Accelerin : the activated form(s) of human blood coagulation factor V

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Accelerin

**The activated form(s) of human
blood coagulation factor V**

Harry M. Bakker

Accelerin

The activated form(s) of human

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blood coagulation factor V

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volgens het besluit van het College van Dekanen,
in het openbaar te verdedigen op vrijdag, 17 juni 1994
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Harm Marten Bakker

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Aan mijn ouders

voor Marijke, Jeroen en Maarten

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Chapter 1

INTRODUCTION

The basic defence mechanism for preventing the loss of blood involves several cellular and plasma components, as well as physiological effects upon the blood vessels involved. When the wall of a blood vessel is damaged blood is lost to the surroundings. Spontaneous arrest of bleeding by vasoconstriction, can be effective in stopping bleeding from small blood vessels. In larger blood vessels, however, formation of a solid plug derived from blood platelets and fibrin is the most important mechanism in haemostasis. The haemostatic plug is formed initially by aggregation of platelets; later fibrin deposition occurs and the fully formed blood clot contains also red cells and leukocytes within the fibrin meshwork. Blood coagulation is the conversion of the soluble protein fibrinogen into the insoluble protein fibrin, by means of thrombin. The biochemical process that leads to blood coagulation essentially is a series of consecutive activations in which (inactive) zymogens are converted to proteolytic enzymes. Typical of this so-called cascade type of process is the large overall amplification resulting from sequential enzymatic activation which is of at least an order of magnitude at each stage.

The enzymes involved in blood coagulation are circulating in plasma in their zymogen form. The zymogens are converted to active serine proteases by limited proteolysis of one or two peptide bonds when the coagulation cascade is activated. The catalytic domains of the coagulation enzymes are all homologous to trypsin and chymotrypsin and contain the enzyme active site, the 'catalytic triad' typical for serine proteases. Coagulation factors are represented by roman numerals. The activated coagulation factors are designated by addition of lowercase 'a'. Thrombin can be formed via two routes, the intrinsic and the extrinsic pathway. The extrinsic pathway is considered to be important in the initiation of fibrin formation (Davie *et al.*, 1991). The (extrinsic) coagulation cascade is initiated when blood makes contact with tissue factor (TF, Figure 1). Tissue factor is exposed to blood when the layer of endothelial cells (inner lining of blood vessels) is damaged. Extravascular tissue constitutively exposes tissue factor molecules on the cell membranes. Tissue factor complexes factor VII from the blood. Complexed factor VII is autoactivated to its active two chain form, factor VIIa (Nakagaki *et al.*, 1991). Factor VIIa complexed with tissue factor activates the zymogens factor X and factor IX. Factor X is not only

Figure 1. A simplified view of the extrinsic pathway of blood coagulation. When tissue factor (TF) is exposed it binds factor VII, the zymogen factor VII is autocatalytically converted to its active serine protease factor VIIa. Factor VIIa activates the zymogens factor IX and X. Factor IXa activates factor X and represents the major link between the intrinsic and extrinsic pathway. Factor Xa activates prothrombin into thrombin which converts fibrinogen into insoluble fibrin. Thrombin activates the protein cofactors factor V and VIII (adapted from Hemker and Lindhout, 1984).

activated by VIIa-TF complex but also by factor IXa. In 1965, Jossen already suggested that factor VIIa-TF complex could activate factor IX, a component of the intrinsic pathway, forming a reinforcement loop for factor Xa generation at low TF concentration (Jossen and Prou-Wartelle, 1965; Østerud and Rapaport, 1977). Factor Xa activates prothrombin (factor II) to thrombin (factor IIa) and thrombin is the enzyme responsible for the conversion of soluble fibrinogen into insoluble fibrin. It is thought that the intrinsic pathway, known as contact activation route, plays a role in sustaining the thrombin generation (Davie *et al.*, 1991). Current data suggest that thrombin produced through the initial action of factor VIIa-TF could activate factor XI and thereby sustain thrombin generation via the intrinsic pathway (Gailani and

Broze, 1991). Both pathways converge at factor Xa generation (Figure 1). The function of the cofactors factor V and factor VIII (Figure 1) are discussed below.

The coagulation proteins circulating in plasma are mainly synthesized in the liver. The zymogens prothrombin, factor VII, IX, X and protein C are posttranslationally modified by the action of a vitamin K-dependent carboxylase (Suttie *et al.*, 1985). Depending which of the coagulation factors is to be carboxylated, 9 to 12 specific Glu residues within the first 45 N-terminal residues are converted to Gla-residues (γ -carboxy Glu) by this vitamin-K dependent carboxylase. (Stenflo and Suttie, 1977; Jackson and Nemerson, 1980).

An important feature of coagulation is the fact that the reactions are confined to membrane surfaces. The membrane surfaces that support the coagulation reactions must contain anionic phospholipids. These negatively charged membranes are provided by activated blood platelets and disrupted endothelial cell membranes. The above mentioned Gla residues are required for binding of the vitamin K-dependent clotting factors to negatively charged (procoagulant) membranes. It remains as yet uncertain whether some of the Gla residues directly bind to membrane surfaces through calcium ion bridges (Resnick and Nelsestuen, 1980; Rosing *et al.*, 1987), or whether metal binding to γ -carboxyglutamic acid in the Gla domain alters the structure of adjacent domains, exposing a membrane-binding site (Nelsestuen, 1976; Prendergast and Mann, 1977; Borowski *et al.*, 1986; Liebman *et al.*, 1987; Furie and Furie, 1988).

The primary structure of most of the human coagulation proteins has been determined by either amino acid and/or cDNA sequencing techniques and the structure and organization of the genes have been resolved (Yoshitake *et al.*, 1985; Leytus *et al.*, 1986; Friezner and Davie, 1987; O'Hara *et al.*, 1987; Jenny *et al.*, 1987; Furie and Furie, 1988; Cripe *et al.*, 1992; Furie and Furie, 1993).

PROTEIN COFACTORS

In the expression of activity of the active proteases involved in blood coagulation, cofactors play a central role (Hemker *et al.*, 1967; Hemker and Kahn, 1967; Rosing *et al.*, 1980; van Dieijen *et al.*, 1981; reviewed by Mann *et al.*, 1988). These cofactors are required for efficient proteolytic activity of the serine proteases of the blood coagulation cascade. The effect of the cofactor on the reaction can be regarded to result of at least three separate effects: 1) An increase in the maximum turnover rate (k_{cat}) of the reaction. 2) Localization of the enzyme-cofactor complex to membrane surfaces. 3) Stimulation of the binding of the substrate to the

membrane bound enzyme-cofactor complex. By virtue of the latter two effects, the cofactor can be conceived as forming a receptor on the membrane both for the serine protease and for the substrate.

Factor V is a single-chain glycoprotein with a MW of 330,000 Da. In 1950, the term proaccelerin and accelerin, for its activated form, were introduced. In the 1960's the present name, factor V, was gradually adapted. Factor V has been purified to homogeneity from canine, bovine and human plasma (Nesheim *et al.*, 1979; Esmon 1979; Bolhuis *et al.*, 1979; Dahlback, 1980; Kane and Majerus, 1981; Katzmann *et al.*, 1981; Suzuki *et al.*, 1982). The cDNA sequences for human, and recently, bovine factor V have been determined (Kane and Davie, 1986; Kane *et al.*, 1987; Jenny *et al.*, 1987, Guinto *et al.*, 1992) and from this the amino acid sequences could be deduced. Human factor V contains 2196 amino acid residues (Jenny *et al.*, 1987). Factor V has a domain structure similar to that of factor VIII (Figure 2). The homology between the factor V domains involved in the cofactor activity, A1, A2, A3, C1 and C2 (see below, Figure 2) is 40% with its factor VIII counterparts (Church *et al.*, 1984; Jenny *et al.*, 1987). The connecting region, the B domains of factor V and VIII, share a 14% amino acid sequence homology (Jenny *et al.*, 1987).

Figure 2. Comparison of the structural domains in factor V and VIII. Cleavage sites of thrombin, factor Xa, Activated protein C, Russell's viper venom factor V activator, and platelet associated protease (PAP) are represented by respectively IIa, Xa, APC, RVV-V and N.

Homologies are also found with ceruloplasmin ($\approx 35\%$) and discoidin I, the lipid binding lectin from *Dictyostelium discoideum* (Jenny, 1987). Plasma factor V is synthesized in liver parenchyma cells (Wilson *et al.*, 1984). Platelet α -granules (Holmsen and Weiss, 1979) and (aorta) endothelial cells also contain factor V molecules. However, factor V mRNA was not detected in human umbilical vein endothelial cells (Jenny *et al.*, 1987). Factor V is an inactive procofactor with less than 0.27% of the activity of the thrombin activated factor V (Nesheim *et al.*, 1979). Thus in order for factor V to exert its cofactor activity factor V must be activated during coagulation. That factor Va is indeed an essential cofactor for prothrombin activation is clear from the data shown in Table 1. Without factor Va the $V_{\max}$ in thrombin formation is far too low to account for coagulation. The mechanism by which factor Va bring about the three factors of magnitude increase in the $V_{\max}$ of thrombin formation is not known. In addition it has been shown that factor Va

Table 1. Kinetic properties of the prothrombinase and tenase complex components. In both cases it is clear that phospholipids (PL) decrease the K_m for the substrate and the cofactor increases the k_{cat} . (Data taken from Rosing *et al.*, 1980; van Dieijen *et al.*, 1981)

Component	K_m (μM)	$V_{\max}$ $\text{mol IIa}\cdot\text{s}^{-1}\cdot\text{mol Xa}^{-1}$	Relative catalytic Efficiency
Xa	84	0.011	1
Xa, Va	34	6.22	1,400
Xa, PL	0.06	0.038	4,800
Xa, Va, PL	0.21	32.0	1,130,000

Component	K_m (μM)	$V_{\max}$ $\text{mol Xa}\cdot\text{s}^{-1}\cdot\text{mol IXa}^{-1}$	Relative catalytic Efficiency
IXa	181	$1.8\cdot 10^{-4}$	1
IXa, PL	0.058	$4.1\cdot 10^{-5}$	730
IXa, VIIIa, PL	0.063	8.3	140,000,000

causes a considerable decrease of K_m when prothrombin is activated at procoagulant surfaces with low affinity for prothrombin (Rosing *et al.*, 1980). Prothrombin can be activated to thrombin via two pathways in which prethrombin 2 or meizothrombin occur as reaction intermediate. Factor Va causes a shift in prothrombin activation from a process in which mainly prethrombin 2 and small amounts of thrombin and meizothrombin are produced into one in which only thrombin and meizothrombin are formed (Rosing and Tans, 1988).

