in whole plasma [14].
Figure 5.1 Effect of D5Q1,2 on the cleavage of factor V by α-thrombin.

A, activation of factor V (500 nM) by α-thrombin alone (2 nM). Factor V was incubated with α-thrombin. B, α-thrombin was preincubated with D5Q1,2, and the mixture was added to factor V. At selected time intervals, aliquots of the mixtures were removed, mixed with 2% SDS, heated for 5 min at 90 °C, and analyzed on a 4–12% SDS-PAGE followed by immunoblotting. Fragments were identified following staining with a mixture of monoclonal antibodies HFV#17 that recognizes an epitope between amino acid residues 307–506 of factor V and HFV#9 that recognizes an epitope on the light chain of the active cofactor. Immunoreactive bands were visualized with chemiluminescence. Lane 1 in both panels depicts aliquots of the mixture withdrawn from the reaction before the addition of thrombin or α-thrombin/peptide mixture, whereas lanes 2–9 show aliquots of the reaction mixture withdrawn at 30 s, 1 min, 5 min, 10 min, 15 min, 30 min, 1 h, and 2 h following the addition of -thrombin alone or of -thrombin/peptide mixture. The positions of the light and heavy chain of factor V as well as of single chain factor V are indicated at the right.

(Bukys et. al J. Biol. Chem. 2006 281:18569-80)
5.2.2 Effect of D5Q1,2 on factor V cleavage by α and β-thrombin.

Activation of factor V to factor Va by α-thrombin occurs after 15 min (see figure 5.1 lanes 2-9). Preincubation of α-thrombin with 100 µM D5Q1,2 decreased considerably the cleavage rate at Arg709 while the cleavage at Arg1545 was severely impaired (see figure 5.2, panel B). Similar conclusions were drawn using the non-sulfated version of the peptide, albeit the inhibition of factor Va, under similar experimental conditions, was reduced [14]. A thrombin molecule cleaved at Arg67 and Arg77 results in the formation of β-thrombin [15]. Experiments designed to use β-thrombin instead of α-thrombin but in the absence of D5Q1,2 showed a similar result as in figure 5.2 A – a slower cleavage rate at Arg709. In the case of the β-thrombin the Arg1545 was not observed during the maximum 2 h time interval. When β-thrombin was incubated with D5Q1,2 a slight decrease of the cleavage rate at Arg709 was observed [14]. These results demonstrate the requirement of ABE-I of thrombin for the timely cleavage of factor Va at Arg1545. Similar experiments performed using factor VIII demonstrate that the exposure of ABE-I of thrombin is required and sufficient for efficient activation of the two cofactors, factor V and factor VIII [14].

5.2.3 Activation of factor V by meizothrombin.

To test the hypothesis that exposure of the ABE-I of thrombin is sufficient alone to efficiently activate factor V we used a meizothrombin molecule with only one cleavage site for factor Xa. This meizothrombin molecule rMZ-IIa has three important Arg (155, 284, and 271) residues changed to Ala [16, 17]. Membrane bound
meizothrombin was capable to activate factor Va to factor Va. The reaction was strongly inhibited by D5Q1,2 because of reduced cleavages at Arg709, Arg1018, and Arg1545. The activation rate of factor V by meizothrombin was found to be 5-fold faster as compared with the activation rate using α-thrombin. This result also demonstrates that exposure of ABE-II (not exposed in meizothrombin) is not required for the activation of factor V.

5.2.4 Activation of factor V during clot formation.

Incorporation of factor Va into prothrombinase directs prothrombin activation by factor Xa through the meizothrombin pathway, characterized by initial cleavage at Arg320. We have recently shown that a pentapeptide with the sequence DYDYQ specifically inhibits the factor Va-induced change in the cleavage pathway of prothrombin by prothrombinase. It has been also established that Hir$^{54-65}$ (SO$_3^-$) is a specific inhibitor of prothrombinase. To understand the role of factor Va within prothrombinase at the molecular level, we have studied thrombin formation by prothrombinase in the presence of various prothrombin-derived fragments alone or in combination.

