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**DEPARTMENT OF MATHEMATICS AND STATISTICS**

# On a Minimal Mathematical Model for Regulation of Factor Va by Activated Protein C

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**Abstract:**

Mathematical modeling and simulation on computers offer an efficient and innovative approach to the study of biomedical phenomena, such as blood coagulation kinetics. Information from the *in silico* experiments can be combined with that of *in vivo* and *in vitro* studies to improve our knowledge of coagulation and provide insights into disorders that cause the deaths of millions each year. We have developed a minimal mathematical model for the regulation of factor Va by activated protein C (APC)[1]. To validate our model, we use the same initial conditions and model rate constants for an existing, extensive model that was proposed by Hockin, Cawthern, Kalafatis and Mann (*Biochemistry* 1999, **38**:6918-6934) [1]. We demonstrate that the results from our minimal model compare very well with those of the existing model. We also develop a minimal mechanism and mathematical model to simulate the effect of factor Va Leiden and propose that our minimal models could also be used to efficiently study the regulation of factor Va and factor Va Leiden by APC.

**Introduction:**

Blood coagulation is a cascade of enzymatic reactions and cellular events that result in the rapid production of thrombin [2-10]. Thrombin is arguably the most essential coagulation protein. It activates platelets and cleaves fibrinogen to fibrin, which forms the insoluble fibers of the blood clot. Furthermore, thrombin amplifies its own production by activating factors V and VIII and regulates its production by activating protein C, a potent inhibitor of factor V and VIII [11]. Consequently, far from being a simple linear mechanism, the formation of a blood clot is a result of several positive and negative feedback loops. Furthermore, a normal haemostatic response is dependent upon a delicate balance of procoagulant and anticoagulant reactions. Tipping the balance can result in either bleeding disorders (hemophilia) or clotting disorders (thrombophilia)[8, 12].

The coagulation cascade is triggered by vascular injury (Figure 1). Upon exposure to blood, subendothelial tissue-factor (TF) binds to factor VIIa on a membrane surface and forms the extrinsic tenase complex (TF=VIIa). The extrinsic tenase complex is approximately four

[1] Abbreviations: 'a': active form of a species; 'APC': Activated Protein C; 'ATIII': Antithrombin III; 'ODES': Ordinary Differential Equations; 'TF': Tissue Factor; 'TFPI': Tissue Factor Inhibitor; '=': binding between species

orders of magnitude more efficient in converting zymogens factor IX and X to their active forms than factor VIIa alone[13]. The resulting factor Xa catalyses the initial production of thrombin. Although at this point the concentration of thrombin is low, it begins to activate platelets, factor V and factor VIII [3]. The resulting factor VIIIa serves as the cofactor to factor IXa and forms the intrinsic tenase complex (VIIIa=IXa) on a membrane surface. Intrinsic tenase has been estimated to be $10^9$ times more efficient in the activation of factor X than the serine protease factor IXa alone[13]. In a similar fashion, factor Va serves as cofactor to factor Xa and forms the prothrombinase complex (Va=Xa), also on a membrane surface[14]. The prothrombinase complex is five orders of magnitude more efficient in converting prothrombin to thrombin than Xa alone[15]. Through a positive feedback loop, the resulting thrombin further activates cofactors V and VIII, whose complexes ultimately lead to an explosion in thrombin concentration. The derived thrombin continues to activate more platelets, factors V, VIII, XIII and also cleaves fibrinogen to form the protein scaffolding of the blood clot.

Although clot formation is necessary to stem the loss of blood flow, if production of thrombin were to occur without regulation, there would be major haemostatic complications. Consequently, the anticoagulant processes operate throughout the cascade and are largely responsible for the threshold to thrombin production and thus clot formation[6, 8]. There are two main types of negative regulators: those which inactivate enzymes and those which inactivate cofactors. Circulating inhibitors, such as TFPI and ATIII, specifically inhibit serine proteases, such as TF=VIIa, factor IXa, factor Xa, meizothrombin (mIIa) and thrombin. Protein C, on the other hand, targets cofactors Va and VIIIa and regulates thrombin production by inhibiting the essential enzymatic complexes (intrinsic tenase and prothrombinase).

Therefore, the formation of a blood clot is said to occur in three stages: initiation, propagation and termination (Figure 2)[3, 4, 6]. During initiation, the concentrations of factors Va, VIIIa and their respective enzymatic complexes are low, and thrombin production is minimal. However, once the concentrations of these procoagulants surpass the threshold, rapid thrombin production is seen in the propagation phase. (The start of the propagation phase is generally taken as clot time.) Eventually a variety of regulatory and inhibitory mechanisms result in the termination phase, when thrombin production halts.

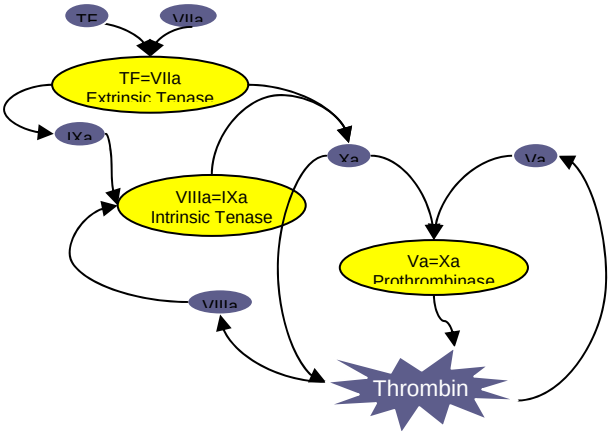


Figure 1 – The Coagulation Pathway: The formation of a blood clot results from a cascade of enzymatic reactions. For simplicity only the key enzymes and complexes are shown here.

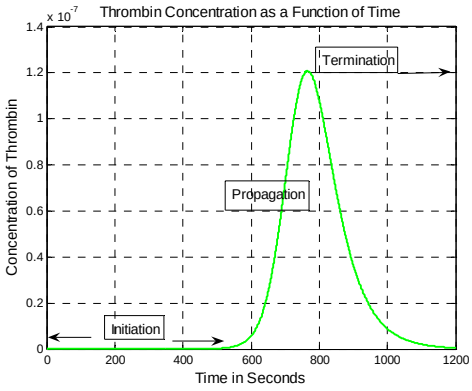


Figure 2 – Predicted Thrombin Concentration as a function of time: The three stages of thrombin production and thus clot formation are shown.

A proper haemostatic response is dependent on the balance of procoagulant and anticoagulant activities. However, it has been shown that both genetic and environmental risk factors can tip the haemostatic system and result in a hemorrhagic or thrombotic event or lead to a disorder. For instance, hemophilia A and hemophilia B are inherited bleeding disorders that are caused by a deficiency in factor VIII or in factor IX, respectively[2, 12]. On the other hand, factor V Leiden is the most prevalent genetic risk factor for thrombophilia and results from a mutation that protects factor Va from inactivation [8, 11, 16-22]. Furthermore, activities of the essential coagulation proteins and inhibitors have been shown to be affected by age, blood type, cigarette smoking, oral contraceptive use and hormone replacement therapy[23-30]. A few of the pathologic conditions that can result from the interplay of genetic and environmental factors are coronary artery disease, myocardial infarction, stroke, deep venous thrombosis and pulmonary embolism. According to the World Health Organization, cardiovascular disease alone was responsible for the deaths of 17.5 million people worldwide in 2005 [31].

Clearly, the study of the coagulation cascade and associated pathology merits our attention. Over the years, *in vitro* and *in vivo* studies have contributed significantly to our understanding of the mechanism, kinetics and disorders associated with the formation of a blood clot. Likewise, *in silico* studies and mathematical models have become powerful tools providing insights into the system and associated pathology.

Mathematical models of blood coagulation consist of a set of equations that describe the change in concentrations or activities of haemostatic species as a function of time. They are usually derived from the reaction mechanism with the use of the Law of Mass Action and

Michaelis-Menten enzyme kinetics [10, 32-34]. For instance, consider the elementary reaction of two species A and B that react to form product C: $aA + bB \xrightarrow{k} cC$. The Law of Mass Action states that the rate of collision between reactants (or the rate of a reaction) is proportional to the product of the concentrations of reactant species. Therefore, the rate of this reaction can be expressed as $v = \frac{d[C]}{dt} = k[A][B]$, where k is the constant of proportionality and has units dependent on the order of the reaction. In this case the reaction is first order with respect to A, first order with respect to B and second order overall. Since the rate of reaction is given in M/s, the rate constant, k, must have units $M^{-1}s^{-1}$. Furthermore, since the loss in the concentrations of reactants must be proportional to the gain in the concentration of product, the differential change in the concentrations can be related to the overall reaction rate by $v = \frac{-1}{a}\frac{d[A]}{dt} = \frac{-1}{b}\frac{d[B]}{dt} = \frac{1}{c}\frac{d[C]}{dt}$.

Blood coagulation, however, does not occur in a single step. Instead, the coagulation cascade can be described by an overall sequential mechanism or series of elementary reactions that ultimately result in the production of thrombin. At each step in the mechanism, certain species are formed and others are consumed. Consequently, the differential change in the concentration of a particular species is the sum of the rates of the reactions in which it is formed and those in which it is consumed.

For instance, consider the mechanism for a general enzyme-substrate reaction (that is repeatedly seen throughout the coagulation cascade): $E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$ The reaction begins by the enzyme E and substrate S forming the enzyme-substrate complex (ES) with a rate constant of $k_1$ (units: $M^{-1}s^{-1}$). Then, in a first-order reaction, the intermediate ES can either break into its components with a rate constant of $k_{-1}$ (units: $s^{-1}$) or be converted into product P (and regenerate the enzyme catalyst) with a rate constant of $k_2$ (units: $M^{-1}s^{-1}$). The rate constants $k_1$, $k_{-1}$ and $k_2$ describe the relationship between rate of product formation and the concentration of reactant species. Thus, the mathematical model describing this enzyme-substrate reaction would typically consist of the following nonlinear system of first order ordinary differential equations (ODEs):

$$\frac{d[E]}{dt} = -k_1[E][S] + k_{-1}[ES] + k_2[ES]$$
$$\frac{d[S]}{dt} = -k_1[E][S] + k_{-1}[ES]$$
$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES]$$
$$\frac{d[P]}{dt} = k_2[ES]$$

Since analytical solutions to these systems are often unknown, numerical methods are used to approximate the behavior of the model. Solving the set of differential equations through numerical integration yields a concentration profile for the reaction (the plot of concentration as a function of time). Although this set of ordinary differential equations is small, the models describing parts of the coagulation cascade are extensive, and numerical solutions are efficiently obtained with a numerical solver on a computer.