In vitro factor V can be activated by thrombin, factor Xa and also by proteases isolated from snake venoms (Esmon and Jackson, 1974; Monkovic and Tracy, 1990; Gerads *et al.*, 1992). The most important physiological activator of factor V, however, is most likely thrombin (Pieters *et al.*, 1989). The structure of the activated factor V is different with various activators. Thrombin activates factor V via hydrolysis of three peptide bonds. In the human system the first cleavage occurs between Arg⁷⁰⁹ and Ser⁷¹⁰, subsequently peptide bonds between Arg¹⁰¹⁸ / Thr¹⁰¹⁹ and Arg¹⁵⁴⁵ / Ser¹⁵⁴⁶ are cleaved. Partially cleaved factor V already possesses full cofactor activity (Monkovic and Tracy, 1990). Fully thrombin activated factor V consists of a 105 kD heavy chain and a 71 and 74kD light chain doublet which are noncovalently associated through interactions that involve a single calcium ion (Nesheim and Mann, 1979; Esmon, 1979; Guinto and Esmon, 1982). Russell's viper (*Vipera russelli*) venom (RVV-V) activated factor V is cleaved between Arg¹⁵⁴⁵ and Ser¹⁵⁴⁶ resulting in a large heavy chain and a light chain similar to the thrombin activated factor V (Suzuki *et al.*, 1982; Dahlback, 1986; see Figure 2). This suggests that a single cleavage within the B region will bring the N- and C-terminal parts of the factor V molecule together resulting in an active cofactor molecule (Dahlback, 1986). It has been generally accepted that the factor Va heavy chain is mainly responsible for the cofactor activity. The heavy chain contains a binding region for prothrombin (Guinto and Esmon, 1984) and the light chain is involved in the interaction with membrane surfaces. Both heavy and light chain are required for binding to factor Xa (Guinto and Esmon, 1984). The location of the lipid binding site on the factor Va light chain is a matter of controversy. In bovine factor Va, the lipid binding site has been localized in the A3 domain to the amino acid residues 1667-1765 (Kalafatis *et al.*, 1990). Analysis of deletion mutants of recombinant human factor V showed that only deletion of the C2 domain resulted in a considerable loss of the phospholipid binding properties of factor Va (Ortel *et al.*, 1992).

Factor VIII is a single-chain glycoprotein of 265,000 Da, which shows marked sequence homology with factor V and ceruloplasmin (Church *et al.*, 1984; Jenny *et al.*, 1987; see above). In plasma factor VIII circulates in complex with the carrier protein, von Willebrand factor (Hoyer, 1981). Factor VIII has been purified from human, porcine and bovine plasma. The cDNA sequence for factor VIII has been determined (Vehar *et al.*, 1984; Toole *et al.*, 1984). This cDNA codes for a peptide of 2332 amino acid residues. In order to exert its cofactor function, factor VIII must also be activated during coagulation. Factor VIIIa can be produced through limited proteolysis by thrombin or factor Xa (Figure 2). Factor VIIIa exerts effects in factor IXa-catalyzed factor X activation similar to the effects of factor Va in prothrombin activation. Bound to the membrane surface, factor VIIIa enhances the assembly of factor IXa into the tenase (IXa-VIIIa) complex, and factor VIIIa increase the k_{cat} of Xa formation (van Dieijen *et al.*, 1981; Griffith *et al.*, 1982; van Dieijen *et al.*, 1985; Table 1).

Tissue factor, is an integral membrane protein expressed on the surface of subendothelial cells. It is a 53kDa glycoprotein, rich in carbohydrates (Broze *et al.*, 1985; Guha *et al.*, 1986). In contrast to factors V and VIII, tissue factor needs not to be activated in order to perform its function. Tissue factor has no proteolytic activity and is thought to serve as a receptor for factor VII. It was recently shown that the single-chain zymogen, factor VII is converted to two-chain factor VIIa in an autocatalytic manner following complex formation with tissue factor (Nakagaki *et al.*, 1991; Yamamoto *et al.*, 1992). The complexed factor VIIa activates factor X (Nemerson, 1988; Gemmel *et al.*, 1988) and factor IX (Østerud and Rapaport, 1977).

MEMBRANE SURFACE

Catalysis of reactions at membrane surfaces is a characteristic feature of the coagulation cascade (Rosing and Tans, 1988; Mann *et al.*, 1990). A suitable membrane surface is a surface containing negatively charged phospholipids (*i.e.* phosphatidylserine). These surfaces are provided by activated platelets and disrupted endothelial cells. The presence of membranes profoundly changes the kinetic parameters of coagulation factor activations and inactivations (see Table 1). The K_m for the substrate is greatly decreased in the presence of phospholipid membranes and appears to be a function of the phospholipid concentration present in the reaction medium. When the concentration of phospholipid is increased there is a parallel

increase of the K_m which is explained by the fact that the membrane-bound substrate molecules are diluted or bind to non-functional sites due to the increased amount of surface. Also the V_{max} of phospholipid-dependent reactions often appears to be a function of the phospholipid concentration. In general, the V_{max} increases with the amount of phospholipid present and reaches a maximum at high phospholipid concentrations. The lipid dependency of V_{max} is a reflection of the binding of the enzyme to the membrane surface which increases at increasing surface concentration until at high surface concentration all enzyme is bound and participates in coagulation enzymatic reactions. Another aspect of membranes is the receptor function of cofactors. All coagulation proteins bind to negatively charged membrane surfaces. The cofactors factor Va and factor VIIIa have relatively low dissociation constants (in the order of 0.1 nM; Pusey *et al.*, 1982; Kop *et al.*, 1989; Andree, 1992), thus tend to bind with relatively high affinity. The vitamin K-dependent zymogens have dissociation constants 2 to 4 orders of magnitude higher, which suggest a 'receptor' function for the activated cofactors. Furthermore membrane bound molecules have an increasing probability of collision. Activated cofactors bound to a membrane, therefore can assemble faster with the appropriate enzyme. Enzyme(complex) and substrate also have a higher probability of collision. A probability of collision between reacting molecules in two dimensional space, as on a membrane surface, is one to two orders of magnitude greater than in three dimensional space (Berg and von Hippel, 1985; Giesen, 1992).

PLATELETS

Platelets play a major role in the initiation of the hemostatic process *in vivo*. Several receptors on its surface enable it to interact with components of the subendothelial matrix that become exposed when the vascular endothelium is damaged (*e.g.* collagen). Upon adhesion a sequence of metabolic events is triggered that cause platelets to aggregate into clumps sealing the damaged vessel. During this process, secretion of substances amplifying aggregation and blood coagulation occurs.

Platelets are non-nucleated discoid cells, 2 - 5 μm in diameter which originate from budding of giant polyploid megakaryocytes residing in the bone marrow. Immediately following adherence, the platelet changes shape from a discoid structure to a sphere with projecting pseudopodia, which interact with other platelets. Finally, flattening and discharge of the cytoplasmic granules occur, with the release of ATP, ADP, calcium, platelet factor 4 (PF4), fibrinogen, factor V, and

protein S (cofactor of protein C; see below), many of which amplify the aggregation process by causing other platelets to aggregate. Unactivated platelets show an asymmetric membrane composition with negatively charged phospholipids (*i.e.* phosphatidylserine) located inside. Upon activation the phospholipids in the membrane become scrambled. The exposed phosphatidylserine in the activated platelet membrane provide an excellent surface for the coagulation cofactors Va and VIIIa, and the assembly of prothrombinase and tenase complexes responsible for activation of prothrombin and factor X respectively. These surfaces also support the anticoagulant protein C pathway (see below).

ENDOTHELIAL VESSEL WALL

In health, there is a balance between the clot inhibiting and clot promoting activities of the endothelium so that detrimental intravascular clotting is prevented. Endothelial production of prostacyclin (PGI_2) prevents the aggregation of any adhering platelets. Endothelial cells constitutively express thrombomodulin at the cell surface and this thrombomodulin complexes with thrombin if any thrombin should form. Thrombin complexed with thrombomodulin loses its procoagulant properties (*i.e.* cleavage of fibrinogen, stimulation of platelets and activation of the factors V and VIII) and gains anticoagulant properties (activation of protein C). Activated protein C (APC) inactivates any activated factor V or factor VIII. The APC-mediated inactivation of factor Va and VIIIa on endothelial cells is accelerated by protein S. Endothelial cells also possess heparin-like molecules on their surface. These molecules act as binding sites for antithrombin III (ATIII) which inhibits especially thrombin and factor Xa. All these properties of endothelium help to prevent local thrombus formation. Endothelial cells can, however, also actively promote blood coagulation. This only occurs if there is some 'stress' to the endothelium, but it does not necessarily require endothelial cell disruption or death. If, for instance, endothelial cells are exposed to endotoxin or tumor necrosis factor (TNF), they actively produce tissue factor molecules which promote clotting activity via factor VII binding (extrinsic pathway). In addition, thrombomodulin is internalized. Also prostacyclin production may drop after serious endothelial stress, platelets may adhere and aggregate releasing platelet factor IV, which is an antiheparin and prevents (local) ATIII activity. The balance of clot preventing and promoting activities is in favor of clot prevention by a fully healthy and non-stressed endothelium.

CONTROL OF COAGULATION

Clearly, uncontrolled coagulation would be fatal, therefore, modulating mechanisms exist to control and limit intravascular coagulation. Two systems control coagulation. One of the systems involves the presence of serine protease inhibitors (serpins) in plasma (see Table 2). These proteins inactivate serine proteases by acting as a suicide substrate. The other system is the protein C pathway. The protein C pathway, involving activated protein C and its cofactor protein S, attenuate the activity of the coagulation pathway by the selective inactivation of factors Va and VIIIa (Esmon, 1984).

SERPINS

Table 2 summarizes the most important protease inhibitors characterized from human plasma. Antithrombin III appears to be the most significant inhibitor of the coagulation process (Travis and Salvesen, 1983). Antithrombin III reacts mainly with thrombin and factor Xa. It functions by acting as a substrate for the serine protease forming an irreversible complex with the protease and its function is considerably enhanced in the presence of heparin. Inhibition of the factor Xa/factor VIIa/tissue factor complex involves a plasma inhibitor named tissue factor pathway inhibitor (TFPI) (Rao and Rapaport, 1987; Broze, 1987; Rapaport, 1989). In addition, other inhibitors of the proteases in the coagulation cascade are found, i.e. α 2-macroglobulin, α 2-antiplasmin and C1 inhibitor (Travis and Salvesen, 1983).

Table 2. Properties of the major serine protease inhibitors (serpins) in plasma. Data taken from Travis J, and Salvesen GS, 1983; Tollfsen DM *et al.*, 1982; Heeb MJ *et al.*, 1989; Novotny WF *et al.*, 1989; Rapaport SI, 1991.