Activation of prethrombin 1 is slow with cleavages at Arg320 and Arg271 occurring with similar rates (see Appendix A5.3). Addition of purified fragment 1 to prethrombin 1 accelerates both, the rate of cleavage at Arg320 and thrombin formation. Both reactions were inhibited by Hir$^{54-65}$ (SO$_3^-$) while DYDYQ had no effect on prethrombin 1 cleavage in the absence or presence of fragment 1. Similarly, activation of prethrombin 2 by prothrombinase, which is notably slow and inhibited by Hir$^{54-65}$ (SO$_3^-$), is not affected by DYDYQ. Addition of purified fragment 1 to prethrombin 2 accelerates
the rate of cleavage at Arg320 resulting in a rate of thrombin formation comparable to the rate of prothrombin activation by prothrombinase. Further, addition of fragment 1•2 to prethrombin 2 results in a significant inhibition of thrombin formation by prothrombinase by DYDYQ which is concurrent with the elimination of the inhibitory effect of Hir^{54-65} (SO_3^-) on the same reaction.

Finally, a ternary complex composed of prethrombin 2/fragment 1•2 /Hir^{54-65} (SO_3^-) is inhibited by DYDYQ. Altogether these data demonstrate that 1) membrane-bound fragment 1 is required to promote optimum factor Va cofactor activity (i.e. initial cleavage at Arg320); and 2) DYDYQ and Hir^{54-65} (SO_3-) bind to separate sites on the prothrombin molecule. Overall the data suggest that fragment 1•2 precludes Hir^{54-65} (SO_3^-) inhibition of cleavage at Arg320 because it interacts with amino acids in or around (pro)exosite I through its fragment 1 component.

5.3 Methods

5.3.1 Conformational search for DYDYQ.

We used molecular dynamics simulations, performed with the GROMACS 3.2.1 package [18, 19] to propose a conformer with the longer-life in water. The peptide, initial structure as a strait coil, was built using Pymol. Next, the peptide was inserted in a box of solvent so that the periodic images were at 20 Å apart. Single point charge (SPC) water model [20] was used as solvent. The negatively charged system was neutralized using two Na^+ ions. The Na^+ ions were added using the “genion” tool from the GROMACS package by replacing two SPC water molecules. The system composed of DYDYQ peptide, SPC water molecules, and Na^+ ions was energy minimized using the steepest-descent method. Long-range electrostatics was evaluated using the particle mesh Ewald
summation method [21, 22]. Simulations time was set to 50 ns.

5.3.2 Free energy perturbation of the DYDYQ molecule.

The free energy perturbation procedure was performed using the Gromacs package [18, 19]. For detailed theory about the free energy perturbation see Chapter I, section 1.4.3. Specific parameters are described in the topology file in GROMACS format of the DYDYQ and DFDYQ molecules (see figure 5.4, panels A, B).

5.4 Results and discussion

5.4.1 DYDYQ and D5Q1,2 conformations.

The peptide appears to interact with ABE-I of the enzyme impairing several ABE-I-related functions. Sulfation of DYDYQ on both tyrosines significantly increases its potency with respect to α-thrombin inhibition when compared with the non-sulfated peptide [9]. To ascertain the differences in peptide conformation following sulfation of DYDYQ, a 50-ns molecular dynamics simulation of the peptides in aqueous solution was performed, and snapshots of each molecule are shown in figure 5.2.

Although the distance between the carbon of Asp1 and the carbon of Gln5 was always greater than 1 nm, DYDYQ was found to interchange conformations periodically between a linear and a packed conformation. In 40% of the simulation time, DYDYQ adopted a packed conformation with Tyr2 and Tyr4 facing each other (see figure 5.2).
Figure 5.2 Molecular dynamics simulations of the DYDYQ and D5Q1,2 peptides.

The two panels show a representation with schematics and sticks for DYDYQ (A) and D5Q1,2 (B). Snapshots for both peptides were taken at 10 ns.
Although the distance between the hydroxyl groups of Tyr2 and Tyr4 varies between 3.5 and 5 Å, which is approximately twice the minimal distance allowed for a typical hydrogen bond to occur (~1.8 Å), the packed conformation appeared to be the preferred conformation for DYDYQ approximately half the time in solution. Thus, the fact that the two-phenyl groups in DYDYQ face each other periodically, may explain the insolubility problems encountered when working with high concentrations of DYDYQ. In contrast, D5Q1,2 preferred a linear rather than a packed conformation (Fig. 10B). D5Q1,2 was found in the latter conformation only 1% of the total simulation time. In addition, no solubility problems were encountered when working with D5Q1,2.