For instance, one of the first mathematical models of blood coagulation was proposed in 1984 by Nesheim, Tracy and Mann, who used a "desk-top computer and a program designated 'Clotspeed'" to study the prothrombinase complex[35]. Since then, advances in both computers and mathematical software have significantly increased the power and efficiency of using mathematical models to study blood coagulation. In 1994, Jones and Mann proposed a model of 20 differential equations to examine the procoagulant reactions leading to the production of thrombin[5]. The simulated concentration profiles were first validated with empirical data and then used to study the dynamics of the overall process and to examine the importance of varying the initial concentrations of certain species. Since then, the model, known as "Hockin I", has grown to a system of 34 ordinary differential equations with 42 rate constants and now accounts for inhibition by ATIII and TFPI [6]. The results from the Hockin I model were also rigorously compared with experimental data and contributed to our understanding of the threshold to thrombin production.

The reaction progress diagrams generated from these initial models illustrate the advantage of *in silico* experiments over *in vitro* and *in vivo* studies. First of all, the mathematical models lend themselves to quantitative and statistical analysis. One can easily study the dynamics of the entire system or examine the importance of a single species at a single step in the reaction. These methods also allow for simulation of conditions that could not be

examined empirically. Furthermore, these quantitative and statistical methods are efficient means of hypothesis testing and have the potential to be useful in clinical scenarios[36-38].

However, there are several notable challenges to modeling, and in the words of George Box , "all models are wrong; some models are useful"[39]. First, it is impossible for the model to account for all known proteins, reactions and components of coagulation. For instance, many models assume the presence of phospholipids or fully activated platelets even though the presence of a membrane surface significantly alters the rates of reactions [21, 22, 40-43]. Nonetheless, narrowing the focus to a manageable set of species and reactions and using clearly defined assumptions allows for the development of a biochemical mechanism through a system of stiff ordinary differential equations, which are highly dependent on initial conditions (concentrations) and rate constants.

Precise and accurate measurements of initial concentrations and estimations of rate parameters are necessary, but difficult to obtain. Furthermore, the plasma concentrations of coagulation proteins and inhibitors range from $1*10^{-10}$ M (factor VIIa) to $7.6*10^{-6}$ M (fibrinogen)[3]. Moreover, empirically derived rate constants tend to be highly dependent on experimental conditions and assay methods. A typical example is the inactivation of factor Va by APC. After cleavage at $Arg^{506}$, factor Va appears to retain 40% activity under one set of conditions and to be completely inactivated under a different set[22]. It is common practice that after the creation of the mathematical model, the rate parameters are then "adjusted" to fit experimental data[5]. Therefore, validation of the model from a variety of sources is extremely essential if we are to understand the etiology of hemophilia and thrombosis, for example.

Another challenge to modeling blood coagulation is the lack of a steady state system. Indeed, the only steady states seem to be before initiation or after termination when the concentrations of reactant species are 0 M. However, after the triggering event of the exposure of TF to blood, the concentrations of all species change on widely different timescales and the range of rate constants spans many orders of magnitudes. For instance, the rate of dissociation of Xa=TFPI is $3.6*10^{-4}s^{-1}$ while the rate of formation of the prothrombinase complex is $4.0*10^{8}$ $M^{-1}s^{-1}$ [6]. Prototype model systems with varying timescales are difficult to solve, and although the use of stiff numerical solvers can alleviate some of these problems, significant challenges still remain.

Other difficulties in modeling blood coagulation include spatial heterogeneity, diffusion, and the presence of background flow [44]. Nonetheless, mathematical models of blood coagulation have contributed to our understanding of the blood biochemical system and clotting disorders. Consequently, I had two main objectives of my research: (i) to understand the enzymatic activities and processes that lead to the production of thrombin and thus the formation of a blood clot; (ii) through the process of creating my own model, learn how a mathematical model can be formulated, validated, improved and applied with the objective of contributing to the understanding of the blood biochemical systems and clotting disorders.

**Methods**:

We initially began by reviewing the literature in order to better understand how a blood clot is formed. Although we did not perform any biochemical experiments, it was important for us to understand how the reaction mechanisms and related kinetics were determined experimentally. Likewise, as an initial approach to modeling, we reviewed published models from the literature on blood coagulation and reproduced their results. At the onset, we must admit to being very critical of the assumptions used to develop the models and to estimate the rate "constants". Therefore, with the objective of contributing to the understanding of the blood biochemical system and associated pathology, we began our own attempts at modeling blood coagulation.

Our original research plan was to reduce the Hockin I model of 34 ordinary differential equations with 42 rate parameters to a more manageable system[6]. From earlier studies on a model for regulation by APC proposed by Qiao, Liu and Zeng, we understood that a small system of equations lends itself to equilibrium and stability analysis, and that such analyses could provide insights into important parameter relationships[45]. However, we remained adamant that using biologically unrealistic assumptions to reduce the model may result in an interesting math problem but not necessarily improve the model or contribute to our understanding of biological phenomena. Therefore, we hoped that a critical examination of the chemical expressions used to derive the Hockin I model might reveal cooperative phenomena and inhibitory mechanisms (suicide substrates) and thus change the description of the reaction kinetics[33]. Unfortunately, due to the many feedback loops, a smaller system of equations could not be extracted for equilibrium and stability analysis.

Although our attempts to improve the full model through general model reduction, examination of cooperativity, inhibitory and suicide substrate mechanisms, and nondimensionalization to unravel parameter relationships were fruitless, our work on a simple (minimal) model for the inactivation of factor Va by APC was successful. Currently, the Hockin I model simulation of the change in concentration of both procoagulant and anticoagulant species as a function of time has been validated with empirical studies and has led to a greater understanding of normal clotting behavior and certain pathologies[37].

Of late the inactivation of factor Va has gained a lot of prominence in the literature (see, for example, [1, 13, 14, 21, 22, 41-43, 53, 54]). We have developed a minimal mathematical model describing the inactivation of factor Va by APC. The numerical system consists of only 7 ordinary differential equations and 3 algebraic expressions. The model was developed in four recursive steps. First, we extensively studied the literature and publications from many laboratories across the world in order to develop biologically realistic assumptions. Although we acknowledged the importance of other species, such as protein S, prothrombin and Xa, to the regulatory system, we limited our focus to factor Va, APC and products of cleavage and dissociation. Based upon our assumptions, a minimal mechanism describing the elementary reactions in inactivation of factor Va by APC was proposed and analyzed. Using the Law of Mass Action, we then derived our minimal mathematical model which consisted of 10 ordinary differential equations. After eliminating the redundant equations, the minimal model consists of 7 ODEs and 3 algebraic expressions. Numerical simulations were offered by Matlab's stiff ODE solver (ode15s) and resulted in reaction progress diagrams that were highly correlated with a model, which has three times as many degrees of freedom. (See Table 1 below.)

Along with simulating concentration profiles, we developed a method to predict the loss in activity of factor Va as a function of time. The equations for the cofactor activity loss were derived directly from the mechanism by assuming that after each elementary reaction (cleavage at $Arg^{506}$, cleavage at $Arg^{306}$, or dissociation of the A2 domain) the activity of factor Va was reduced to a certain percentage. The simulated activity profiles allowed for a quantitative comparison in assuming the inactivating event was cleavage at $Arg^{306}$ or dissociation of the A2 domain.

Next, we modified the mechanism to account for Factor $Va_{Leiden}$, which is characterized by a single amino acid substitution at $Arg^{506}$ [8, 11, 16-22, 46]. The replacement with $Gln^{506}$

results in resistance to APC and a delay in inactivation of factor Va. Consequently, factor $Va_{Leiden}$ poses the greatest genetic risk for thrombosis. Using similar biologically plausible assumptions, we proposed a minimal mechanism with cleavage occurring solely at $Arg^{306}$ and derived the mathematical model of 5 ODEs and 3 algebraic expressions.

**Maximum Percentage Difference in Model Concentrations for each Species**

Comparing the Simulations for the Inactivation of Factor Va by APC

| Va | Va=APC | $Va_5$=APC | $Va_5$ | $Va_{53}$=APC | $Va_{53}$ | A1LC=APC | A1LC | A2 | APC |
|---|---|---|---|---|---|---|---|---|---|
| 1.00% | 3.62% | 6.16% | 6.34% | 17.14% | 15.02% | 4.25% | 4.26% | 2.73% | 4.71 E-9 % |

Comparing the Simulations for the Inactivation of Factor Va Leiden by APC

| Va | Va=APC | $Va_3$=APC | $Va_3$ | A1LC=APC | A1LC | A2 | APC |
|---|---|---|---|---|---|---|---|
| .213 % | 0.20% | 0.40% | 0.45% | 0.57% | 0.57% | N/A | 6.43E-11 % |

**Table 1: The Maximum Percentage Difference in Model Concentrations for each Species:** In order to compare the minimal model to the HCKM model, the absolute difference in the concentrations were calculated at each time-point. To normalize these differences, we divided by the average of the maximum concentration of the species under consideration. We then found the maximum percentage difference for each species. Please note that percentage difference in the simulated concentration of the A2 domain is not available because the HCKM model was not able to simulate the change in concentration of the A2 domain. The HCKM model predicts that the maximum concentration of the A2 domain in the Leiden system is 0 nM.

For validation, we compared the simulated concentration profiles and inactivation profiles from our minimal models (the model for factor Va and the model for factor Va Leiden) to a model for regulation of factor Va by APC that was proposed by Hockin, Cawthern, Kalafatis and Mann ("HCKM model") in 1999[1]. The HCKM model consists of 30 ordinary differential equations and has been validated by empirical studies. To compare the models, we used the same rate constants and initial conditions and solved both systems with Matlab's stiff ordinary differential equation solver. The reaction progress diagrams and activity profiles from our minimal models were nearly indistinguishable from those of the HCKM model (See Table 1). Furthermore, our Leiden model accurately predicted the delay in inactivation of Factor Va that is seen in Leiden patients.

Our work on modeling the regulation of factor Va by APC has resulted in a draft manuscript, which will be submitted for consideration for publication in a refereed journal. A copy of the draft manuscript follows.