<i>Serine protease inhibitors</i>	Molecular weight	Plasma concentration (μ M)	Enzymes inhibited
Antithrombin II	58 000	2	Thrombin, Xa
C1 inhibitor	105 000	2	C1, Kallikrein
α 2 Macroglobulin	725 000	3.5	Thrombin, kallikrein
α 1 Antitrypsin	55 000	25	XIa, APC
α 2 Antiplasmin	67 000	1	Plasmin
Heparin cofactor II	66 000	1	Thrombin, Xa
TFPI	33 000	0.0025	TF/VIIa/Xa-complex
PCI	57 000	0.010	APC, XIa

PROTEIN C PATHWAY

Already in 1960, Seegers and coworkers described an inhibitor of blood coagulation thought to be derived from prothrombin and named it autoprotease II-A (Mammen *et al.*, 1960). In 1976, protein C was purified from bovine plasma (Stenflo, 1976) and was shown to be the zymogen of autoprotease II-A (Seegers *et al.*, 1976). Protein C is activated by thrombin when complexed with an endothelial cell membrane protein, thrombomodulin (Esmon *et al.*, 1982). It exerts its regulatory function by inactivating factors Va and VIIIa. The inactivation of these factors is accelerated by a protein cofactor for activated protein C, protein S (Walker, 1980). In recent years, however, doubt has arisen as to the cofactor function of protein S (see also below) and, recently, Dahlbäck *et al.* (1993) have provided evidence for the existence of another as yet unidentified cofactor for activated protein C.

The activated protein C pathway is regulated by an inhibitor of activated protein C (PCI) which has been demonstrated in plasma (Marlar and Griffin, 1980). This inhibitor forms heparin-dependent complexes with activated protein C when tested in a purified system (Suzuki *et al.*, 1984). Human activated protein C is also inhibited by α 1-antitrypsin (Heeb and Griffin, 1988; Heeb *et al.*, 1989). Both protein C inhibitors seem to be of physiological importance *in vivo* (Heeb and Griffin, 1988).

Protein C is a multidomain glycoprotein circulating in plasma as a precursor of a serine protease. Human protein C has an apparent molecular weight of 62,000 Da and a carbohydrate content of approximately 23 % (Kisiel, 1979). The concentration of human protein C in plasma is approximately 65 nM (Comp *et al.*, 1984; Sala *et al.*, 1984; Seligsohn *et al.*, 1984; Ikeda and Stenflo, 1985). Protein C has been purified from bovine and human plasma (Stenflo, 1976; Kisiel, 1979). The primary structure of human protein C was determined from the cDNA sequencing (Foster and Davie, 1984). In addition, the structure of the gene for human protein C has been determined (Foster *et al.*, 1985). Like the other vitamin K-dependent proteins, protein C is synthesized in the liver (Fair and Marlar, 1986).

As for the other vitamin K-dependent Gla-containing blood coagulation proteins, it has been shown that bovine protein C is capable of interaction with Ca^{2+} (Stenflo, 1976). It has been found that protein C with the Gla domain removed by limited proteolysis is much slower activated by thrombin bound to thrombomodulin on the surface of endothelial cells than is intact protein C (Esmon *et al.*, 1983). An

intact Gla domain is thus required for normal activation of protein C on cell surfaces, as well as for the protein to exert its anticoagulant function.

The anticoagulant effect of activated protein C has been described by several investigators (Marciniak, 1970; Kisiel *et al.*, 1977; Walker *et al.*, 1979; Seegers, 1981). Activated protein C has a narrow substrate specificity and exerts its anticoagulant effect by selectively inactivating factors Va and VIIIa (Kisiel *et al.*, 1977; Vehar and Davie, 1980). Factor Va and factor VIIIa degradation have been studied in detail (Kisiel *et al.*, 1977; Vehar and Davie, 1980; Marlar *et al.*, 1982; Suzuki *et al.*, 1983; Fulcher *et al.*, 1984; Odegaard and Mann, 1987; Chapter 5). Figure 2 shows the cleavage sites of activated protein C in the cofactors Va and VIIIa. Activated protein C inactivates factor Va by proteolytic cleavages in both chains of factor Va. The heavy-chain cleavages are probably the important ones in destroying the cofactor activity (Odegaard and Mann, 1987). Bovine factor Va inactivated by activated protein C loses its ability to bind to prothrombin and factor Xa (Guinto and Esmon, 1984). Cleavage of the Va light chain by APC in the bovine system appears to be much faster than in the human system.

Protein S is thought to act as a cofactor in the APC-catalyzed degradation of the factors Va and VIIIa (Walker, 1980; Walker *et al.*, 1987; Heeb and Griffin, 1988; Koedam *et al.*, 1988). The physiological significance of protein S is demonstrated by the fact that there is a relationship between deficiency of protein S and venous thromboembolism (Briet *et al.*, 1988; Walker, 1992). Protein S is a vitamin K-dependent protein, which is not a zymogen of a serine protease. The primary structure of human protein S has been determined (Lundwall *et al.*, 1986; Hoskins *et al.*, 1987). It has been reported that protein S is absolutely required for activated protein C to exert its anticoagulant function on the surface of activated bovine platelets (Harris and Esmon, 1985). Binding of protein S to human endothelial cells was reported recently to be required for expression of cofactor activity for activated protein C (Hackeng *et al.*, 1993). On human platelets, however, this absolute requirement has not been shown (Solymoss *et al.*, 1988; Tans *et al.*, 1990). In the past few years data have become available that indicate that protein S must at least also have other functions in the protein C pathway in addition to the cofactor function for activated protein C. *In vitro* experiments, for instance, show that protein S stimulate the inactivation of factor Va by APC maximally a factor 2 (Solymoss *et al.*, 1988; Tans *et al.*, 1990; Chapter 5.). Furthermore, factor Xa was shown to protect factor Va against proteolytic inactivation by activated protein C. Protein S

was found to abrogate this protective effect (Nesheim *et al.*, 1982; Jane *et al.*, 1991). Salem and coworkers reported anticoagulant properties of protein S in the absence of activated protein C (Michell *et al.*, 1988). This suggested a direct interaction between protein S and the prothrombinase complex. Recently, it was indeed demonstrated that protein S inhibits the prothrombinase complex via interaction with prothrombinase components (Heeb *et al.*, 1993).

Chapter 2

Purification of factor V and Va

Factor V is a single chain plasma glycoprotein that circulates in plasma as an inactive cofactor at a concentration of approximately 8 $\mu\text{g/ml}$ ($\sim 25 \text{ nM}$). Factor V is a rather labile protein and this has complicated the isolation of this factor from plasma for many years. By the end of the seventies, procedures were developed to isolate single chain factor V from bovine plasma (Nesheim *et al.*, 1979; Esmon 1979). This work opened the way to the isolation of the even more labile human factor V (Bolhuis *et al.*, 1979; Dahlbäck, 1980; Kane and Majerus, 1981; Katzmann *et al.*, 1981; Suzuki *et al.*, 1982). Factor V is activated by thrombin releasing two activation peptides of 71 and 150 kD. Factor Va is a heterodimer which consists of a 105kD heavy chain and a 71-74 kD light chain doublet. Guinto and Esmon (1984) have demonstrated that factor Va separated from factor V and the activation peptides by affinity chromatography over Prothrombin-Sepharose.

Isolation procedures of human factor V usually start with adsorption of plasma with bariumsulfate to remove vitamin K dependent coagulation proteins, this includes removal of prothrombin and factor X which are precursors of the factor V activators thrombin and factor Xa. The first step is followed by polyethylene glycol fractionation, ion exchange chromatography on QAE- or DEAE-based columns and gel filtration chromatography. Essential in the procedures is the abundant use of protease inhibitors. Most common is the use of benzamidine in a concentration of 10 mM. Furthermore, soybean trypsin inhibitor (inhibits factor Xa), phenylmethanesulfonyl fluoride (PMSF), and diisopropyl fluorophosphate (DFP) are frequently used. Since these inhibitors also interfere with factor V activation by thrombin or factor Xa they are omitted in the final step or removed from the final preparations via dialysis.

The purification procedures in general yield reasonable pure factor V preparations ($>80 - 85\%$). However, for highly purified factor V preparations the available procedures are time consuming and result in variable yields and quality of the final material. In this chapter, we describe a factor V and a factor Va purification method that we developed in order to overcome these difficulties. The factor V purification method we use is based on the procedure developed by Dahlbäck (1980) and Suzuki *et al.*, (1982). Changes made in this procedure resulted in a decrease of the isolation time, in higher yields, higher concentration of factor V and in much less variability and quality of the final preparation. The factor Va purification method we used is based on the method of Guinto and Esmon (1984).

EXPERIMENTAL PROCEDURES

Materials. Bovine serum albumin, chicken egg albumin (ovalbumin), egg-yolk PC, bovine brain PS, HEPES and Tris were purchased from Sigma Chemical Co., St. Louis, MO, USA. The chromogenic substrate S2238 and the thrombin inhibitor I-2581 were obtained from AB Kabi Diagnostica, Stockholm, Sweden. PPACK was from Calbiochem, La Jolla Ca. p-NPGB was purchased from Nutritional Biochemicals.

Proteins. Proteins used in this study were purified from fresh frozen human plasma. Prothrombin was purified according to DiScipio *et al.*, (1977). Thrombin was isolated from prothrombin activation mixtures in which purified prothrombin (20 mg) was activated at 37 °C in 10 ml of a buffer solution containing 50 mM Tris (pH 7.5 at 37 °C), 100 mM NaCl, 2 mM CaCl₂, 50 µM phospholipid vesicles (brain PS/egg-yolk PC; 20/80, mol/mol), 2.5 nM factor Xa, 40 nM factor Va and 20 µM I-2581. The reversible thrombin inhibitor I-2581 was present to prevent autocatalytic degradation of thrombin. Prothrombin activation was complete within 10 min after which the thrombin was purified from the reaction mixture as described by Pletcher and Nelsestuen (1982). Bovine factor Xa, which was used in the factor Va assay (see below), was purified as described by Fujikawa *et al.*, (1972). RVV-X was purified from the crude venom of Russell's viper by the method of Schiffman *et al.*, (1969). Protein preparations were homogeneous and >95% pure as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate according to Laemmli (1970).

Protein concentrations. Protein concentrations were routinely determined according to Lowry *et al.*, (1951) using BSA as a standard. Molar concentrations of thrombin and factor Xa were determined by active site titration with p-NPGB (Chase and Shaw, 1969; Smith, 1973). Concentrations of factor Va were determined kinetically as described for bovine factor Va by Lindhout *et al.*, (1982).

Phospholipid vesicle preparations. The phospholipid vesicles used in the factor Va assay were prepared from a mixture of 10 mole % brain-PS and 90 mole % egg PC. Vesicles were made by mixing the desired quantities of lipids, dissolved in CHCl₃/CH₃OH (1/1,v/v), in a glass test tube. After drying under mild N₂ flow, buffer containing 50 mM Tris-HCl (pH 7.9 at 20 °C) and 175 mM NaCl was added. After vigorous vortexing for 1 minute the formed liposomes were sonicated for 10 minutes at 4 °C using a MSE Mark II 150-W ultrasonic disintegrator set at 8 µm peak to peak amplitude. These vesicles were stored at -80 °C prior to use.