The data suggest that the two sulfate moieties in D5Q1,2 are repulsive leading to an open conformation most of the time, which in turn, must favor interaction of D5Q1,2 with -thrombin compared with DYDYQ. Moreover, D5Q1,2 has two additional negative charges that can interact with basic amino acids from ABE-I of -thrombin and meizothrombin. In sum, D5Q1,2 has an advantage over DYDYQ, because it possesses overall more negative charges available to interact with more positive charges from ABE-I of -thrombin. All these facts may explain the increased potency for inhibition of -thrombin function by D5Q1,2 when compared with inhibition of the enzyme by DYDYQ.

This enhanced potency is most likely the result of a more linear conformation of the sulfated pentapeptide compared with the non-sulfated form, allowing more contact points with ABE-I of -thrombin.

5.4.2 Free energy perturbation of DYDYQ to DFDYQ.

Figure 5.3 shows the result of the result of 22 simulations (11 for each perturbation direction, i.e., forward DYDYQ ->DFDYQ in red (°) and backward DFDYQ
->DEDYQ in blue (×)). The significant overlapping between the two curves demonstrates the validity of the procedure meaning that the same profile is obtained regardless of the direction of the perturbation.
Figure 5.3. Free energy perturbation.

$\Delta G$ of the two states 0->1 represents DYDYQ -> DFDYQ whereas 1->0 represents the perturbation of DFDYQ -> DYDYQ. The charge of the OH group of the Tyr residue is reduced from the initial value to 0 by the $\lambda$ factor ($\lambda \in [0,1]$, and $\lambda_{\text{step}}=0.5$)
5.5. References


17. Cote, H.C., et al., *Functional characterization of recombinant human meizothrombin and Meizothrombin(desF1). Thrombomodulin-dependent*


CHAPTER VI

Overall conclusions

6.1 Overall conclusions

The activation of prothrombin to thrombin by the prothrombinase complex has a central role in the blood clotting process. It has been demonstrated that the C-terminus of human coagulation factor Va is required for expression of the cofactor’s function within prothrombinase. The recent X-ray structure of Adams et al. provided a factor Va molecule where the A2 domain was not present [1]. Our model provides for the first time a complete factor Va molecule where the missing residues are included using the folded model based on molecular dynamics simulations. The factor Va model was first validated and then used to study several important regions from the C-terminus. Amino acid residues located at 334-335 were found to be important for the cofactor’s function. Several mutant molecules having D334 → K and Y335 → F were found to be impaired in their capability to activated prothrombin to thrombin within prothrombinase. Molecular dynamic simulations performed on the mutant factor Va molecules revealed
that after the amino acid change secondary structure elements from the mutant molecules were compromised. On the other hand, in the simulation of the wild type factor Va molecule the secondary structures were preserved throughout the simulation. The factor Va model was also used to answer a long-standing question about the inactivation of factor Va\textsuperscript{LEIDEN}. Simulations performed on several model systems were able to demonstrate that the slower inactivation of Va\textsuperscript{LEIDEN} is due to a slower cleavage at Arg306.

We also proposed the first model of a mixed/asymmetric phospholipid bilayer composed of POPC and POPS. This phospholipid bilayer model provided the long needed framework to study the interaction of coagulation factors with phospholipids. Several molecular dynamics simulations performed on large time scales (the orders of tens of nanoseconds) were able to provide an atomic view of the interaction between the proteins and phospholipids. The prothrombin fragment 1 and prothrombin simulations provided a mechanism of interaction of phospholipids with the regions that contain \(\gamma\)-carboxyglutamic residues of the vitamin K dependent proteins. These results, were confirmed using similar studies performed by Ohkube et al. [2]. Another study that we performed followed the dynamics of interaction of factor Va with phospholipids was able to offer for the first time a glimpse of the factor Va insertion process.

The X-ray structures of prothrombin components were successfully solved. Several good quality X-ray structures of prothrombin fragment 1, meizothrombin that lacks fragment 1, and thrombin exist to date. We constructed for the first time an atomistic model of prothrombin based on the X-ray structures of the fragments and FRET measurements. This model of prothrombin is able to provide a general view of the
molecule in light of the new functional studies of prothrombin, especially concerning the role of fragment 1 as an important mediator of prothrombin’s function.