**On a Minimal Mathematical Model for Regulation of Factor Va by Activated Protein C** [**]

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**We propose a minimal mathematical model to study the inactivation of factor Va by activated protein C (APC)* based upon the assumptions that cleavage at $Arg^{506}$ precedes cleavage at $Arg^{306}$ and that complete activity loss can be attributed to subsequent dissociation of the A2 domain (residues 307-709). Our minimal model encapsulates steps in the enzymatic pathway that are crucial to predicting the time-dependent rates of change in the concentrations of factor Va, APC, and products of cleavage and dissociation. We find that the simulated reaction progress diagrams are highly correlated with an existing model. We also develop a method of simulating inactivation profiles and use our predictions to quantify the difference in assuming the inactivating event is cleavage at $Arg^{306}$ or is the dissociation of the A2 domain. The minimal model is also adapted to study factor $Va_{Leiden}$, which is characterized by the elimination of the $Arg^{506}$ cleavage site. We find that the delay in inactivation of factor $Va_{Leiden}$ is a result of kinetic differences in the rates of cleavage at $Arg^{506}$ and at $Arg^{306}$. The proposed mathematical models could be used for quantitative studies of the inactivation of factor Va and factor $Va_{Leiden}$ by APC.**

Running head: Modeling Factor Va Regulation by Activated Protein

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## Introduction

The coagulation cascade has often been described as a delicate balance of procoagulant and anticoagulant activities resulting in the rapid production of thrombin [3, 4, 6-8, 29]. Thrombin is arguably the most essential coagulation protein. It activates platelets and cleaves fibrinogen to fibrin, which forms the insoluble fibers of the blood clot. Furthermore, thrombin amplifies its own production by activating factors V and VIII and regulates its production by activating protein C, a potent inhibitor of both factor V and VIII.

In particular, factor Va is comprised of two non-covalently associated subunits: heavy chain (HC) (residues 1-709) and light chain (LC) (residues 1546-2196) [47-50]. On a membrane surface, factor Va forms the prothrombinase complex with factor Xa[13, 14]. The prothrombinase complex is estimated to accelerate the rate of conversion of prothrombin to thrombin by five orders of magnitude[15]. The formed thrombin down-regulates its own production by forming the thrombin-thrombomodulin complex and rapidly activating protein C [7, 11]. Activated protein C then binds to factor Va through its light chain (residues 1546-2196) and subsequently cleaves three amide bonds at $Arg^{506}$, $Arg^{306}$ and $Arg^{679}$, which are located in its heavy chain subunit [41, 42, 50-53]. After cleavage of $Arg^{306}$, the A2 domain is believed to dissociate and result in complete activity loss of the factor Va molecule[1, 54].

The importance of the regulation of factor Va is demonstrated in individuals who express resistance to APC. Deficiencies in key anticoagulants, such as protein C or protein S, have been shown to increase the risk of thrombosis [8]. However, factor $V_{Leiden,}$ which is characterized by an amino acid substitution at $Arg^{506}$, is the most prevalent genetic risk factor for thrombosis [16-20]. The replacement of $Arg^{506}$ with $Gln^{506}$ eliminates a critical cleavage site for APC and results in a significant delay in the inactivation of factor Va. The use of purified factor V obtained from Leiden patients and the creation of recombinant ($rVa^{506Q}$) proteins have greatly increased our understanding of the regulation of factor $Va_{Leiden}$ by APC [21, 22, 46, 55]. However, due to varying experimental conditions and types of assays used, characterizing the effect of the amino acid substitution has proved difficult. Nonetheless, increasing our understanding of the regulation of factor Va through the clinical, experimental and mathematical modeling viewpoints has the potential to greatly improve our understanding of APC resistance caused by factor $Va_{Leiden}$.

We propose a mathematical model for the regulation of factor Va by APC. The model consists of seven ordinary differential equations and three algebraic expressions that describe the rate of change in the concentrations of factor Va, APC and products of cleavage and dissociation as a function of time. The reaction progress diagrams and inactivation profiles generated by our model are highly correlated with results from an existing model. Therefore, our minimal model encapsulates steps in the enzymatic pathway that are crucial in quantifying the inactivation of factor Va by APC. We also adapt our model to simulate the effect of eliminating the cleavage site at $Arg^{506}$ and thus to study the resistance to APC that is exhibited by Leiden patients. We find our minimal mathematical models to be efficient methods for quantitative studies on the inactivation of factor Va and factor $Va_{Leiden}$ by APC.

**The Model**

With the use of biologically plausible assumptions, we propose a minimal mechanism consisting of a series of elementary reactions that we deem critical to describing the inactivation of factor Va by APC. Given our interest in the regulation of factor Va by APC, we consider APC, Va and the products of

cleavage and dissociation as the key components in the model formulation. Although we acknowledge the importance of other species, such as protein S, prothrombin and Xa, to the regulatory system, we limit the focus with the understanding that our future work will incorporate these species. From the proposed mechanism, we then derive a mathematical model that hinges on a set of ordinary differential equations. Given initial conditions (the concentration of reactants), the mathematical model predicts the time-dependent variation in concentrations of reactants, intermediates and their products. The mechanism and mathematical model are based upon the following assumptions:

1. We assume that factor Va is membrane-bound. Phospholipids are required for both the formation of the prothrombinase complex and the complete inactivation of factor Va[13, 14]. Without a membrane surface, the cofactor has been shown to remain between 60% and 80% active after 2 hours of incubation with APC [41-43]. Furthermore, phospholipids have been shown to enhance cleavage rates at $Arg^{506}$ and $Arg^{679}$ and are necessary for cleavage at $Arg^{306}$ [21, 22, 54]. Our assumption is also consistent with derivations of the model rate constants, which are based on kinetic data from experiments performed with phospholipids.
2. We also assume that equilibrium governing the association and dissociation of APC from all species containing the light chain subunit is uniform. Our assumption is based upon studies showing that APC binds equally with all species containing the membrane-bound light chain ($K_d$=7 nM) [1, 14, 51-54].
3. Since cleavage at $Arg^{506}$ occurs about ten times faster than cleavage at $Arg^{306}$, we assume that cleavage first occurs at $Arg^{506}$ and then occurs at $Arg^{306}$ [1, 21, 22, 41-43, 53, 54]. We acknowledge the primary cleavage at $Arg^{306}$ does occur, albeit very infrequently, and remains pivotal to understanding Factor $V_{Leiden}$. However, by comparisons with an existing model, we show that this assumption does not significantly alter the kinetics or concentration profile of the reaction and serves to greatly simplify our mechanism and mathematical model. Therefore, we assume the first cleavage occurs rapidly at $Arg^{506}$ and results in an intermediate with reduced activity. The second cleavage at $Arg^{306}$ further reduces the activity of the cofactor.
4. We also assume that after cleavage at $Arg^{306}$, the A2 domain (residues 307-709) dissociates as non-covalently associated fragments and leaves behind the membrane-bound A1 domain and the light chain (A1LC: residues 1-306 and 1546-2196) [1, 54]. Prior to dissociation, the doubly-cleaved intermediate ($Va_{53}$=APC) is estimated to retain approximately 20% of its activity [54]. However, upon dissociation, the remaining cofactor is functionally inactivated.
5. We assume that cleavage at $Arg^{679}$ is irrelevant to the model formulation. Although there has been speculation regarding the importance of cleavage at $Arg^{679}$ in Leiden patients, it has been repeatedly shown that cleavage at $Arg^{679}$ has a negligible effect on factor Va activity especially in the presence

of phospholipids [21, 41, 42, 55]. More importantly, cleavage at $Arg^{679}$ occurs so slowly ($5.2*10^{-4}s^{-1}$) that it is likely to occur after the inactivating event, which we take to be the dissociation of the A2 domain [1, 22]. We also find that this assumption does not significantly alter the kinetics or reaction concentration profile and does reduce the complexity of the mechanism and mathematical model.

Using the assumptions presented above, the proposed mechanism for factor Va inactivation by APC is shown in Figure 1. The corresponding rate constants are given in Table 1.

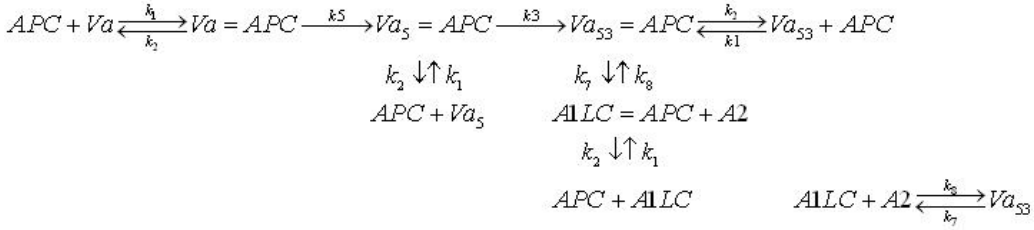



**Figure 1: Proposed Mechanism of Inactivation of Factor Va by APC.** APC is assumed to associate with rate $k_1$ and dissociate with rate $k_2$ from all LC containing species (Va, $Va_3$, $Va_{53}$ and A1LC). At the onset of inactivation, APC binds with membrane-bound factor Va and forms the Va=APC complex. The initial cleavage occurs at $Arg^{506}$ with a rate of $k_5$ to yield the intermediate $Va_5$=APC. Subsequent cleavage at $Arg^{306}$ occurs with a rate of $k_3$ and yields the doubly cleaved intermediate $Va_{53}$=APC. The A2 domain then rapidly dissociates at a rate of $k_7$ to yield A1LC=APC and the A2 domain. The reassociation rate of the A2 domain is given by $k_8$. Free A1LC and A2 can also associate to or dissociate from $Va_{53}$. Upon dissociation of the A2 domain, the remaining factor Va molecule (A1LC) is functionally inactive.