Preparation of Prothrombin-Sepharose. Bovine prothrombin (100 ml, 1 mg/ml) in buffer containing 0.1 M NaHCO_3 pH 9.0 and 0.5 M NaCl was coupled overnight at 4 °C to 4 g CNBr-activated Sepharose 4B. After several washing steps with alternating 1 M Ethanolamine pH 8.0 and 0.1 M Na-acetate buffer pH 5.0, 1 M NaCl, the gel was stored in 25 mM HEPES pH 7.5, 50 mM NaCl and 0.02% Na-azide. The final concentration on the gel was 6.5 mg prothrombin/ml gel. The efficiency of coupling was 97%.

Isolation of Human factor V. Factor V was purified by modification of the procedure as described by Dahlbäck (1980) and Suzuki *et al.*, (1982). Only plastic labware was used throughout the whole procedure. All buffer solutions used in the isolation procedure contained the irreversible thrombin inhibitor PPACK. The gel permeation chromatography on FPLC Superdex-200 was performed with a buffer solution that did not contain PPACK. The presence of PPACK was necessary to obtain reproducible preparations of homogeneous and single chain factor V (Factor Va was stable for several months at -80 °C and lost about 10% of its activity when kept on ice for 6 hours). The purification is essentially as follows: Six units fresh frozen citrate plasma (approximately 1.5 litre) were rapidly thawed in warm water while gently shaking and benzamidine, STI (Soybean Trypsin Inhibitor), and PPACK were added to a final concentration of 10 mM, 50 mg/l, and 2 μM respectively. The inhibitors were allowed to dissolve and react with proteases for ten minutes after which the mixture was transferred to 4 °C. All further steps were performed in the cold room at 4 °C. A 1 M BaCl_2 solution was added dropwise to the plasma to result in a final concentration of 74 mM BaCl_2 while stirring with a magnet bar. After 30 minutes the barium-citrate precipitate was removed by centrifugation for 10 minutes at 1250*g. The supernatant was brought to 7% polyethylene glycol 6000 by adding dropwise 250 ml of a 50% stock solution. The precipitate was removed by centrifugation (15 minutes at 3600*g) after which the supernatant was brought to 13% polyethylene glycol by adding dropwise 240 ml of the 50 % stock solution. The formed precipitate contained the factor V. The mixture was stirred for 60 minutes and centrifuged for 15 minutes at 3600*g and the supernatant was carefully discarded. The precipitate was dissolved in 350 ml DEAE-start buffer containing 20 mM Tris-HCl (pH 7.5 at 4 °C), 50 mM NaCl, 5 mM CaCl_2 , 10 mM benzamidine and 2 μM PPACK. The dissolved precipitate was centrifuged at 3600*g to remove all non-soluble particles and was loaded overnight on a DEAE-sepharose-CL-6B column (1.5 * 30 cm) equilibrated in DEAE-start buffer. After loading the column was rinsed with the same buffer containing 120

mM NaCl and 0.25 μ M PPACK until the A_{280} in the effluent had reached approximately the buffer value. The column was developed with a 300 ml linear gradient from 120 mM NaCl to 250 mM NaCl. Fractions of 6 ml were collected and screened for factor V cofactor activity (see below). Fractions with factor V activity were pooled. Solid ammonium sulphate was slowly added to the pooled fractions to a final amount of 70%. The ammonium sulphate precipitate was stirred for 1 hour, spun down for 15 minutes at 25000*g and dissolved in a small volume (2 - 3 ml) of HEPES buffer containing 10 mM HEPES pH 7.5, 50 mM NaCl. The dissolved ammonium sulphate precipitate was centrifuged for 15 minutes at 14000*g to remove insoluble material before loading on a FPLC Superdex-200 (16/60) column. The column was equilibrated and developed in HEPES buffer without PPACK or other protease inhibitors. Fractions of 1.0 ml were collected and tested for factor V activity. The fractions containing factor V activity were pooled and stored in aliquots at -80 °C.

Factor V preparations thus obtained were $\geq 85\%$ pure and single chain as judged by SDS-PAGE. Contaminations varied from preparation to preparation between 5%-15% (the most abundant contamination being a small protein running in the buffer front of a Laemmli SDS gel).

Chromatography of factor V on MonoS®. Factor V was chromatographed on a FPLC MonoS® column equilibrated with 25 mM HEPES pH 7.5, 50 mM NH_4Cl and 5 mM CaCl_2 . After loading of the factor V the column was washed with ten column volumes starting buffer. The column was subsequently developed with a 15 ml linear gradient from 50 mM NH_4Cl to 1 M NH_4Cl . Fractions of 1 ml were collected and screened for factor V activity (see below). Factor V preparations thus obtained were pure ($> 95\%$) and single chain as judged by SDS-PAGE (see also Figure 4).

Activation of human factor V. Factor Va was prepared by activation of factor V with 30 nM human thrombin for 30 minutes 10 mM HEPES pH 7.5, 50 mM NaCl, and 5 mM CaCl_2 . After activation, 30 nM PPACK was added to inhibit thrombin. Factor Va was either used as such or further purified by affinity chromatography on Prothrombin-Sepharose. To this end a Prothrombin-Sepharose column (0.5 * 5 cm) was loaded with thrombin activated factor V. The column was equilibrated and after sample application washed with a buffer containing 25 mM HEPES pH 7.5, 50 mM NH_4Cl and 5 mM CaCl_2 . The amount of factor Va loaded on the column ranged between 0.2 -1.0 mg factor Va. The column was developed with a 15 ml linear gradient from 50 mM NH_4Cl to 450 mM NH_4Cl . The eluted

factor Va was used directly or was stored at -80°C after addition of 5 mg BSA/ml.

Factor V assay. Factor V was activated by thrombin and assayed for cofactor activity (see below). In a typical experiment factor V was diluted to a concentration of approximately 1 nM in buffer containing 25 mM HEPES pH 7.5, 126 mM NaCl, 2.68 mM KCl, 5 mM CaCl_2 and 5 mg/ml BSA. To 90 μl of the diluted factor V 10 μl thrombin of a 60 nM stock solution was added. After 10 minutes incubation at 37°C an aliquot of 10 μl was taken from the mixture and assayed for its factor Va activity (see below).

Factor Va assay. Factor Va was determined via its cofactor activity in the activation of prothrombin by factor Xa. For convenience, bovine factor Xa was used in the assay. Amounts of factor Xa, phospholipid vesicles and prothrombin present in the assay were such that the rate of prothrombin activation was linearly dependent on factor Va, was constant in time, and was not influenced by small variations of the factor Xa, phospholipid and prothrombin concentrations. In a typical experiment factor Va was quantitated as follows: to 230 μl of a buffer solution (prewarmed at 37°C) containing prothrombin and phospholipid, 10 μl bovine factor Xa was added and after 15 s prothrombin activation was started with the addition of a 10 μl aliquot containing factor Va. Final concentrations reached in the reaction mixture were: 50 mM Tris (pH 7.9 at room temp), 175 mM NaCl, 2 mM CaCl_2 , 5 nM factor Xa, 50 μM phospholipid vesicles (10 mole% brain PS/90 mole % egg-yolk PC), 0.5 μM prothrombin, 0.5 mg/ml ovalbumin and the factor Va from the aliquot. Prothrombin activation in the assay mixture was determined by measuring the amount of thrombin formed after 1 min using the chromogenic substrate S2238 (Rosing *et al.*, 1980). The amount of thrombin generated in this assay was linearly dependent on factor Va (up to some 30 pM factor Va present in the assay mixture) and from a calibration curve made with known amounts of factor Va the amount of factor Va present in the reaction mixture could be calculated.

RESULTS and DISCUSSION

Isolation of human factor V. Factor V has been referred to in literature as 'Labile Factor'. This lability is caused by the fact that thrombin is a very efficient activator of factor V. When blood is taken from a donor small amounts of tissue factor or contact activation in the receiving container triggers the coagulation reaction. Although this is initially a rather slow process, it is absolute necessary to take appropriate measures to prevent further activation of the coagulation cascade during the isolation procedure otherwise all coagulation factors, including factor V, will become activated. In general, four measures are taken: First, blood is collected in citrate buffer, this slows down the activation of most coagulation proteins since these reactions are calcium dependent and the factors Va and VIIIa show an absolute requirement for calcium. Second, throughout the whole procedure plastic is used to prevent contact activation. Third, a cocktail of protease inhibitors is added to the plasma and kept in the buffers during the whole isolation procedure and, finally, almost the entire procedure is performed at 4 °C without interruption.

Table 1. Purification of human factor V from plasma. Human factor V was purified as described in EXPERIMENTAL PROCEDURES. Total protein was calculated from volume and A_{280} values using an $A_{280}^{1\%}$ of 10.0. Total unit values were obtained from measured cofactor Va activities as described in EXPERIMENTAL PROCEDURES (using a k_{cat} of 6000 for factor Va mediated prothrombin activation).

	volume (ml)	A_{280}	protein (mg)	total units $\text{mol} \cdot 10^{-9}$	specific activity nmol/mg	recovery %	purification -fold
plasma	1600	56	89600	20.7	0.00023	-	1
Ba-Citrate plasma	1650	56	92400	30.7	0.00033	100	1.4
7% PEG supernatant	1850	30	55500	33.3	0.00060	108	2.6
13% PEG precipitate (resuspended)	450	54.6	24600	27.1	0.0011	88	4.8
DEAE resuspended ($(\text{NH}_4)_2\text{SO}_4$ precipitate	4	58	232	13.1	0.056	43	244
Superdex	11	1.8	19.8	6.6	0.33	21	1443
MonoS	20.5	0.224	4.6	4.6	1.0	15	4329

Figure 1. Chromatography of human factor V on FPLC Superdex-200. 4 ml of dissolved ammoniumsulphate precipitate (precipitation was made after DEAE-Sepharose chromatography) was applied to a Superdex-200 column in a buffer containing 10 mM HEPES (pH 7.5), and 50 mM NaCl and eluted with the same buffer. Fractions of 1.0 ml were collected at a flow rate of 0.8 ml/min. **(A)** The absorbance at 280 nm was continuously recorded (solid line). Samples from the column fractions were incubated for 10 minutes at 37 °C with thrombin (final concentration 6 nM) and the factor Va generated (●) was assayed as described under EXPERIMENTAL PROCEDURES. **(B)** 20 μl samples were subjected to SDS-PAGE on 5% gels according to Schägger and von Jagow (1987). Protein was visualized with coomassie brilliant blue R250. The fractions applied to the gel are indicated in the figure. The starting material applied to the column is shown in lane 1. Fractions 28-32 were pooled and further chromatographed on MonoS as shown in figure 2.