It needs to be pointed out that the studies described in this thesis are purely theoretical and are based on the current knowledge of the field of structural computational biology and experimental results from the coagulation field.

6.2. Future directions

Now that the prothrombin, factor Va, and the phospholipids were characterized alone and in the presence of negatively charged phospholipids, the docked factor Va-factor Xa-prothrombin macromolecule on phospholipids would be an interesting modeling project. However, before one embarks on such a challenging project that consists of the modeling of at least 1 million atoms and looking at the dynamics over several tens of nanoseconds, it is necessary that the current models are further tested and validated/optimized. One immediate direction of research, that could fully benefit from the modeling work performed in this thesis, would be to test whether the peptide fragments (see chapter III, sections 3.4.6-8, figure 3.11) found to be located in the phospholid binding sites of factor Va/pf1/prothrombin are able to inhibit the function of prothrombinase. Several peptides that have sequence identity with factor Va-factor Xa interactive sites were recently characterized and were shown to have high chances to become efficient prothrombinase inhibitors [3-5]. However, there are no peptides that would specifically inhibit the interaction of the factor Va/prothrombin with the negatively charged phospholipid surface.
5.5. References


APPENDIX

TABLES

Table 2.1

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Table 3.1 Table shows the rotamers and best scores (a good score is a negative or 0 value) for the changed amino acids in the bovine C1 and C2 domain three-dimensional structure that match the sequence of human C1 and C2 domains. For each residue the best rotamer is the one with the lowest score. The score is calculated using the formula: \( S = (4 \times \text{# of Clash with backbone N CA and C atoms}) + (3 \times \text{# of Clash with backbone O atoms}) + (2 \times \text{# of Clash with sidechains atoms}) - \text{# of Hbonds} - 4 \times \text{# Ssbonds} \).

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\(^1\) Low energy side-chain conformations. First value represents the rotamer number and the second the total number of rotamers.
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Table 3.2 Hydrogen bonds in the folded 46 amino acid peptide.

Hydrogen bonds are the bonds found in the final structures of the folded peptide and are represented in donor – H – acceptor format with bond distance. Atoms nomenclature is used as described in [51].

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<th>Angle (°)</th>
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Figures

A3.1. Equilibration of the factor Va molecule.

RMSD (nm) is shown as a function of simulation time (ps) of Cα compared with the starting model (at the beginning of the simulation) of factor Va.
A3.2 Superposition of the Cα from the bovine crystal structure of factor Va with the corresponding Cα from the new model of human factor Va.

Red lines represent the Cα trace from the human factor Va model whereas the blue lines represent the Cα trace from the bovine crystal structure. RMSD is shown together with the percent similarity and identity.

\[
\begin{array}{l}
\text{Human Ala1-Ser16} \\
\text{Bovine Ala1-Asn16} \\
\text{AQLRQFYVAAQGWS} \\
\text{AAAAAAAAAAAAAAAAAAAA} \\
\text{ARKRQFYVAAQGWSIN} \\
\end{array}
\]

- **Identity**: 12/16 (75.0%)
- **Similarity**: 14/16 (87.5%)
- **RMSD**: 1.6 Å
b.

Human Thr31-Ala221
Bowine Thr31-Ala221

TSFKIVYREPEFKEPKQSTISGLLGPFTLYAEVEGDIKIVHKKNADK
TSFKIVYREAYFQKEPKQSTISGLLGPFTLYAEVEGDIKIVHKKNADK

PLSIHPQGIRYSKLELAGYLDHTFPAEMDDAVAPGRETTYEWSISED5
PLSIHAQGIRYSKLESEGASYSDHTLPMERKDDAVAPQRETTYEWSISED5

GPTHDPPCLTHYYSHELDFNSGLICPLICKGTLEGTQKTFD
GPTHDPPCLTHYYSYANLVEDFNSGLICPLICKGTLEGTQKTFD

KOIVLLFAVFDESKMSQSSSLMYTVNYVNTMDITVCA
KQHLMLFAVFDESKSNNQTSSLMYTVNYVNTMDITVCA

Identity : 166/191 (86.9%)
Similarity : 176/191 (92.1%)
RMSD : 2.4 A
### c.