First, the Va=APC complex is formed by binding of APC to membrane-bound factor Va ($k_1$). The Va=APC complex can either dissociate into its constituents ($k_2$) or cleavage at $Arg^{506}$ can occur to yield $Va_5$=APC ($k_5$). The intermediate $Va_5$=APC can also dissociate into APC and $Va_5$ or be cleaved at $Arg^{306}$ to yield $Va_{53}$=APC ($k_3$). After cleavage at $Arg^{306}$, the A2 domain rapidly dissociates from $Va_{53}$=APC, generating A1LC=APC ($k_7$). The remaining membrane-bound factor Va (A1LC=APC) is completely inactivated. Throughout the mechanism, APC is assumed to associate ($k_1$) and dissociate ($k_2$) equally with all LC containing species (Va, $Va_5$, $Va_{53}$ and A1LC). Although the reassociation of the A2 domain is slow ($k_8$), APC-bound and APC-free A1LC species can rebind with the A2 domain to yield $Va_{53}$=APC and $Va_{53}$, respectively.

| Rate Constant | Model Rate | Rate Description |
|---|---|---|
| $k_1$ | $1.0*10^{-8}$ $M^{-1}s^{-1}$ | Association of APC to LC species |
| $k_2$ | 0.7 $s^{-1}$ | Dissociation of APC from LC species |
| $k_3$ | 0.064 $s^{-1}$ | Cleavage at $Arg^{306}$ |
| $k_5$ | 1.00 $s^{-1}$ | Cleavage at $Arg^{506}$ |
| $k_7$ | 0.028 $s^{-1}$ | Dissociation of the A2 domain |
| $k_8$ | $2.57*10^{5}$ $M^{-1}s^{-1}$ | Association of A2 domain |

**Table 1: Rate Constants Used in the Minimal Models:** The rate constants used to simulate reaction progress diagrams and activity loss as a function of time were taken directly from a model proposed by Hockin, Cawthern, Kalafatis and Mann in 1999 [1]. Please see their paper for details on the derivation of model rate constants.

With the assumptions presented above and the proposed mechanism, we used the Law of Mass Action to derive our minimal mathematical model. The mathematical model describes the rate of change in the concentration of species as a function of time and is given by the following system of ordinary differential equations:

$$\frac{d[Va_5]}{dt} = k_2[Va_5 = APC] - k_1[Va_5][APC] \tag{1}$$

$$\frac{d[Va_{53}]}{dt} = k_2[Va_{53} = APC] - k_1[Va_{53} = APC][APC] + k_8[A1LC][A2] - k_7[Va_{53}] \tag{2}$$

$$\frac{d[Va = APC]}{dt} = k_1[Va][APC] - k_2[Va = APC] - k_5[Va = APC] \tag{3}$$

$$\frac{d[Va_5 = APC]}{dt} = k_5[Va = APC] - k_3[Va_5 = APC] - k_2[Va_5 = APC] + k_1[Va_5][APC] \tag{4}$$

$$\frac{d[Va_{53} = APC]}{dt} = k_3[Va_5 = APC] + k_1[Va_{53}][APC] - k_2[Va_{53} = APC] - k_7[Va_{53} = APC] + k_8[A1LC = APC][A2] \tag{5}$$

$$\frac{d[A1LC = APC]}{dt} = k_7[Va_{53} = APC] - k_8[A1LC = APC][A2] - k_2[A1LC = APC] + k_1[A1LC][APC] \tag{6}$$

$$\frac{d[A1LC]}{dt} = k_2[A1LC = APC] - k_1[A1LC][APC] - k_8[A1LC][A2] + k_7[Va_{53}] \tag{7}$$

$$\frac{d[A2]}{dt} = k_7[Va_{53} = APC] - k_8[A2][A1LC = APC] + k_7[Va_{53}] - k_8[A1LC][A2] \tag{8}$$

$$\frac{d[Va]}{dt} = -k_1[Va][APC] + k_2[Va = APC] \tag{9}$$

$$\frac{d[APC]}{dt} = -k_1[APC]([Va_5] + [Va_{53}] + [A1LC] + [Va]) + k_2([Va = APC] + [Va_5 = APC] + [Va_{53} = APC] + [A1LC = APC]) \tag{10}$$

where [$Va_5$], [$Va_{53}$], [Va=APC], [$Va_5$=APC], [$Va_{53}$=APC], [A1LC=APC], [A1LC], [A2], [Va] and [APC] are the concentrations of the reactants, intermediates and products and come with appropriate initial and boundary conditions.

There appears to be redundancy in the model system. Since the sum of some equations equals zero (e.g., Eq (6-8)), we can reduce the total number of differential equations from ten to seven. Indeed, the concentration of A2 at time t can be written as a function of the concentrations of A1LC=APC and A1LC at time t:

$$[A2] = [A1LC = APC] + [A1LC] + c_1 \tag{11}$$

where $c_1$ depends on the initial concentrations. Likewise, we can reduce the total number of equations to seven by eliminating the differential equations describing the rates of change in the concentrations of factor Va and APC:

$$[Va] = -([Va_5] + [Va_{53}] + [Va = APC] + [Va_5 = APC] + [Va_{53} = APC] + [A1LC = APC] + [A1LC]) + c_2 \quad (12)$$

and $$[APC] = -([Va = APC] + [Va_5 = APC] + [Va_{53} = APC] + [A1LC = APC]) + c_3, \quad (13)$$

where $c_2$ and $c_3$ also depend on the initial concentrations.

*Adapting the Minimal Model Simulate Factor $V_{Leiden}$*

In its present form, our proposed model cannot account for the absence of the $Arg^{506}$ cleavage site and cannot be used to efficiently study the regulation of Factor $Va_{Leiden}$ by APC. To adapt our model to simulate this pathology, we once again assume: (a) factor Va is membrane-bound; (b) APC binds equally well with all species containing the light chain; (c) the inactivating event is the dissociation of the A2 domain; (d) cleavage at $Arg^{679}$ is irrelevant. In addition to these assumptions, we assume (a) cleavage occurs solely at $Arg^{306}$; (b) cleavage at $Arg^{306}$ and the dissociation of the A2 domain are sufficient to fully inactivate factor $Va_{Leiden}$; (c) the model rate constants, shown in Table 1, apply equally to the Leiden system. The kinetic reaction pathway for the inactivation of factor $V_{Leiden}$ that we propose is shown in Figure 2.

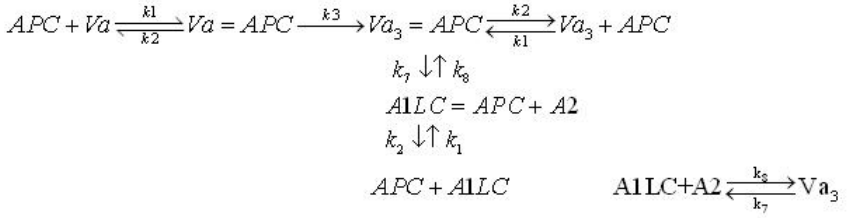



**Figure 2: Proposed Mechanism of Inactivation of Factor $Va_{Leiden}$ by APC.** The equilibrium between free APC and bound APC species is dictated by the rates of association ($k_1$) and dissociation ($k_2$). We assume that after forming the Va=APC complex, cleavage occurs solely at $Arg^{306}$ ($k_3$) to yield the $Va_3$=APC intermediate. Then, the A2 domain rapidly dissociates ($k_7$) from $Va_3$=APC, generating A1LC=APC, which is functionally inactive. Although the re-association of the A2 domain is slow ($k_8$), APC-bound and APC-free A1LC species can rebind with the A2 domain to yield $Va_3$=APC and $Va_3$, respectively.

In our proposed Leiden mechanism, the association and dissociation equilibrium of APC and all species containing the light chain is governed by rate constants $k_1$ and $k_2$. Upon formation of the Va=APC complex, the first and only cleavage occurs at $Arg^{306}$ ($k_3$). After the cleavage, the A2 domain dissociates with a rate of $k_7$, and the reassociation of the A2 domain to APC=A1LC and to A1LC is given by the rate $k_8$. Our mathematical model simulating the inactivation of factor $Va_{Leiden}$ is

$$\frac{d[Va]}{dt} = -k_1[Va][APC] + k_2[Va = APC] \quad (14)$$

$$\frac{d[Va = APC]}{dt} = k_1[Va][APC] - k_2[Va = APC] - k_3[Va = APC] \quad (15)$$

$$\frac{d[Va_3 = APC]}{dt} = k_3[Va = APC] + k_1[Va_3][APC] - k_2[Va_3 = APC] - k_7[Va_3 = APC] + k_8[A1LC = APC][A2] \quad (16)$$

$$\frac{d[Va_3]}{dt} = k_2[Va_3 = APC] - k_1[Va_3][APC] + k_8[A1LC][A2] - k_7[Va_3] \tag{17}$$

$$\frac{d[A1LC = APC]}{dt} = k_7[Va_3 = APC] - k_8[A1LC = APC][A2] - k_2[A1LC = APC] + k_1[A1LC][APC] \tag{18}$$

$$\frac{d[A1LC]}{dt} = k_2[A1LC = APC] - k_1[A1LC][APC] - k_8[A1LC][A2] + k_7[Va_3] \tag{19}$$

$$\frac{d[A2]}{dt} = k_7[Va_3 = APC] - k_8[A2][A1LC = APC] + k_7[Va_3] - k_8[A1LC][A2] \tag{20}$$

$$\frac{d[APC]}{dt} = -k_1[APC]([Va] + [Va_3] + [A1LC]) + k_2([Va = APC] + [Va_3 = APC] + [A1LC = APC]) \tag{21}$$

We can again exploit the redundancies among the equations and reduce the system to five ordinary differential equations and three algebraic expressions:

$$[A1LC] = [Va] + [Va = APC] + [Va_3 = APC] + [Va_3] + [A1LC = APC] + e_1 \tag{22}$$

$$[A2] = [Va] + [Va = APC] + [Va_3 = APC] + [Va_3] + e_2 \tag{23}$$

$$[APC] = [Va = APC] + [Va_3 = APC] + [A1LC = APC] + e_3, \tag{24}$$

where $e_1$, $e_2$ and $e_3$ depend on the initial concentrations.

We use Matlab's numerical solver (ode15s) to approximate solutions to both systems of ordinary differential equations and algebraic expressions. The solver is adept at handling stiff systems of equations and used a maximum time step of 0.05 seconds. The initial concentrations are taken to be 200 nM for factor Va, 10 nM for APC, and 0 nM for the rest of the species. For purposes of modeling comparison, the values for the rate constants are taken directly from an existing mathematical model and are given in Table 1 [1]. See reference [1] for details on the estimation of the model rate parameters.