Table 1 shows a typical example of the results obtained during the various steps of the factor V isolation procedure. The purification is started with a barium citrate adsorption. This removes the vitamin K-dependent proteins from the plasma and reduces the risk of formation of thrombin or other active coagulation factors during subsequent steps. As the vitamin K-dependent proteins are only a small percentage of the total amount of protein present in plasma, this step has no effect on the purification rather than a slight dilution in plasma. Although protein content (by A_{280}) and factor V activity are assayed in dilute samples, the inhibitors present may interfere somewhat with the measurements which may explain the apparent increase

Figure 2. Chromatography of factor V on monoS. 5 ml of purified factor V (5 mg) in 10 mM HEPES (pH 7.5), 50 mM NaCl was applied to a MonoS column (HR 5/5). The column was washed with 15 ml of a buffer containing 25 mM HEPES (pH 7.5), 50 mM NH_4Cl and 5 mM CaCl_2 and subsequently developed with 30 ml of a linear gradient from 50 to 1000 mM NH_4Cl in 25 mM HEPES (pH 7.5) and 5 mM CaCl_2 . Samples from the column fractions were incubated for 1 hour at 37 °C with thrombin (final concentration 25 nM) and the factor Va generated (●) was assayed as described under EXPERIMENTAL PROCEDURES.

Figure 3. Affinity chromatography of thrombin activated factor V on prothrombin-sepharose gel. A. Factor Va (4 ml 0.4 A_{280} in 10 mM HEPES (pH 7.5) and 50 mM NaCl) was applied to a prothrombin-sepharose column (0.5×5 cm). After sample application, the column was washed with 15 ml column buffer containing 25 mM HEPES pH 7.5, 50 mM NH_4Cl and 5 mM CaCl_2 . Elution was accomplished with a 15 ml linear gradient from 50 - 450 mM NH_4Cl in 25 mM HEPES (pH 7.5), 5 mM CaCl_2 . 1 ml fractions were collected during the gradient elution. The A_{280} signal is indicated by a solid line. Samples from the column fractions were assayed for factor Va activity (●) as described under EXPERIMENTAL PROCEDURES. The NH_4Cl gradient is indicated by the interrupted line.

in total protein and activity observed during the first few steps. The results obtained up to and including the DEAE-Sepharose are in reasonable agreement with the results reported for these steps by Dahlbäck and coworkers. The following step reported by these authors involves gel permeation chromatography over classical media such as AcA-34. The use of such columns is, however, very time consuming and in our hands resulted in variable yield and quality of the final factor V obtained. In order to improve on this, we used a Superdex-200 column mounted in a Pharmacia FPLC system. Figure 1 shows a typical elution pattern obtained together with a SDS slabgel of the various column fractions. Factor V pools thus obtained still contained some contaminants and were usually some 85-90% pure as judged by SDS-gel electrophoresis. When factor V was chromatographed over a Mono-S column on a FPLC system (Figure 2) these contaminants were lost and the specific activity increased some three-fold as compared to the material obtained from the Superdex column. This large increase in specific activity as compared to the relatively minor decrease in contaminants observed in SDS-gels (Table 1, cf. also Figures 1B and 4) suggests that the contaminating protein removed had a large extinction coefficient or that part of the protein band identified as factor V was in fact non-active protein. The purified factor V was stored at -80 °C without additions and was stable for at least several months as judged from its activity and by SDS-PAGE. The procedure described here has been repeated several times and each time preparations of comparable high quality were obtained.

Purification of factor Va on a Prothrombin-Sepharose column. Factor Va has been demonstrated to bind to immobilized prothrombin (Guinto and Esmon, 1984). We used bovine prothrombin covalently coupled to CNBr activated sepharose to separate human factor Va from its activation peptides. In our preparations the factor Va activities increased 80- to 100- fold after activation by thrombin which is 3-fold higher than the preparations reported by Kane and Majerus (1981) and Suzuki *et al.*, (1982). During loading and washing of the Prothrombin-sepharose column, small amounts of factor Va leaked from the column at low salt concentrations. After elution of the column with a linear gradient, approximately 60 - 70% of the initial factor Va activity was eluted from the Prothrombin-Sepharose at 200 mM NH₄Cl. This resulted in highly purified factor Va. An additional advantage of the use of this column is that small contaminations present in the initial factor V preparations are also lost after this step. Figure 3 shows a typical chromatogram of a factor Va purification on the Prothrombin-Sepharose column. Polyacrylamide gel analysis of the factor Va eluted from the Prothrombin-Sepharose column showed that no

activation peptides eluted in the gradient (not shown). Figure 4 shows an SDS-gel of the final preparations (factor V and factor Va) obtained using the procedures described here. Factor Va lost 10% of its activity after freezing and thawing. Presence of 5 mg/ml BSA proved to be essential.

Figure 4. *SDS gelelectrophoretic analysis of factor V and factor Va.* 20 μ l of final preparations of factor V after MonoS and factor Va after affinity chromatography were subjected to SDS-PAGE on 7.5% gels according to Schagger and von Jagow (1987). Protein was visualized with coomassie brilliant blue R250.

Protein C activation by an activator purified from the venom of *Agkistrodon halys halys*

ABSTRACT

The protein C activator from *Agkistrodon halys halys* venom has been purified 533-fold by ion-exchange chromatography on QAE-Sephadex A-50, affinity chromatography on aprotinin-Sepharose and Mono-Q fast protein liquid chromatography. The purified enzyme is a single chain protein with an apparent molecular weight of 36,000 that activates protein C by proteolytic removal of a small fragment from the heavy chain. The protein C activator exhibited a high amidolytic activity towards the tripeptide substrates D-Pro-Phe-Arg-pNA (S2302) and D-Phe-(pipecolyl)-Arg-pNA (S2238). The activity of the activator was not affected by thiolprotease or metalloprotease inhibitors. The activator was inhibited, however, by benzamidine, Phe-Pro-Arg chloromethyl ketone, *p*-nitrophenyl *p*-guanidinobenzoate and soy bean trypsin inhibitor, which classifies the enzyme as a serine protease. The purified protease was capable of activating both human and bovine protein C. Activation of human protein C only occurred at an appreciable rate in a calcium-free reaction medium at low ionic strength. Ca^{2+} ions inhibited the activation of human protein C with an apparent K_i of 0.8 mM. Addition of NaCl to the reaction medium also strongly inhibited human protein C activation (50% inhibition at 20 mM NaCl). Kinetic analysis of human protein C activation by the venom activator (in a calcium-free medium) revealed an apparent K_m for protein C of 0.52 μM and a k_{cat} of 0.17 s^{-1} at $I = 0.05$ ($k_{\text{cat}}/K_m = 3.3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$). At $I = 0.15$ rates of human protein C activation became linear with protein C indicating a strong increase in K_m with increasing ionic strength. Activation of bovine protein C was hardly affected by variation of Ca^{2+} and NaCl concentrations in the reaction medium. The apparent K_i 's for calcium ion and NaCl inhibition of bovine protein C activation were >10mM and 220 mM, respectively. At $I = 0.1$ and in the absence of Ca^{2+} ions bovine protein C was activated with a K_m of 0.056 μM and a k_{cat} of 0.24 s^{-1} ($k_{\text{cat}}/K_m = 4.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$). Our data are indicative for a rather large conformational and/or structural difference between human and bovine protein C at physiological ionic strength.

INTRODUCTION

Protein C is a vitamin K-dependent glycoprotein that circulates in the blood as the zymogen of the serine protease, activated protein C (Stenflo, 1976). Protein C (MW = 62,000Da) consists of a heavy chain (MW = 41,000Da) and a light chain (MW = 21,000Da) linked via a disulfide bridge (Kisiel, 1979). After activation protein C is converted into activated protein C (APC), which is an efficient inhibitor of blood coagulation whose anticoagulant properties have been attributed to its ability to inactivate factor V (Kisiel *et al.*, 1977; Walker *et al.*, 1979) and factor VIII (Vehar and Davie, 1980; Fulcher *et al.*, 1984; Fay *et al.*, 1982) and to promote fibrinolysis (Comp and Esmon, 1981). In vivo protein C activation occurs at the endothelial cell surface and is catalyzed by the thrombin-thrombomodulin complex (Esmon and Owen, 1981) which in case of human protein C removes a dodecapeptide from the aminoterminal end of the heavy chain (Kisiel, 1979).

Protein C is also activated by trypsin (Kisiel *et al.*, 1976), the factor X activator from Russell's viper venom (Kisiel *et al.*, 1976) and by proteases from the venoms of the Southern copperhead, *Agkistrodon contortrix contortrix* (Klein and Walker, 1986; Stocker *et al.*, 1987; Kisiel *et al.*, 1987a; Kisiel *et al.*, 1987b; Orthner *et al.*, 1988; Exner and Vaasjoki, 1988) and the tropical moccasin, *Agkistrodon bilineatus* (Nakagaki *et al.*, 1990). Finally, an activator from *Agkistrodon halys* was reported which displayed *in vivo* antithrombotic properties in a rat model that were attributed to protein C activation (Strukova *et al.*, 1989).

Protein C activation by non-physiological activators is of special interest since such activators can be used as a tool in studies on the structure-function relationship of protein C, in the preparation of APC from protein C and in the development of functional protein C assays. With respect to the latter application the protein C activator from *Agkistrodon contortrix contortrix* received considerable attention (Martinoli and Stocker, 1989). Initially conflicting reports appeared concerning the physical and functional properties of the *Agkistrodon contortrix contortrix* activator (Klein and Walker, 1986; Kisiel *et al.*, 1987a; Kisiel *et al.*, 1987b; Orthner *et al.*, 1988; Martinoli and Stocker, 1986). However, the primary structure that became available in 1989 showed the activator to be a serine protease with a MW of 31,000Da with a high degree of sequence identity to other snake venom (thrombin-like) proteases such as batroxobin, flavoxobin and the factor V activator from Russell's viper (McMullen *et al.*, 1989). Thus, it is now generally accepted that *Agkistrodon contortrix contortrix* venom contains a single protein C

activator which is a serine protease with an apparent molecular weight of 37,000-40,000Da as judged by SDS-PAGE and with an isoelectric point in the basic region (Kisiel *et al.*, 1987b; Orthner *et al.*, 1988; McMullen *et al.*, 1989; Meier *et al.*, 1988). The activator readily activates both human and bovine protein C in a reaction that is strongly inhibited by calcium ions (Kisiel *et al.*, 1987a; Orthner *et al.*, 1988) and (at least in the case of human protein C) by NaCl (Orthner *et al.*, 1988). This phenomenon is of special importance for the use of this activator in a quantitative protein C assay which because of this sensitivity requires well-controlled reaction conditions with respect to the ionic strength and the free CaCl_2 concentration.