<table>
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<td>H.LQMGSGPELSIHFNQQLHQQKVSATLYSATTTT</td>
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**Identity**: 38/40 (95.0%)

**Similarity**: 40/40 (100.0%)

**RMSD**: 2.52 Å
d.

Human  Gly276-Ile296
Bovine  Gly276-Ile296

GKWISSLTPKHLQAGMQAYI
GRWTLASLPRMFQAGMQAYI

Identity : 15/21 (71.4%)
Similarity : 18/21 (85.7%)
RMSD : 2.05 Å
e.

Human  Asn1547-Val1560
Bovine Asn1538-Val1560

NNGNRRNYIAAEEISWDYSEFV
.\.
NTGRKYYYYAEEISWDYSKFV

Identity :  19/23 (82.6%)
Similarity :  21/23 (91.3%)
RMSD     :  1.59 Å
f.

Human Pro1582-Ser1648  
Bovine Pro1569-Ser1635

PEDTTYKKVFRKYLDSTFTKRDPRGEYEEHLGILGPPIRAEVDVIQVR
PEDTTYKKVFRKYLDSTFTKLDQGEYEEHLGILGPVIRAEVDVIQVR

FKNLASRPYSLHAHGLS  
FKNLASRPYSLHAHGLS

Identity : 63/67 (94.0%)  
Similarity : 65/67 (97.0%)  
RMSD : 1.64 Å
g.

**Human**  Asn1670-Glu1757
**Bovine**  Asn1657-Asp1774

NAVQPNSYTYYWHATERSGPESPGSACRAWAYYSVNPEDKIHGSLIGP

NAIPNKTYYWHATTRSGPENPGSACRAWAYYSVNPEDKIHGSLIGP

LLICQKGLHKSNNPVDREVFLLFMTFDEKKSWYYE

LLICRGTLDKEINPVDREVFLLFMVFDEKKSYYD

**Identity**  :  76/88 (86.4%)
**Similarity**  :  83/88 (94.3%)
**RMSD**  :  4.77 Å
Human Cys1879-Ile2195
Bovine Cys1866-Met2182

206
Human  Lys1773-Ile2195
Bovine Asn1788-Met2182

KSHEFAGHNYLGPLKHYEQENWRLHLLNIGSDQIIHQVFHGHQTL
NSHEFAGHNYLGPLKHYEQENWRLHLLNIGSDQIIHQVFHGHQTL
ENGIQGHQLGVWPLLPQSKTLEMKASKPOQWNLNTEVGENQBADMTPF
ENGTQGHQLGVWPLLPQSKTLEMKASKPOQWNLNTEVGEIQBADMTPF
LIDMKDQRIAPMGLGSTGISDQIKASEFGLYEPFLRLLNIGSGSYANKV
LIDMKDQRIAPMGLGSTGISDQIKASEFGLYEPFLRLLNIGSGSYANKV
EKLAEEASKPWWQDMDVMSYGTQKGCYHKSLSETPYVAYSSN
EKLAEEASKPWWQDMDVMSYGTQKGCYHKSLSETPYVAYSSN
QTMQIFKGNSTRNNMYFNGNSDASTIKENQDPVARYIRISFTRAYN
QTMQIFKGNSTRNNMYFNGNSDASTIKENQDPVARYIRISFTRAYN
RKMKMFKGNSTRNNMYFNGNSDASTIKENQDPVARYIRISPTGSYN
RPLRLERGFCEVNGCTPLOMENKIENQITASSFXXSHGYNNEPFR
RPLRLERGFCEVNGCTPLOMENKIENQITASSFXXSHGYNNEPFR
ARLNAQGRVNAWQAHANNNQNLQIDLLELDLLKIKKTITDQCGSLSSMY
ARLNAQGRVNAWQAHANNNQNLQIDLLELDLLKIKKTITDQCGSLSSMY
KSYTIIHSEQEGQMYWQYRLKSKWVDCKFKTNKQNKKNFNPISRF
KSYTIIHSEQEGQMYWQYRLKSKWVDCKFKTNKQNKKNFNPISRF
IRVIRPTKNQSTLLRLEFGCD
IRVIRPTKNQSTLLRLEFGCD