*Cofactor Va Inactivity*

Although simulated reaction progress diagrams are useful for comparing the rates of change of the concentration of reaction species over time, we propose a method to simulate the loss in cofactor activity over time. We note that although similar formulations are suggested by Hockin, Cawthern, Kalafatis and Mann[1], our equations are derived directly from our minimal mechanism. In particular, we assume that after each elementary reaction (cleavage at $Arg^{506}$, cleavage at $Arg^{306}$, or dissociation of the A2 domain) the activity of factor Va is reduced to a certain percentage. Therefore, we approximate the relative remaining activity of factor Va as a function of time by

$$Act(t) = x\frac{[Va] + [Va = APC]}{[Va_{Tot}]} + y\frac{[Va_5] + [Va_5 = APC]}{[Va_{Tot}]} + z\frac{[Va_{53}] + [Va_{53} = APC]}{[Va_{Tot}]} + d\frac{[A1LC = APC] + [A1LC] + [A2]}{[Va_{Tot}]} \tag{25}$$

where x, y, z and d represent a percentage of initial activity. We take the time-dependent total concentration of factor Va species to be

$$[Va_{Tot}] = [Va] + [Va = APC] + [Va_5 = APC] + [Va_5] + [Va_{53} = APC] + [Va_{53}] + [A1LC = APC] + [A1LC] + [A2]. \quad (26)$$

Here, we have assumed that APC bound and APC-free species have the same activity.

Using the Eq (25-26) we can quantify the differences in predicted activity loss that result from assuming the inactivating event is cleavage at $Arg^{506}$ or at$Arg^{306}$ or is the dissociation of the A2 domain. For instance, if we set coefficients y, z and d in Eq (25) to 0 and coefficient x to100 (since factor Va is 100% active prior to cleavage), we can simulate the loss in activity over time as if cleavage at $Arg^{506}$ were the inactivating event: $Act(t)_{Arg506} = 100\dfrac{[Va] + [Va = APC]}{[Va_{Tot}]}$ (27)

Likewise, if we set the coefficient y to be the percent remaining activity after cleavage at $Arg^{506}$, we can simulate time-dependent activity loss if the inactivating event is cleavage at $Arg^{306}$. For comparison purposes[1], we assume that after cleavage at $Arg^{506}$, the cofactor retains y=60 percent of its initial activity and give the remaining activity as

$$Act(t)_{Arg306} = 100\frac{[Va] + [Va = APC]}{[Va_{Tot}]} + 60\frac{[Va_5] + [Va_5 = APC]}{[Va_{Tot}]}. \quad (28)$$

Finally, assuming that the inactivating event is the dissociation of the A2 domain and the remaining activity of both singly and doubly cleaved factor Va is 60%, the percent remaining activity is given by

$$Act(t)_{Disc} = 100\frac{[Va] + [Va = APC]}{[Va_{Tot}]} + 60\frac{[Va_5] + [Va_5 = APC]}{[Va_{Tot}]} + 60\frac{[Va_{53}] + [Va_{53} = APC]}{[Va_{Tot}]}. \quad (28)$$

Similar arguments can be made for $Va_{Leiden}$ system. Here, if we assume the inactivating event is the dissociation of the A2 domain and that after cleavage at $Arg^{306}$, factor $V_{Leiden}$ retains only 60% of its activity, the percent remaining activity is approximated by

$$Act(t)_{Leiden} = 100\frac{[Va] + [Va = APC]}{[Va_{Tot}]_{Leiden}} + 60\frac{[Va_3] + [Va_3 = APC]}{[Va_{Tot}]_{Leiden}} \quad (29)$$

where the total concentration of factor $V_{Leiden}$ species is

$$[Va_{Tot}]_{Leiden} = [Va] + [Va = APC] + [Va_3 = APC] + [Va_3] + [A1LC = APC] + [A1LC] + [A2]. \quad (30)$$

**Results**

The mathematical model is used to simulate the concentration profile for the inactivation of factor Va by APC over a 20 minute interval (1200 seconds). The resulting reaction progress diagrams are shown on the 0 nM to 200 nM scale (Figure 3a) and then again on the 0 nM to 20 nM scale (Figure 3b).

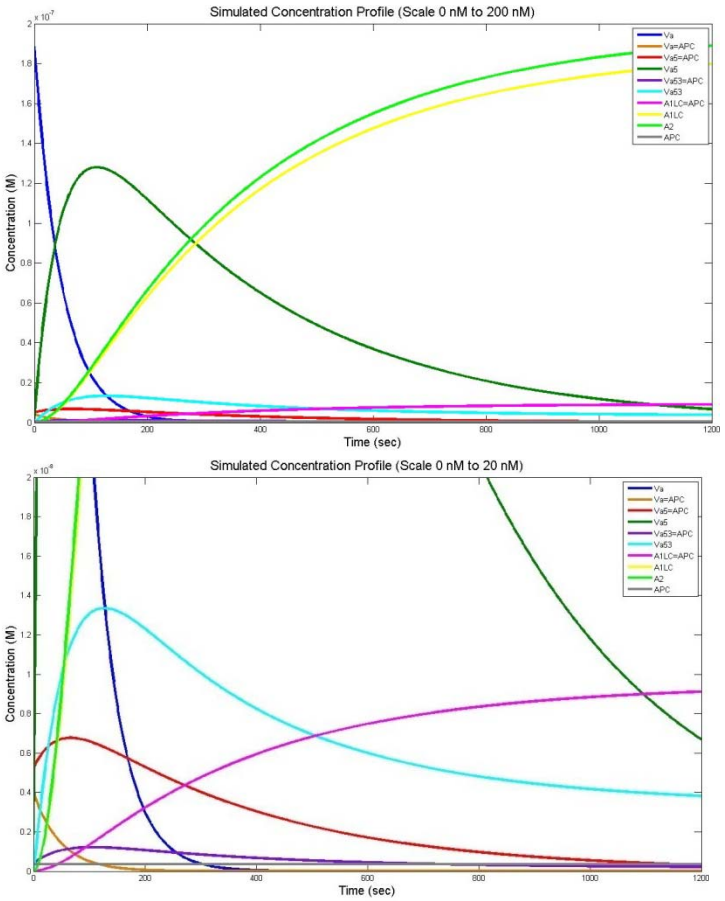



**Figure 3: Simulated Reaction Progress Diagrams.** Theoretical concentrations of factor Va, APC, and products of cleavage and dissociation are shown as a function of time. The mathematical model was initiated with 200 nM Va and 10 nM APC and run 1200 seconds. The model rate constants are shown in Table 1. (a) Concentrations as a function of time on the scale of 0 nM to 200 nM. (b) Concentrations as a function of time on the scale of 0 nM to 20 nM.

Consistent with the mechanism, the predicted concentration of factor Va rapidly decreases as cleavage at $Arg^{506}$ occurs and generates the $Va_5$ product. Quantitatively, the results indicate that concentration factor Va drops to 3 nM within 200 seconds. In the same time interval, the concentration of the cleavage product $Va_5$ rapidly increases to its maximum concentration of 128 nM. These predicted changes in concentration of Va and $Va_5$ correlate well with the results of Western blot analysis. Specifically, several studies have shown that within the first 3 minutes of inactivation, the heavy chain band (Va) loses intensity as cleavage at $Arg^{506}$ generates the Mr=75,000 fragment and a Mr=28,000/26,000 doublet [21, 29, 42, 43, 55].

The kinetic effects of the significant differences in the rates of cleavage at $Arg^{506}$ and $Arg^{306}$ can be quantified by comparing the concentration curves for $Va_5$ and $Va_{53}$. Within the first few minutes, $Va_5$ is formed at a much higher rate and reaches an overall concentration almost 10 times that of $Va_{53}$ (128 nM to 13.3 nM). However, upon reaching their maximums, the concentration of $Va_5$ decays much more rapidly than the concentration of $Va_{53}$, and thus corresponds to the rapid loss in intensity of the Mr=75,000 band after the appearance of the Mr=45,000 and Mr=30,000 fragments that are seen in Western blot analysis [22, 29, 41-43, 55].

Therefore, our model predicts that cleavage occurs predominately in the first three minutes and that the decay in concentrations of singly and doubly-cleaved factor Va is a result of the dissociation of the A2 domain. This is consistent with results from light scattering and immunoprecipitation analyses that suggest the dissociation of the A2 domain and the generation of the Mr=53,000 fragment occur within those three minutes of reaction [1, 13]. Furthermore, our model predicts that the concentrations of the A2 domain and A1LC begin to increase almost immediately, continue to increase throughout the course of the reaction and ultimately approach the initial concentration of factor Va (200 nM). On the other hand, after 20 minutes, the concentrations of factor Va, $Va_5$, and $Va_{53}$ are essentially 0 nM, 0 nM and .4 nM, respectively.

The theoretical inactivation profiles are shown in Figure 4. The biphasic nature of the inactivation is evident in all three methods of simulation [22]. Western blot analyses, clotting assays and prothrombinase assays have shown that the initial rapid stage of inactivation corresponds to cleavage at $Arg^{506}$ and results in a cofactor with significantly depressed activity [1, 21, 22, 29, 41-43, 55]. Our simulations suggest that within the first three minutes of reaction, over 60% of the initial cofactor Va activity is lost. Likewise, the long tails seen in the three inactivation profiles correspond to the second, slower phase of inactivation, when cleavage occurs at $Arg^{306}$ and the A2 domain dissociates.

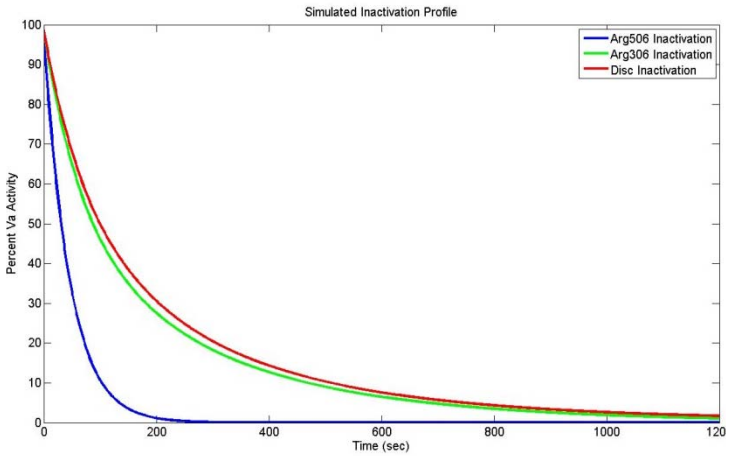



**Figure 4: Simulated Factor Va Inactivation Profiles.** Loss of activity as a function of time was calculated according to the Eq. 25-28.