During a screening of the pro- and anticoagulant actions of Central Asian snake venoms it was observed that the venom from *Agkistrodon halys halys* contained considerable anticoagulant activity (Sadykov *et al.*, 1985; Yukelson *et al.*, 1991). Preliminary experiments in bovine plasma and with bovine protein C indicated that part of the anticoagulant activity of the venom was due to the presence of a protein C activator. The activity of this venom activator on bovine protein C was neither affected by Ca^{2+} ions nor by increasing ionic strength. In this paper we report the purification of this activator which after further characterization appears to differ from the protein C activator from *Agkistrodon contortrix contortrix* with respect to its catalytic properties towards bovine protein C.

EXPERIMENTAL PROCEDURES

Materials - Crude lyophilized venom from *Agkistrodon halys halys* was supplied by the Institute of Zoology and Parasitology, Uzbek Academy of Sciences, Tashkent, Uzbekistan and was dissolved at a concentration of 25 mg/ml in 25 mM Tris/HCl (pH 7.5 at 4 °C), 50 mM NaCl, 1 mM EDTA. Non-soluble material was removed by centrifugation. The chromogenic substrates D-Phe-(piperidyl)-Arg-pNA (S2238), Glp-Pro-Arg-pNA (S2366), D-Pro-Phe-Arg-pNA (S2302), D-Val-Leu-Lys-pNA (S2251), Ile-Glu-Gly-Arg-pNA (S2222), Ile-Glu-(piperidyl)-Gly-Arg-pNA (S2337), N- α -Benzylcarbonyl-D-Arg-Gly-Arg-pNA (S2765), D-Ile-Pro-Arg-pNA (S2288) were obtained from Chromogenix (Mölnådal, Sweden). p-Nitrophenyl-p-guanidinobenzoate (p-NPGB) was from Nutritional Biochemicals, USA. N-Tosyl-Gly-Pro-Arg-NH-Np (Chromozym-TH) was from Boehringer, Mannheim, Germany. Agarose (Indubiose A37-HAA) was from IBF Biotechnics, France. Ovalbumin, bovine serum albumin, o-phenantroline, 2-iodoacetamide, n-ethylmaleimide, p-amidino-PMSF, benzamidin/HCl, soybean trypsin inhibitor and aprotinin were from Sigma Chemicals (St. Louis, MO, USA). D-Pro-Phe-Arg-CH₂Cl (PPACK) was from Calbiochem-Behring Corp, USA. Materials used for the purification of proteins were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Protac, a partially purified protein C activator preparation from *Agkistrodon contortrix contortrix*, was obtained from Pentapharm, Basel, Switzerland. Bovine protein C was a kind gift of Dr. R. Wagenvoort (Department of Biochemistry, CARIM, Maastricht).

Proteins - Human protein C was isolated from citrated human plasma as follows. A barium precipitate was prepared by adding 8 ml 1 M BaCl₂/100 ml plasma. The precipitate was collected by centrifugation, washed with 1/3 volume of 0.15 M BaCl₂, 10 mM benzamidin/HCl and resuspended in 1/3 vol 10% saturated NH₄SO₄. The precipitate was removed by centrifugation and the supernatant was subsequently brought to 40% NH₄SO₄ and then to 70% NH₄SO₄. The 40-70% NH₄SO₄ precipitate was dissolved in 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM benzamidin/HCl and dialysed extensively against the same buffer before it was passed through a column of C-9 Sepharose (1.3 mg anti-protein C monoclonal C-9 coupled/ ml sepharose). The column was washed with the same buffer containing 0.3 M NaCl till the effluent was essentially protein free. Protein C was then eluted with 0.1 M glycine (pH 2.45) and collected in Tris (pH 8.0) followed by dialysis against a suitable buffer. SDS-PAGE analysis of non-reduced and reduced samples

showed this protein C to be more than 95% pure. Molar concentrations were calculated by measuring the protein C antigen concentration by specific ELISA and using a MW of 56,000Da.

The protein C activator present in crude *Agkistrodon halys halys* venom was purified as follows. 722 mg crude venom dissolved in 35 ml 25 mM Tris/HCl (pH 7.5 at 4 °C), 50 mM NaCl, 1 mM EDTA was applied to a QAE-Sephadex column (2.5 x 21 cm) equilibrated in the same buffer and the column was washed with three column volumes 25 mM Tris/HCl (pH 7.5 at 4 °C), 100 mM NaCl, 1 mM EDTA. Venom proteins that adhered to the column were eluted with a linear salt gradient of 2 x 250 ml from 100 to 500 mM NaCl. Column fractions were tested for the presence of a protein C activator as described below. The activator adhered to the column and eluted in the gradient at about 200 mM NaCl. Fractions containing the protein C activator were pooled and dialysed against 20 mM Hepes (pH 7.5) and subsequently applied to an aprotinin-Sepharose column (35 mg aprotinin coupled to 10 ml CNBr-Sepharose according to the manufacturers instructions). The protein C activator bound to the aprotinin-Sepharose column which was then washed with five column volumes 20 mM Hepes (pH 7.5), 50 mM NaCl and developed with a linear salt gradient (2 x 40 ml) of 50-300 mM NaCl in the same buffer. The activator eluted at about 125 mM NaCl. The fractions containing protein C activator were pooled, dialysed against 25 mM Tris (pH 7.5 at room temp), 50 mM NaCl, 1 mM EDTA and subjected to ion-exchange chromatography on a Mono-Q column connected to an FPLC-system of Pharmacia. The activator adhered to the column and was eluted with a linear salt gradient of 50-300 mM NaCl. To remove the final contaminants the fractions containing activator were pooled, dialysed against 25 mM Tris (pH 7.5 at room temp), 50 mM NaCl, 1 mM EDTA and rechromatographed on Mono-Q. The column was washed with 10 column volumes start buffer and developed with a linear gradient of 50 to 150 mM NaCl (5 ml total volume). Elution with 150 mM NaCl containing buffer was continued during which the activator eluted as a single peak.

Protein C activation - During the purification of the *Agkistrodon halys halys* activator protein C activation was routinely determined as follows. 40 µl column fraction, appropriately diluted in 20 mM Hepes (pH 7.5), 1 mM EDTA, 0.5 mg/ml ovalbumin, was preincubated at 37 °C for 5 minutes and protein C activation was started by adding 10 µl of 1µM bovine protein C (prewarmed) in the same buffer. After an additional five minutes a 25 µl aliquot of the activation mixture was transferred to a cuvette with 475 µl of a buffer containing 50 mM Tris (pH 7.9 at

room temp), 175 mM NaCl, 20 mM EDTA, 0.5 mg/ml ovalbumin and 192 μ M S2366. From the $\Delta A_{405-500\text{ nm}}$ determined on a dual wavelength spectrophotometer the amount of activated protein C was calculated using the kinetic parameters for S2366 conversion by bovine APC given by the manufacturer.

Initial rates of protein C activation by the purified activator were determined in a total reaction volume of 250 μ l as follows. Varying amounts of protein C were preincubated for 5 minutes at 37 °C in a reaction buffer (composition given in the legends to the figures) and protein C activation was started by the addition of 10 μ l of purified activator in the same buffer. Aliquots of 25 μ l were removed at 1, 3 and 5 minutes from the reaction mixture and assayed for activated protein C with S2366 as described above. Quantitation of human APC was based on the kinetic parameters of S2366 conversion by human APC reported by Sala *et al.* (1984). Rates of protein C activation were linear with time and proportional to the amount of activator added and expressed in nM APC formed per minute. Further experimental details are given in the legends to the figures.

Gel electrophoresis - Electrophoresis of proteins was carried out as described by Laemmli (1970) on 10% polyacrylamide gels (6% stacking gel) in the presence of SDS. After electrophoresis the gels were stained for protein with Coomassie Brilliant blue R250. Zymographic detection of the protein C activator was carried out using the amidoblot procedure described earlier Tans *et al.*, 1989). To this end the activator was transblotted onto nitrocellulose and SDS was removed by soaking the nitrocellulose sheet for 3 hrs at room temperature in 25 mM Tris (pH 7.9), 90 mM NaCl containing 2.5% Triton X-100 followed by four washes (7.5 minutes) in 25 mM Tris (pH 7.9). The nitrocellulose sheet was subsequently placed on an agarose gel of 1.25% agarose in 25 mM Tris (pH 7.9), 1 mM EDTA, 480 μ M S2366 and 0.5 μ M bovine protein C. If there is protein C activator present on the nitrocellulose sheet APC is formed which liberates p-nitroaniline from the chromogenic substrate in the agarose and generates a yellow band on the nitrocellulose sheet. This band can be photographed by transillumination with UV-A light to achieve optimal contrast.

RESULTS

Purification of the protein C activator from Agkistrodon halys halys venom -

The protein C activator was purified from the crude venom by a combination of standard chromatographic techniques involving ion-exchange chromatography on QAE-Sephadex, affinity chromatography on aprotinin-Sepharose and repeated ion-exchange chromatography on a Mono-Q column connected to a Pharmacia FPLC system. Table 1 summarizes the purification procedure (details are given under Experimental Procedures), which resulted in 533-fold purification at a 13% overall yield.

Table 1. Purification of a protein C activator from the venom of *Agkistrodon halys halys*. Protein concentrations were calculated from the absorbance at 280 nm assuming $A_{280}^{1\%} = 10$. Further details are described under EXPERIMENTAL PROCEDURES and in the text.

	Protein ^a (mg)	Specific activity (nmole APC/min/mg)	Yield (%)	Purifi- cation (-fold)
crude venom	722	0.6	100	1
QAE Sephadex	41	3.5	33	5.8
Aprotinin Sepharose 11.4		12.0	31	20
MonoQ 1	0.9	98	20	163
MonoQ 2	0.17	320	13	533

Figure 1A shows a non-reduced SDS-polyacrylamide gel of the crude venom (lane 1), the purified activator (lane 2) and protein standards (lane 3). The purified activator appeared homogeneous and pure (>95%) and migrated as a single band with an estimated MW of 36,000Da. SDS-PAGE of reduced samples also showed a single band at 36,000Da indicating that the activator is a single chain protein (not shown).

The protein C activator on the gel was also identified by a modification of the amidoblot procedure described by Tans *et al.* (1989). The purified protein C activator was subjected to SDS-PAGE and transblotted onto nitrocellulose. The nitrocellulose sheet was washed and subsequently placed on a layer of agarose

containing protein C and the protein C-specific chromogenic substrate S2366 (Figure 1B). The appearance of a yellow band (i.e. paranitroaniline production from S2366 by APC generated in the agarose) at the migrating distance of the protein band (lane 2 Fig. 1A) indicates that the protein visible on the Coomassie blue stained gel and the protein C activator are one and the same protein. In this respect it should be mentioned that the appearance of the yellow band was not due to direct conversion of S2366 by the purified venom protein since omission of protein C from the agarose resulted in a complete loss of p-nitroaniline formation (data not shown).