Identity : 366/424 (86.3%)
Similarity : 398/424 (93.9%)
RMSD : 4.64 Å
A3.3. Factor Xa binding site on factor Va. Factor Va with the newly inserted region (i.e., amino acid residues Ser664 - Arg709 yellow spheres). Blue spheres represent Met410, red spheres represent factor Xa binding site on factor Va (amino acid residues Glu323 - Val331). Panel A shows factor Va model before simulation, while panel B shows factor Va model after 1.4 ns simulation of the completed factor Va model in solution. In both panels green spheres depict amino acid residues Tyr371 - His379 which is the region that partially covers residues Glu323 - Val331 (the factor Xa binding site, red spheres).
A4.1 The figure shows the primary sequence alignment between the target (human prothrombin fragment 1, pf1) and the two templates (1NL1 and 2PF1). The alignment was performed using the EMBOSS server and represented an input parameter for the Modeller package.

```
>P1;pf1
sequence:pf1:1..:155:....:
ANT-FLEEVRKGNLERCVEETCSYEAAFEALESSTATDVFWAKYTACETARTPRDKLAAACLEGNCAEGLGNYR
GHVNIITRSIGECQLWRSRYPHKPEINSTTHPGADLQENFCRNPDSSTTGPWCYTTDPTVRRQEC9IPVCQGDQVT
VAMTPR*

>P1;1NL1
structureX:1NL1:1..:147:....:
ANKGFLEEVKGNLERCLEECPSREEAEFEALELSATDAFWAKYTACESARNPREKLNACLEGNCAGVGMNYR
GNVSVTSGIECQLWRSRYPHKPEINSTTHPGADLRENFCRNPDSITGPWCYTTSSPLRREEC9SVPCQDQ*

>P1;2PF1
structureX:2PF1:36..:156:....:
--------------------SATDAFWAKYTACESARNPREKLNACLEGNCAGVGMNYR
GNVSVTSGIECQLWRSRYPHKPEINSTTHPGADLRENFCRNPDSITGPWCYTTSP4RREEC9SVPCQDQRT
VEVIPR*
```
A4.2 The figure shows the z-values as a function of the human prothrombin fragment 1 residue number for the target (red line) and the templates 1NL1 (green line) and 2PF1 (blue line). The Z-factor corresponding to 0 is shown as a horizontal line (in pink). Z-values were calculated using the PROSAII software package.
A4.3 The figure shows the Ramachandran plot corresponding to the human prothrombin fragment 1 homology model. The plot shows the $\phi$ angles $\psi$. Statistics produced by PROCHECK show that 89.6% of residues are in the most favored regions and 9.6% are in the additional allowed regions.
A4.4 The figure shows the diffusion of pf1 through the XY plane of the lipid bilayer. The trajectory, from 0 ns to 20 ns, is represented as a XY plot of the projection of the Gla domain center of mass.
A4.5 Z-score values for prethrombin 1 modeling. Z-score values were calculated using the PROSAII validation tool. Z-score values for prethrombin 1 are shown in blue, prethrombin 2 in yellow (amino acid residues 285-579\(^2\)), and meizothrombin (desFragment 1) in magenta (amino acid residues 164-287).
A4.6 Alignments of the human prothrombin amino acid sequence with the amino acid sequences of the template structures used in the homology modeling procedure. The alignment was performed using the Emboss server. The template protein databank files used are: 1NL1.pdb, 2PF1, 1A0H, and 1HAG. The alignment is shown in the input format for the MODELLER software package.
A4.7 Modeling of prothrombin started with the crystallographic unit of meizothrombin-des fragment 1 as described by Edwards et al. The crystallographic unit contains two molecules (in red and in blue) (1). The kringle 1 domain of pf1 (in green - see section 4.3.1 for modeling details of pf1) was superposed with the kringle 2 of molecule 2 from the crystallographic unit (molecule in red). The green arrow indicates the phospholipid binding site – N- terminus of pf1) (2). The missing region (amino acid region 156-163 – prothrombin numbering) was modeled using Modeller and is shown as a pink rectangle (3). Step 4 shows the complete prothrombin model (red ribbon) in the same position as described in (3). The amino acid region tat connects the two kringle domains is shows with blue sticks.
A5.1 Effect of synthetic peptides on prothrombin activity during clotting. A, comparison of the three forms of thrombin. The average clotting times obtained in six different measurements in the presence of 10 nM enzyme are shown as follows: white, \(-\)thrombin; black, \(-\)thrombin; and hatched, rMZ-IIa. B, comparison of the activity of the peptides. The average clotting time found in three different measurements in prothrombin-deficient plasma are shown as follows: A, 25 nM prothrombin; B, 25 nM rMZ-II; C, 25 nM prothrombin that was preincubated with 250 \(\mu\)M D5Q1,2; D, 25 nM prothrombin that was preincubated with 250 \(\mu\)M P15H; E, 100 nM rMZ-II; F, 100 nM rMZ-II that was preincubated with 250 \(\mu\)M D5Q1,2; G, 25 nM prothrombin that was preincubated with 50 \(\mu\)M Hir54–65; H, 25 nM prothrombin that was preincubated with 50 \(\mu\)M Hir54–65(SO3).