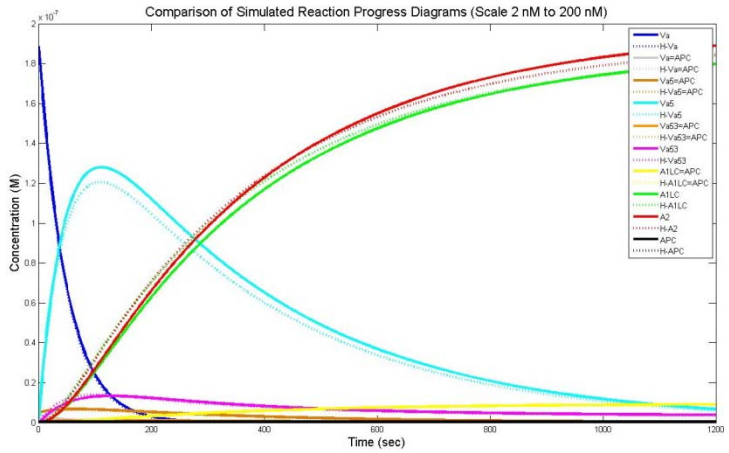
The simulated activity profiles can be used to quantify the differences in assuming the inactivating event is cleavage at $Arg^{506}$, is cleavage at $Arg^{306}$ or is the dissociation of the A2 domain. For instance, if we (incorrectly) assume that cleavage at $Arg^{506}$ is sufficient to inactivate factor Va (as would be seen on a clotting assay), then the simulation shows that factor Va is essentially inactive (<10% activity) after 105 seconds. Furthermore, the deviations between inactivation predictions based on cleavage at $Arg^{306}$ versus the dissociation of the A2 domain grow substantially with time. When 30% activity remains, the time delay between inactivation by cleavage at $Arg^{306}$ and dissociation is almost 25 seconds. Later, the time requirement for inactivation (<10% activity) is 470 seconds (7.83 minutes) for $Arg^{306}$-dependent inactivation and 512 seconds (8.53 minutes) for dissociation-dependent inactivation, a

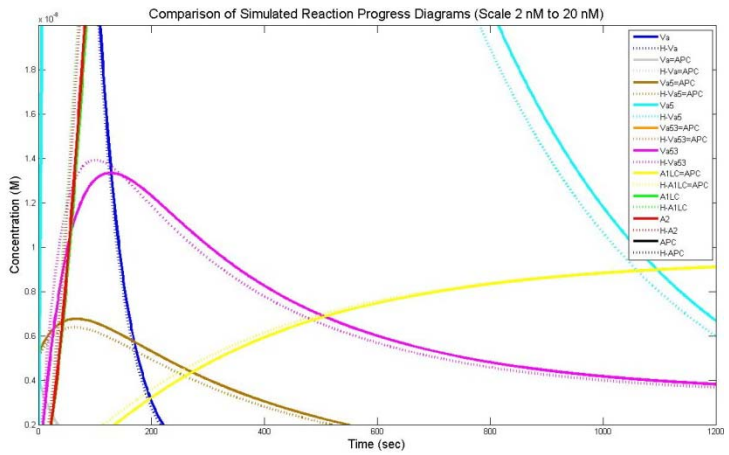
difference of 42 seconds. Although these differences may seem small, unraveling the kinetics regarding cleavage at $Arg^{306}$ and the dissociation of the A2 domain has proven challenging and our mathematical modeling may lead a greater understanding of these events.

***Comparison with Existing Models***

As means of model validation, we compare the reaction progress diagrams and simulated activity profiles to a mathematical model of inactivation of factor Va by APC that was proposed by Hockin, Cawthern, Kalafatis and Mann in 1999. In their model (the "HCKM model"), primary cleavage could occur either at $Arg^{505}$ or $Arg^{306}$ and allowed for secondary and tertiary cleavages at $Arg^{662}$ [1]. (They modeled the inactivation occurring in bovine factor Va, where comparable cleavages occur at $Arg^{306}$, $Arg^{505}$ and $Arg^{662}$.) The HCKM model was validated by comparing simulated cofactor inactivity profiles to experimental data obtained from prothrombinase assays with a high level of factor Xa (200 nM bovine Va, 100 uM PC/PS and 10 nM APC). However, the HCKM model consisted of thirty stiff, non-linear, ordinary differential equations, with several parameters, some of which were intractable. Our minimal mathematical model consists of a system of only seven ordinary differential equations and three algebraic expressions. Our model encapsulates steps in the enzymatic pathway that we think are crucial in describing the rates of change of concentrations of Va, APC and cleavage products as a function of time.

Figure 5 and Table 2 illustrate comparative results for the two model mechanisms. There is strong correspondence between the minimal model and the HCKM model. The average of the maximum percentage difference between the predictions of the two models is 6.052%. The high level of correlation between the reaction progress diagrams suggests our minimal model captures the most important elements of the mechanism and associated kinetics of the regulation of factor Va by APC. Likewise, the results from all three methods of simulating time-dependent cofactor activity loss are nearly indistinguishable. Since our results are similar to those obtained in the HCKM model, we can infer our model would also reproduce experimental results.



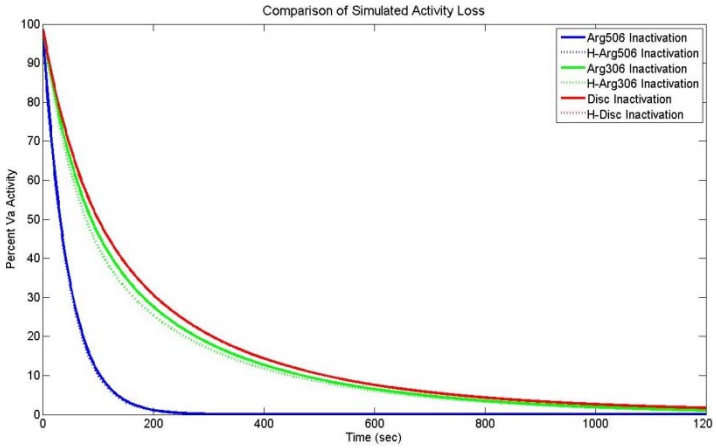




**Figure 5: A Comparison of Modeling Results:** Theoretical concentrations of factor Va, APC and cleavage species are shown as a function of time. Here, a comparison is made between our minimal model (solid lines) and the HCKM model (dotted lines and legend entries denotes with "H-".) Both numerical systems were initiated with 200 nM Va and 10 nM APC and run over 1200 seconds. (a) Concentrations as a function of time on the scale of 2 to 200 nM. (b) Concentrations as a function of time on the scale of 2 to 20 nM. (c) Simulated Inactivation Profiles.

**Maximum Percentage Difference in Model Concentrations for each Species**

Comparing the Simulations for the Inactivation of Factor Va by APC

| Va | Va=APC | $Va_5$=APC | $Va_5$ | $Va_{53}$=APC | $Va_{53}$ | A1LC=APC | A1LC | A2 | APC |
|---|---|---|---|---|---|---|---|---|---|
| 1.00% | 3.62% | 6.16% | 6.34% | 17.14% | 15.02% | 4.25% | 4.26% | 2.73% | 4.71 E-9 % |

**Table 2: The Maximum Percentage Difference in Model Concentrations for each Species:** In order to compare the minimal model to the HCKM model, the absolute difference in the concentrations were calculated at each time-point. To normalize these differences, we divided by the average of the maximum concentration of the species under consideration. We then found the maximum percentage difference for each species.

***Simulation of Factor Va Leiden***

Our minimal mathematical models can also be used to quantify the differences in the reaction progress diagrams for normal factor Va and factor $Va_{Leiden}$ by APC. Starting with the initial concentrations of 200 nM Va (or $Va_{Leiden}$) and 10 nM APC, the change in the concentration of reaction species was simulated over a 20 minute interval (1200 seconds). Comparisons between the concentration profiles resulting from the wild-type factor Va and from factor $Va_{Leiden}$ are made on the 2 nM to 200 nM scale (Figure 6a) and then again on the 2nM to 20 nM scale (Figure 6b).

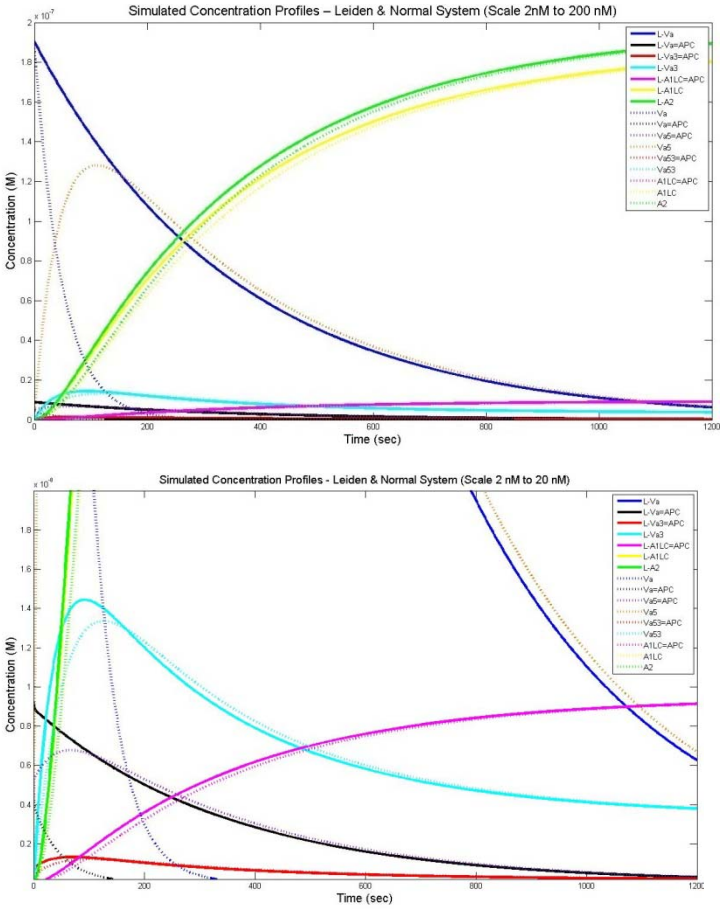



**Figure 6: Simulated Reaction Progress Diagrams to Compare Inactivation in Normal Factor Va and Factor $Va_{Leiden}$.** Theoretical concentrations of factor Va, APC, and products of cleavage and dissociation are shown as a function of time. The mathematical model was initiated with 200 nM Va and 10 nM APC and run over 1200 seconds. The model rate constants are shown in Table 1. The species corresponding to normal factor Va are shown in dashed lines and the species corresponding to the Leiden system are shown in solid lines and denoted with an "L-" in the legend. (a) Concentrations as a function of time on the scale of 2 nM to 200 nM. (b) Concentrations as a function of time on the scale of 2M to 20 nM.