Figure 1. SDS-PAGE analysis of the protein C activator from the venom of *Agkistrodon halys halys*. Crude *Agkistrodon halys halys* venom and purified activator were subjected to SDS-PAGE on 10% slabgels (6% stacking gel) according to Laemmli (1970) under non-reducing conditions. (A) Gel stained with Coomassie Brilliant Blue R250. Lane 1, 13 μ g crude venom; Lane 2, 4 μ g purified activator; Lane 3, molecular weight standards. (B) After electrophoresis a separate part of the gel containing a lane with 4 μ g purified activator was subjected to electrophoretic transfer of the protein onto nitrocellulose and visualization of the protein C activator with protein C and S2366 as described in EXPERIMENTAL PROCEDURES.

The product of protein C activation by the protein C activator - Human protein C is a protein with a molecular weight of 62,000 that consists of a heavy chain (41,000Da) linked via a disulfide bridge to a light chain (21,000Da). Activation of human protein C results from a specific cleavage in the heavy chain of the molecule (Kisiel, 1979; Kisiel *et al.*, 1987b; Nakagaki *et al.*, 1990). In the case of protein C activation by its physiological activator, thrombin this cleavage has been identified to result in the removal of a dodecapeptide of 1,400Da from the aminotermius of the heavy chain (Kisiel, 1979).

Figure 2 shows that the protein C activator purified from *Agkistrodon halys halys* venom very likely activates human protein C via the same pathway. Activation of protein C by our activator was accompanied by the cleavage of a small polypeptide from the heavy chain of protein C as indicated by the small increase in

Figure 2. SDS-PAGE of protein C activated by the activators from the venoms of *Agkistrodon halys halys* and *Agkistrodon contortrix contortrix*. Human protein C (16 µg) was activated in 100 µl of a reaction buffer containing 50 mM Tris/HCl (pH 7.9 at room temp), 0.5 mM EDTA and 0.08 µg of the purified activator from *Agkistrodon halys halys* or 0.12 µg Protac (partially purified activator from *Agkistrodon contortrix contortrix*). After complete reaction 20 µl aliquots were taken and subjected to SDS-PAGE both under non-reduced (lanes 1-3) and under reduced (lanes 4-6) conditions. A lane with molecular weight standards (identified as MW) was included on the gel. After electrophoresis the gel was stained with Coomassie Brilliant Blue R250. Lanes 1 and 4, protein C, Lanes 2 and 5, protein C activated with activator purified from the venom of *Agkistrodon halys halys*; Lane 3 and 6, protein C activated with Protac, the activator from *Agkistrodon contortrix contortrix*.

electrophoretic mobility of the heavy chain of protein C after complete activation. For comparison we have also electrophoresed the product of protein C activation by the activator from *Agkistrodon contortrix contortrix*, which appeared to be indistinguishable from the APC generated by the activator from *Agkistrodon halys halys*. It is, therefore, highly probable that activation of protein C by the activators from both *Agkistrodon* species is due to removal of the same dodecapeptide that is also removed by thrombin (Kisiel, 1979).

Chromogenic substrate conversion by the protein C activator - The purified protein C activator from the venom of *Agkistrodon halys halys* possesses amidolytic activity towards a number of commercially available synthetic peptide substrates (Table 2). High activities were observed on the kallikrein substrate S2302 and on the thrombin substrate S2238, whereas other substrates tested were converted at considerably lower rates. In Table 2 we have also included the rates of substrate conversion by the crude venom. From the fact that there was a considerable difference between the substrate specificities of the purified activator and the crude venom it can be concluded that the crude venom from *Agkistrodon halys halys*

Table 2. Amidolytic activities of crude *Agkistrodon halys halys* venom and of the purified protein C activator. Amidolytic activities were determined at 37 °C in 1 ml cuvettes (1 cm pathlength) on an Aminco DW2-C spectrophotometer set in the dual wavelength mode at 405 minus 500 nm. Final reaction conditions were: 50 mM Tris/HCl (pH 7.9 at room temp), 175 mM NaCl, 20 mM EDTA, 0.5 mg/ml ovalbumin, 192 μ M chromogenic substrate and appropriate amounts of crude venom or purified activator. From the $\Delta A_{405-500}/\text{min}$ the rate of p-nitroaniline formation was calculated using a molar extinction coefficient of 9900 $\text{cm}^{-1}\text{M}^{-1}$ Hemker (1983).

Substrate	Rate of substrate hydrolysis	
	Crude venom ($\mu\text{M pNA}/\text{min}/\text{mg}$)	Purified activator ($\mu\text{M pNA}/\text{min}/\text{mg}$)
S2238	0.371	0.703
Chromozym TH	0.346	0.062
S2222	0.036	0.029
S2337	0.051	0.009
S2765	0.039	0.011
S2366	0.129	0.027
S2302	4.024	1.290
S2288	0.271	0.048
S2251	0.286	0.043

contains other enzymes with amidolytic activity. Since the crude venom contains less than 0.2% protein C activator (as can be concluded from the 533-fold purification of the activator, Table 1) it can be calculated that the protein C activator has a negligible contribution to the amidolytic activity of the crude venom.

The effect of protease inhibitors on the activity of the protein C activator - Table 3 summarizes the effects of inhibitors of different classes of proteolytic enzymes on the activity of the protein C activator from *Agkistrodon halys halys*. Inhibitors of metallo- and thiol proteases did not affect the amidolytic activity of the protein C activator as determined with S2302. Considerable inhibition was observed when the protein C activator was incubated with the serineprotease inhibitors benzamidin, soybean trypsin inhibitor, PPACK and p-NPGB. The virtually complete inhibition by pNPGB and PPACK suggests that the protein C activator from *Agkistrodon halys halys* is a serine protease in which both histidine- and serine residues are involved in the catalytic mechanism.

Table 3. Inhibition of the purified *Agkistrodon halys halys* activator by protease inhibitors. 0.77 μ g Protein C activator purified from *Agkistrodon halys halys* venom was incubated for 1 hr at 37 °C in 450 μ l buffer containing 50 mM Tris/HCl (pH 7.9 at room temp), 175 mM NaCl, 0.5 mg/ml ovalbumin and inhibitor as indicated. The residual amidolytic activity was determined after 1 hr by adding 50 μ l 3.84 mM S2302 in water. The reaction with chromogenic substrate was allowed to proceed (usually between 5 and 25 minutes) until sufficient p-nitroanilide was formed for accurate measurement of the absorbance at 405-500nm after which 300 μ l 1 M citric acid was added to stop p-nitroaniline formation. From the measured absorbance the amidolytic activity was calculated as $\Delta A_{405-500}$ nm/min. The percentage remaining activity was obtained by taking the activity measured in the absence of inhibitors as 100%.

Inhibitor		% activity
none		100
o-phenantrolin	(4 mM)	94
EDTA	(10 mM)	107
2-iodoacetamide	(1 mM)	99
n-ethylmaleimide	(1 mM)	97
p-amidino PMSF	(100 μ M)	98
antithrombin III	(60 nM)	117
benzamidin	(10 mM)	48
soybean trypsin inhibitor	(10 mg/ml)	66
p-NPGB	(300 μ M)	6
PPACK	(50 μ M)	4

Figure 3. Effect of NaCl on the rate of protein C activation by the purified activator from *Agkistrodon halys halys* venom. Protein C was preincubated in 240 μ l reaction buffer for 5 minutes at 37°C after which reaction was started with the addition of 10 μ l buffer containing purified *Agkistrodon halys halys* activator. Final concentrations of reactants were: 50 mM Tris/HCl (pH 7.9 at room temp), NaCl as indicated in the figure, 5 mM EDTA, 0.5 mg/ml ovalbumin, 130 nM human protein C and 25 ng purified activator (●) or 150 nM bovine protein C and 5 ng activator (□). After 1, 3 and 5 minutes 25 μ l aliquots were removed from the reaction mixture and assayed for activated protein C using the chromogenic substrate S2366 as described under EXPERIMENTAL PROCEDURES. From the amounts of activated protein C thus determined the initial rate of protein C activation was calculated. The rate of protein C activation determined in the absence of NaCl was taken as 100% (5.9 nM human APC formed per minute and 5.4 nM bovine APC formed per minute).

Figure 4. The effect of CaCl_2 on the rate of protein C activation by the activator purified from the venom of *Agkistrodon halys halys*. Protein C was preincubated in 240 μ l reaction buffer for 5 minutes at 37 °C after which reaction was started with the addition of 10 μ l buffer containing purified *Agkistrodon halys halys* activator. Final concentrations of reactants were: 50 mM Tris/HCl (pH 7.9 at room temp), 10 mM NaCl, 0.5 mM EDTA, 0.5 mg/ml ovalbumin, 130 nM human protein C with 25 ng activator (●) or 150 nM bovine protein C with 4 ng activator (□) and amounts of CaCl_2 in excess over EDTA to obtain the concentrations indicated in the figure. After 1, 3 and 5 minutes 25 μ l aliquots were removed from the reaction mixture and assayed for activated protein C using the chromogenic substrate S2366 as described under Material and Methods. From the amounts of activated protein C present the initial (steady-state) rate of protein C activation was calculated and expressed as percentage of the rate determined in the absence of added CaCl_2 (5.7 nM human APC formed per minute and 4.2 nM bovine APC formed per minute).

Effect of CaCl₂ and NaCl on the activation of protein C - During our studies it became clear that the activator isolated from the venom of *Agkistrodon halys halys* activated both bovine- and human protein C. In this respect it resembles the protein C activator from the venom of *Agkistrodon contortrix contortrix*. Our protein C activator differs, however, in one important aspect from the latter activator. Activation of both bovine- and human protein C by the activator from *Agkistrodon contortrix contortrix* is strongly inhibited by Ca²⁺ ions (Kisiel *et al.*, 1987; Orthner *et al.*, 1988) and (at least for human protein C) by NaCl (Orthner *et al.*, 1988). When we tested protein C activation with Protac (the activator from *Agkistrodon contortrix contortrix* venom) under our experimental conditions, both bovine and human protein C activation by Protac were strongly inhibited by CaCl₂ ($K_{i \text{ app}} < 1 \text{ mM}$) and by NaCl ($K_{i \text{ app}} \approx 50 \text{ mM}$) (data not shown). However, Ca²⁺ ions and increasing ionic strength had different effects on the activation of bovine and human protein C by the *Agkistrodon halys halys* protein C activator. Figure 3 shows that activation of human protein C by the activator from *Agkistrodon halys halys* was strongly inhibited at increasing NaCl concentrations ($K_{i \text{ app}} = 20 \text{ mM}$) whereas the activation of bovine protein C was inhibited at much higher NaCl concentrations ($K_{i \text{ app}} = 220 \text{ mM}$). A similar difference between bovine and human protein C activation was also observed when the effect of Ca²⁺ ions was studied (Figure 4). Activation of human protein C was already inhibited at low Ca²⁺ concentrations ($K_{i \text{ app}} = 0.8 \text{ mM}$), whereas bovine protein C activation was even slightly stimulated at this calcium concentration. At much higher Ca²⁺ concentrations there was some inhibition of bovine protein C activation by the activator from *Agkistrodon halys halys*. The $K_{i \text{ app}}$ for inhibition by Ca²⁺ ions was, however, much higher than 10 mM.