(Bukys et al. J. Biol. Chem. 2006 281:18569-80)
A5.2 Effect of D5Q1,2 on factor V activation by meizothrombin. A, activation of factor V (500 nM) by membrane-bound rMZ-IIa (2 nM). B, membrane-bound meizothrombin was preincubated with D5Q1,2, and the mixture was added to factor V. At selected time intervals, aliquots of the mixtures were removed and treated as described in the legend to figure 5.1 Lane 1 in both panels depicts aliquots of the mixture withdrawn from the reaction before the addition of rMZIIa or rMZIIa/peptide mixture; whereas lanes 2–8 show aliquots of the reaction mixture withdrawn at 30 s, 1 min, 3 min, 5 min, 10 min, 15 min, and 20 min following the addition of rMZ-IIa alone or of rMZ-IIa/peptide mixture. The positions of the light and heavy chains of factor V as well as of single chain factor V are indicated at the right.

(Bukys et. al J. Biol. Chem. 2006 281:18569-80)
A5.3 Proteolysis of factor V and prothrombin during clot formation. Normal plasma (lanes 1–7) was diluted 10-fold in a CaCl2-containing buffer following by the addition of PCPS vesicles. Prothrombin-deficient plasma (lanes 8–14) was also diluted 10-fold in a CaCl2-containing buffer, supplemented with rMZ-II (140 nM) and clotting was initiated with PCPS vesicles. Clotting in both samples occurred at 32 min (arrowheads on top). Lanes 1 and 8 represent control samples prior to the addition of PCPS vesicles. Lanes 2–7 and 9–14 depict aliquots of the reaction mixture at 5, 15, 25, 35, 45, and 60 min following the addition of PCPS vesicles. All samples were analyzed by SDS-PAGE (4–12% linear gradient) under non-reducing conditions followed by transfer to polyvinylidene difluoride membranes. A, results of an immunoblot probed with a mixture of monoclonal antibodies to human factor Va (i.e. HFV-17 that recognizes an epitope on the heavy chain of the cofactor and HFV-9 that recognizes an epitope on the light chain of factor V. The arrows at right depict the position of single chain factor V (FV), the Mr 105,000 heavy chain of factor Va (HC), the Mr 74,000 light chain of the cofactor (LC), the Mr 75,000 fragment deriving from cleavage of the heavy chain at Arg506 (amino acids 1–506, 75,000APC), the Mr 30,000 fragment obtained from cleavage of factor Va at Arg506/Arg306 (amino acids 307–506, 30,000APC), and the Mr 54,000 fragment deriving from initial cleavage of the heavy chain by APC at Arg306 amino acids (306–679, 54,000APC). The positions of the molecular weight markers are indicated at the left. B, result of an immunoblot probed with a polyclonal monospecific antibody to human prothrombin made in sheep. The arrows at the right represent the position of fragment 1·2 (amino acids 1–271) and of α-thrombin.

(Bukys et. al J. Biol. Chem. 2006 281:18569-80)
A5.4 A. Topology for the free energy perturbation of the tyrosine 1 residue in DYDYQ to a phenylalanine in DFDYQ. The topology is written for the ffGmx force field available in GROMACS 3.2.1

[ atoms ]

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B. Atom types in DYDYQ and naming.

POPS:

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