From the reaction progress diagrams, it is evident that the rate of decay of the uncleaved species is much more rapid in normal factor Va than in factor $Va_{Leiden}$. For instance, after 200 seconds, the remaining concentration of uncleaved $Va_{Leiden}$ is 36 times that of normal factor Va (108 nM to 3 nM). Furthermore, the simulated concentration curve of factor $Va_{Leiden}$ is very close to singly-cleaved wild-type factor $Va_5$ (after 200 seconds). Likewise, the predicted concentration curve for factor $Va_{Leiden}$ that has been cleaved at $Arg^{306}$ (L-$Va_3$) is very similar to the curve of the doubly-cleaved $Va_{53}$ in normal factor Va. In particular, the maximum concentration of both species is less than 0.15 nM and occurs after just over 2 minutes. The simulations, however, do indicate the elimination of the $Arg^{506}$ cleavage site has little effect on the predicted time-dependent concentrations of A2 domain, A1LC and A1LC=APC.

Our simulated inactivation profiles predict the resistance to inactivation by APC characteristic of Leiden patients (Figure 7). Within 1 minute, more than 35% of activity is lost in normal Va while factor $Va_{Leiden}$ retains over 80% of its initial activity. Likewise, after 200 seconds, which roughly corresponds to the time required for complete cleavage, the activity of factor Va has dropped to 30% while factor $V_{Leiden}$ still maintains 50% activity. Our model predicts that the time requirement for inactivation (<10%) would be more that 2 minutes longer for factor $Va_{Leiden}$ (636 seconds) than for normal factor Va (511 seconds). Furthermore, the theoretical inactivation profile for the minimal Leiden system is compared to the HCKM system ($k_5=0s^{-1}$). These inactivation profiles are nearly identical. Moreover, the reaction progress diagrams for the Leiden system are compared directly to the HCKM model in Figure 8 and Table 3. The results are striking. The maximum percentage difference between the two model predictions is less than one percent (0.57%). There is no meaningful difference in the predicted rates of change in the minimal Leiden model (5 ODEs) and the HCKM model (30 ODEs). This further emphasizes the appropriateness of our model system in describing the observed reaction mechanism and its use in studying factor $Va_{Leiden}$.

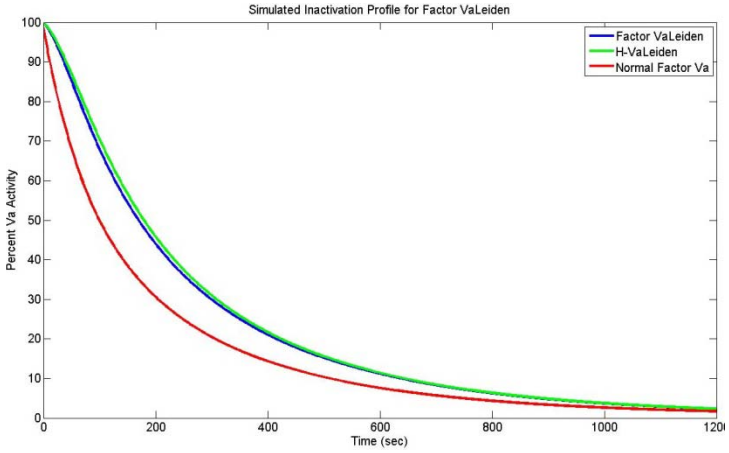



**Figure 7: Simulated Factor Va Inactivation Profile in Normal Factor Va and Factor $Va_{Leiden}$.** To compare the loss in activity of cofactor Va over time, we assume that the inactivating event is the dissociation of the A2 domain. The models that we have derived reflect the delayed inactivation by APC that is characteristic of Leiden patients. Also, a comparison is made between our minimal model (blue) and the HCKM model (green). Since the minimal model results were well-correlated with the HCKM model, it can be inferred that the minimal model would also strongly fit experimental data.

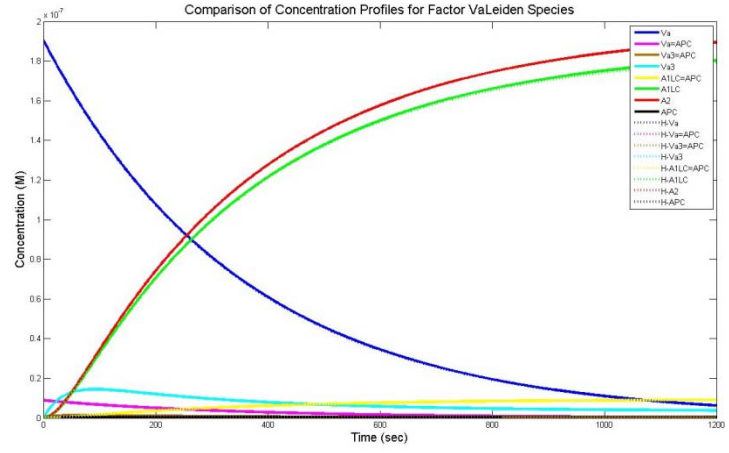



**Figure 8: A Comparison of Modeling Results:** Theoretical concentrations of factor $Va_{Leiden}$, APC and cleavage species are shown as a function of time. Here, a comparison is made between our minimal Leiden model (solid lines) and the HCKM model (dotted lines and legend entries denotes with "H-".) Both numerical systems were initiated with 200 nM VaLeiden and 10 nM APC and run over 1200 seconds. Clearly, the minimal model is well-correlated with the HCKM model system.

**Maximum Percentage Difference in Model Concentrations for each Species**

Comparing the Simulations for the Inactivation of Factor Va Leiden by APC

| Va | Va=APC | $Va_3$=APC | $Va_3$ | A1LC=APC | A1LC | A2 | APC |
|---|---|---|---|---|---|---|---|
| 0.213 % | 0.20% | 0.40% | 0.45% | 0.57% | 0.57% | N/A | 6.43E-11 % |

**Table 3: The Maximum Percentage Difference in Model Concentrations for each Species:** In order to compare the minimal model to the HCKM model, the absolute difference in the concentrations were calculated at each time-point. To normalize these differences, we divided by the average of the maximum concentration of the species under consideration. We then found the maximum percentage difference for each species. Please note that percentage difference in the simulated concentration of the A2 domain is not available because the HCKM model was not able to simulate the change in concentration of the A2 domain. The HCKM model predicts that the maximum concentration of the A2 domain in the Leiden system is 0 nM

## Discussion

Through the use of biologically plausible assumptions, a minimal mechanism and mathematical model for the inactivation of factor Va by APC have been proposed (Figure 1). Our mechanism is limited to factor Va, APC and cleavage species and assumes that factor Va is membrane-bound and that binding between APC and all LC containing species is equivalent. The minimal mechanism required to quantify the regulation of factor Va by APC is given by the following steps: (1) an initial cleavage reaction at $Arg^{506}$, resulting in the $Va_5$=APC intermediate with partial activity; (2) the subsequent cleavage reaction at $Arg^{306}$ that generates $Va_{53}$=APC with further reduced activity; (3) the rapid dissociation of A2 domain; (4) the remaining factor Va molecule is inactive.

From our proposed mechanism, we derive a minimal mathematical model of only seven ordinary differential equations and three algebraic expressions. Although the reduced system is not directly compared with empirical studies, our predictions correlate well with results from Western blot, light-scattering and immunoprecipitation analyses. Further, the predictions of the minimal model correlate very well with results from the celebrated HCKM model, which, in contrast, consists of a system of thirty ordinary differential equations. In particular, the reaction progress diagrams and simulated inactivation profiles are almost identical. Therefore, we find that our minimal mathematical model captures the essential reactions and kinetics required to quantify the inactivation of factor Va by APC and that our minimal model may be a more manageable and efficient method to study the regulatory system. For instance, determining the kinetic consequences of cleavage at $Arg^{306}$ and the dissociation of the A2 domain has proven difficult since these reactions are occurring on the same time scale. However, we show that mathematical modeling may be an efficient method to examine these events. In particular, we quantify the difference in assuming that either cleavage at $Arg^{306}$ or dissociation of the A2 domain is the inactivating event.

The minimal model is also adapted to account for factor $Va_{Leiden}$, the most prevalent genetic risk factor for thrombosis. The Leiden system's reaction progress diagram and simulated inactivation profile are indistinguishable from the HCKM model (Figures 7-8). Furthermore, the creation of the Leiden model allowed for direct comparison of the simulated concentration profiles for the inactivation of normal factor Va and factor $Va_{Leiden}$ by APC. From the reaction progress diagrams shown in Figure 6, it is

evident that time-dependent change in concentration of the A2 domain, A1LC or A1LC=APC are not affected by the mutation at $Arg^{506}$. Instead, we conclude the APC resistance, characteristic of Leiden patients, is due to the kinetic differences in the rates of cleavage at $Arg^{506}$ and $Arg^{306}$ and is manifested by a slower loss in concentration of uncleaved factor Va species over time.

Mathematical modeling and computer simulation are efficient quantitative tools for studying the blood coagulation proteome. In particular, numerical systems allow us to examine the importance of an individual species at a particular step in a reaction. Furthermore, computer models are adept at handling stiff systems, where the concentrations of species are changing on widely different time scales. In the past, they have been used to further our understanding of blood coagulation as a threshold-limited, cascade and of the role played by membranes [44]. In the future, we hope to expand our minimum model to account for the effects of protein S, Xa and prothrombin in the regulation of factor Va by APC [21, 29, 41]. It is also our hope that the minimal model can be further validated by empirical studies, and then incorporated into a larger model of blood coagulation. A more complete model for blood coagulation dynamics can be used to advance our knowledge of the biochemical system and its network. It may also to provide quantitative tools for clinical studies involving the dynamics of thrombosis, and testing of new therapies, thus minimizing the need for extensive clinical protocols.

**FOOTNOTES**

*The abbreviations used are: APC, activated protein C; Va, factor Va; $Va_5$, factor Va cleaved at $Arg^{506}$; $Va_{53}$, factor Va cleaved at $Arg^{506}$ and then at $Arg^{306}$; HC, heavy chain; LC, light chain; =, indicates binding; ODEs, ordinary differential equations; DAPA, dansylarginine-*N*-(3-ethyl-1,5-pentadiyl)amide.

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**Conclusion**:

For several decades mathematicians and scientists have been working together to study blood coagulation with mathematical modeling, more recently, using computers. Several mathematical models of thrombin production have been developed and have contributed greatly to our understanding of the mechanism, kinetics and pathology associated in the formation of a blood clot. With this in mind, we proposed a model for the regulation of factor Va by APC, which is one of the essential regulatory mechanisms in the clotting cascade.

The importance this regulation has in maintaining the haemostatic balance is manifested in individuals with Factor $V_{Leiden}$, who exhibit resistance to APC. In these individuals, a single amino acid substitution at $Arg^{506}$ eliminates the most kinetically important cleavage site and thus delays inactivation of factor Va by APC. Consequently, the Leiden patients are at a much greater risk of thrombosis.

With the use of biologically realistic assumptions, we proposed a reduced mechanism for the regulation of factor Va by APC. From the mechanism and reported rate constants, we then derived a mathematical model of 7 ODEs and 3 algebraic expressions. The reaction progress diagrams from the minimal model were highly correlated with the simulations from the HCKM model, which consisted of 30 ordinary differential equations and had been validated with empirical assays. We also derived equations to describe the activity loss of factor Va as a function of time. Our simulated inactivation profiles were also highly correlated with those of the HCKM model. We also adapted our minimal model to account for Factor $Va_{Leiden}$. The reaction progress diagrams from our Leiden system, which consisted of 5 ODEs and 3 algebraic expressions, were nearly indistinguishable from those of HCKM model. (See Table 1.) The simulated inactivation profiles also accurately predicted the resistance to inactivation by APC that is characteristic of Leiden patients.

Significant work still remains in validating and in improving our model mechanism. The Dahlback laboratory in Malmo, Sweden, has generously shared their experimental data from their most recent publication on the role played by prothrombin in the inactivation of factor Va [29]. However, the initial comparisons of simulated activity profiles to their experimental data suggest that both our minimal model and the full HCKM model are a poor fit and that a modification of our model may be necessary.

However, there are several explanations for the models' inability to reproduce the experimental data from the Dahlback lab. As discussed previously, the derivation of model rate constants is subject to a great deal of uncertainty and highly dependent on experimental conditions. In particular, the experiments performed by the Dalhback lab to examine the inactivation of factor Va were run in the presence of protein S. Protein S also serves as a cofactor to APC and thus greatly accelerates the rate of inactivation. However, the rate parameters used in our mathematical model were obtained or estimated from experiments that did not involve protein S. Therefore, it is unclear how our model rate constants would need to be adjusted to account for the acceleration in the inactivation rate that is due to the presence protein S. Future work will address this issue.

Furthermore, direct comparison of the experimental data to the models required the use of simulated inactivation profiles. (The empirically derived reaction progress diagrams were not available for comparison.) In their original formulation, the equations used to simulate activity loss assumed that with an initial concentration of 200 nM factor Va and 10 nM APC, factor Va retained 60% of its activity after cleavage at $Arg^{506}$ and at $Arg^{306}$. (The initial concentrations and percentages of remaining activities were chosen for purposes of model validation with the HCKM model.) Unfortunately, it has been repeatedly shown that the remaining activity is highly dependent on experimental conditions (initial concentrations) and the type of assay used. Consequently, there is little agreement in the literature on the reported remaining activities after cleavage and/or dissociation. (See Table 2.) Although we did attempt to fit our model to experimental data by testing various combinations of reported percent activities, we were determined at the onset of our research to develop a model that was biologically significant. Therefore, it seems that until a concentration-independent and assay-independent method of analyzing remaining activity is developed, accurately simulating inactivation profiles will be difficult.

| Remaining Activity As a Function of Species, Time, Experimental Conditions and Assay Type | | | | | | |
|---|---|---|---|---|---|---|
| **Species** | % **Remaining Activity** | **Assay Used** | **Reaction Conditions** | | | **Source** |
| | | | **[Va]** | **[APC]** | **[Xa]** | |
| Va | <20% initial | Prothrombinase | 200 nM | 10 nM | | [43] |
| | 40% intial | Prothrombinase | .8 nM | .1 nM | 5 nM | [29] |
| | 50% after 3 min | Prothrombinase | .3 nM | .15 nM | 5 nM | [22] |
| | 30% after 5 min | Clotting | 1 nM | .1 nM | | [21] |
| | 0% after 5 min | Prothrombinase | 280 nM | 5.4 nM | 10 nM | [55] |
| | 60% after 6 min | Prothrombinase | 20 nM | 5 nM | 10 nM | [1] |
| | 15% after 6 min | Clotting | 20 nM | 5 nM | | [43] |
| | 10% after 12 min | Clotting | 20 nM | 5 nM | | [43] |
| | 32% after 12 min | Prothrombinase | 20 nM | 5 nM | 10 nM | [1] |
| | 15% after 15 min | Prothrombinase | 200 nM | 10 nM | | [43] |
| | 20% after 20 min | Prothrombinase | .8 nM | .1 nM | 5 nM | [29] |
| | 5% after 90 min | Clotting | 1 nM | .1 nM | | [21] |
| | 40% after cleavage at 506 | Prothrombinase | <5nM | .15 nM | 5nM | [22] |
| Va Leiden | 0% after cleavage at 506 | Prothrombinase | <5nM | .15 nM | 0.3nM | [22] |
| | 20% after full cleavage | Prothrombinase | 1 uM | 1.7-5 uM | 10 nM | [54] |
| | 50% after 5 min | Prothrombinase | 280 nM | 5.4 nM | 10 nM | [55] |
| | 50% after 5 min | Prothrombinase | 280 nM | 5.4 nM | 10 nM | [55] |
| | 50% 45 min | Prothrombinase | .3 nM | .15 nM | 5 nM | [22] |
| | 20% after 1 hr | Prothrombinase | 280 nM | 5.4 nM | 10 nM | [55] |
| | 10% after 1 hr | Prothrombinase | 280 nM | 5.4 nM | 10 nM | [55] |
| rVaWT | 70% after 5 min | Clotting | 1 nM | .1 nM | | [21] |
| | 10% after 90 min | Clotting | 2 nM | .1 nM | | [21] |
| rVa306A | 25% after 90 min | Clotting | 3 nM | .1 nM | | [21] |
| rVa506Q | 40% after 90 min | Clotting | 4 nM | .1 nM | | [21] |
| rVa306A/r506Q | 100% after 90 min | Clotting | 4 nM | .1 nM | | [21] |

**Table 2**: Remaining Activity As a Function of Species, Time, Experimental Conditions and Assay Type. Abbreviations: 'r': recombinant; 'WT': wild-type; 'A': alanine substitution of Arg; 'Q': glutamine substitution of Arg. Sources are shown in the right hand column.

A possible improvement to our models would be the incorporation of stochasticity. In their present forms, our minimal models are deterministic. There are two problems with the deterministic models [40, 56, 57]. First, all reaction species are not treated as discrete proteins but instead modeled as continuous entities. Secondly, deterministic solvers ignore the randomness inherent to biological systems. Randomness or stochasticity can cause divergent reaction trajectories when given the same initial conditions. The Gillespie method is a stochastic simulation algorithm that was developed to account for the randomness inherent in real chemical systems. In the algorithm, the reaction progress diagrams are dependent on the probability of reactions occurring (in general) and the probability of a particular reaction occurring[57]. Lo, Denney and Diamond have suggested that stochastic modeling of blood coagulation may result in more accurate predictions especially when the concentrations of species are low and are more amenable to statistical analysis (variances and standard deviations)[40]. Therefore, an

incorporation of stochastic elements into our model could be an avenue for improvement, and this is an area for future research.

Despite its many challenges, mathematical modeling of blood coagulation is a powerful tool that has and will continue to contribute to our understanding of the blood coagulation proteome and associated pathology. For instance, *in vitro* and *in vivo* methods to study Factor $V_{Leiden}$ are costly and error prone. They either require homozygous carriers of the genetic defect or the use of recombinant proteins. However, the minimal mathematical model with only 5 ODES described above can be used efficiently to study Factor $V_{Leiden}$. For instance, we were able to quantitatively compare the clotting properties of Leiden patients to normal individuals through the initial loss in concentration of uncleaved factor Va, the maximum concentrations of cleavage species and the simulated inactivation profiles. We found that the delay of inactivation in Leiden patients is largely attributable to the kinetic differences in the rates of cleavage at $Arg^{506}$ and $Arg^{306}$.

In conclusion, the principal goal of modeling is to simplify the complex system under study, and that goal was achieved. Reducing the mechanism and simplifying the numerical system are powerful ways to gain insights to the biological phenomena. The minimal models that we have proposed substantially reduce the complexity of the mechanism and numerical system without a significant loss of information. However, our initial comparisons to experimental data remind us of George Box's saying, "don't fall in love with a model"[39]. Thus, we plan to continue and extend our modeling efforts with the hope of contributing significantly to blood coagulation studies.

**Glossary**:

Catalytic efficiency: the ratio of reaction rates.

Cofactor: a non-enzymatic molecule that is essential for the activity of its enzyme.

Cooperative phenomena: occurs when an enzyme binds one substrate molecule at one site and then can bind another substrate molecule at a different site.

Enzyme: a biomolecule that increases the rate of reaction (catalysis).

Mechanism: a series of elementary reactions (single-step reactions) that describe the overall reaction.

Procofactor: the precursor to a cofactor.

Reaction Order: describes the dependency of the reaction rate on the concentrations of reactant species. Consider the reaction: $aA + bB \xrightarrow{k} cC$ whose rate law is $\frac{d[C]}{dt} = k[A]^1[B]^1$. The reaction is first order with respect to reactant A, first order with respect to reactant B and second-order overall.

Serine protease: an enzyme that cleaves an amide bond joining two amino-acids and is characterized by the presence of a serine residue in its active site.

Suicide substrates: an inhibitor that binds irreversibly to the enzyme and results the enzymes permanent inactivation.

Zymogen: the inactive form of an enzyme.

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