Kinetic parameters of protein C activation by the protein C activator - To assess the efficiency by which the purified *Agkistrodon halys halys* enzyme activated human protein C we determined the kinetic parameters of protein C activation by measuring initial rates of activation at varying protein C concentrations. The reaction appeared to be saturable with respect to protein C (Figure 5A) and obeyed Michaelis-Menten kinetics as evidenced by the straight Lineweaver-Burk plot (Figure 5B). The latter plot yields a V_{max} of 14.2 nM protein C activated per minute and K_m for human protein C of 0.52 μM . From the concentration of activator present in this experiment (0.051 $\mu\text{g/ml}$) and the molecular weight of the activator (36,000) it can be calculated that the k_{cat} is 0.17 s^{-1} and $k_{\text{cat}}/K_m = 3.3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$. These kinetic parameters were determined in a

Figure 5. Kinetic analysis of human protein C activation by the activator purified from the venom of *Agkistrodon halys halys*. Varying amounts of human protein C were incubated for 5 minutes at 37 °C in 140 μ l buffer after which reaction was started by addition of 10 μ l purified *Agkistrodon halys halys* activator. Final reaction conditions were: 50 mM Tris/HCl (pH 7.9 at room temp), 0.5 mM EDTA, 1 mg/ml ovalbumin, 7.7 ng purified activator and amounts of protein C as indicated in the Figure. The initial rate of protein C activation was determined as described in the legend to figure 3. (A) rate of protein C activation as a function of the protein C concentration. (B) Lineweaver-Burk plot of the same data. Further details are given under EXPERIMENTAL PROCEDURES.

Table 4. Kinetic parameters of protein C activation by the activator purified from *Agkistrodon halys halys* venom. Kinetic parameters of protein C activation were obtained at 37 °C as described under EXPERIMENTAL PROCEDURES and under the legend to Figure 5. In the case of human protein C activation at $I = 0.15$ rates of APC formation were linear with respect to protein C concentration and from the observed reaction rates the second-order rate constant, which equals k_{cat}/K_m , was calculated. Further details are given in the text.

Substrate	k_{cat} ($s^{-1} \pm s.e.$)	K_m ($\mu M \pm s.e.$)	k_{cat}/K_m ($M^{-1}s^{-1} \pm s.e.$)
Human protein C			
$I = 0.05$	0.17 ± 0.007	0.52 ± 0.04	$3.3(\pm 0.4) \times 10^5$
$I = 0.15$	n.d.	n.d.	$3.4(\pm 0.3) \times 10^4$
Bovine protein C			
$I = 0.10$	0.24 ± 0.006	0.056 ± 0.004	$4.3(\pm 0.4) \times 10^6$

reaction medium that did not contain NaCl. It was not possible to obtain kinetic parameters at high NaCl concentrations since we were not able to saturate the activator with human protein C ($K_m > 2 \mu\text{M}$) and from the observed rate of APC formation the second-order rate constant (k_{cat}/K_m) was calculated to be $4.3 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ at 50 mM NaCl.

The activation of bovine protein C has more favorable kinetic parameters. At 50 mM NaCl a V_{max} of 10.4 nM APC/min and a K_m of 0.056 μM were obtained at 0.026 $\mu\text{g/ml}$ activator which yields a k_{cat} of 0.24 s^{-1} and a k_{cat}/K_m of $4.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$. The latter value indicates that at 50 mM NaCl bovine protein C is activated with a 100-fold higher catalytic efficiency than human protein C. The kinetic parameters obtained in the experiments discussed above are summarized in Table 4.

DISCUSSION

In the present paper we have described the purification and the characterization of a protein C activator from the venom of *Agkistrodon halys halys*. The activator is a single chain protein with an apparent molecular weight of 36,000. The protein C activator and the purified protein were positively identified to be one and the same protein by transblotting the purified protein after SDS-PAGE to nitrocellulose and subsequently visualizing of the protein C activator on the nitrocellulose with protein C and the protein C-specific substrate S2366 (Figure 1). This positive identification is important since in case of the protein C activator from *Agkistrodon contortrix contortrix* there has been some discussion about the molecular weight of the activator and the number of activators present in the venom (cf. refs Klein and Walker, 1986 and Orthner *et al.*, 1988). Moreover, in our case protein C-activating activities eluted in single peaks during the various steps of the purification procedure and thus we did not find evidence that *Agkistrodon halys halys* venom might contain more than one protein C activator.

The protein C activator from *Agkistrodon halys halys* hydrolysed several tripeptide-p-nitroanilide substrates and its activity was readily inhibited by p-NPGB and PPACK which identifies the activator as a serine protease. The activator converted both human and bovine protein C into APC by removal of a small polypeptide fragment from the heavy chain of protein C. It is very likely that protein C activation by the venom activators from different *Agkistrodon* species is the result of cleavage of the same peptide bond in protein C that is also cleaved by thrombin (Kisiel, 1979) yielding products with the same functional activity.

Whereas the rate of hydrolysis of tripeptide-p-nitroanilide substrates by the activator was not affected by variation of the Ca^{2+} and NaCl concentrations in the reaction medium there was considerable inhibition of human protein C activation in activation mixtures which contained Ca^{2+} ions or high NaCl concentrations. Fifty percent inhibition of human protein C activation by the venom activator was observed at 0.8 mM CaCl_2 and 20 mM NaCl, respectively. Kinetic studies at low ionic strength ($I = 0.05$) in the absence of Ca^{2+} ions revealed an apparent K_m for human protein C of 0.52 μM and a k_{cat} of 0.17 s^{-1} from which a catalytic efficiency (k_{cat}/K_m) of $3.3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ can be calculated. Increasing the ionic strength to 0.1 caused a 10-fold decrease of k_{cat}/K_m ($4.3 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) which is at least partially due to an effect of ionic strength on the K_m for protein C which becomes $> 2 \mu\text{M}$.

The properties discussed thus far indicate that the protein C activator from

Agkistrodon halys halys is remarkably similar to the protein C activators from *Agkistrodon contortrix contortrix* (Kisiel *et al.*, 1987a; Kisiel *et al.*, 1987b; Orthner *et al.*, 1988) and *Agkistrodon bilineatus* (Nakagaki *et al.*, 1990) with respect to molecular weight, catalytic efficiencies of human protein C activation and the inhibitory effects of Ca^{2+} ions and high NaCl concentrations. Differences between the amidolytic activities on tripeptide-p-nitroanilide substrates and different sensitivities towards serine protease inhibitors shows, however, that there are still some differences between the protein C-activating proteases from the various *Agkistrodon* species. Such intra and subspecies variation of the structure and function of venom components are very typical for snakes belonging to the genus *Agkistrodon* (Chippaux *et al.*, 1991).

A major difference between the protein C activators from *Agkistrodon halys halys* and *Agkistrodon contortrix contortrix* was observed in studies of bovine protein C activation. In the case of the activator from *Agkistrodon contortrix contortrix* activation of both human and bovine protein C were strongly inhibited by Ca^{2+} ions and by high NaCl concentrations under our experimental conditions. Human protein C activation by the activator from *Agkistrodon halys halys* was also very sensitive to the presence of Ca^{2+} ions ($K_i = 0.8 \text{ mM}$) and to the NaCl concentration ($K_i = 20 \text{ mM}$). However, with bovine protein C much higher CaCl_2 and NaCl concentrations are required in order to observe inhibition (*cf.* Figs 3,4). These observations indicate that there are considerable differences between human and bovine protein C on one hand and between the venom protein C activators on the other hand.

Orthner *et al.* (1988) advanced the hypothesis that Ca^{2+} ions and NaCl change the conformation of protein C in such a way that it becomes a less favorable substrate for the protein C activator. Considering the experiments reported in the present paper and pursuing the proposal of Orthner and coworkers results in a complex picture with different conformations for human and bovine protein C at high Ca^{2+} and NaCl that are recognised by one activator (*Agkistrodon halys halys*) and not by the other activator (*Agkistrodon contortrix contortrix*). It is, however, also possible that other effects contribute to the observed calcium and ionic strength dependence of venom-catalyzed protein C activation. Strong effects of ionic strength on chemical reactions are often taken to implicate that ionic (electrostatic) forces play an essential role in the interactions between the reacting substances. In the case of protein C activation by the venom activators this would mean that ionic interactions between protein C and the venom activator may have an important

contribution to the formation of the enzyme-substrate complex and the subsequent proteolysis. Considering the properties of protein C and the venom activators from the various *Agkistrodon* species this will likely be an ionic interaction between a negatively charged domain on protein C and a positively charged protein domain on the venom activator. Such an interaction will be prevented both at increased ionic strength and also by Ca^{2+} ions, since this will reduce the negative charge of protein C by binding to the γ -carboxyglutamic acid residues. Depending on the net electrostatic charges of the protein domains involved in the interactions between protein C and the activator this may result in different Ca^{2+} ion and ionic strength effects on reactions between protein C's and venom activators from different species. In this respect it is interesting to mention that there is a high degree of divergence in the amino acid sequences of human and bovine protein C around the peptide bond that is cleaved during protein C activation (Foster and Davie, 1984). It is obvious, however, that more detailed studies will be required to gain more insight in these differences between human and bovine protein C.

Finally we would like to emphasize that it is of interest to study the functional properties of the protein C activators present in the venoms of other *Agkistrodon* species. Our experiments indicate that these protein C activators may exhibit a number of important functional differences. It is, therefore, possible that other *Agkistrodon* species contain a protein C activator whose activity on human protein C is not inhibited by Ca^{2+} ions and high NaCl concentrations. Such an activator will be the preferred enzyme in a diagnostic test for the quantitation of plasma protein C.

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Chapter 4

The effect of phospholipids, calcium ions and protein S on rate constants of human factor Va inactivation by activated human protein C

ABSTRACT

Rate constants for human factor Va inactivation by activated human protein C (APC) were determined in the absence and presence of Ca^{2+} ions, protein S and varying concentrations of phospholipid vesicles of different lipid composition. APC-catalyzed factor Va inactivation in free solution (in the presence of 2 mM Ca^{2+}) was studied under first order reaction conditions with respect to both APC and factor Va and was characterized by an apparent second order rate constant of $6.1 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$. Stimulation of APC-catalyzed factor Va inactivation by phospholipids was dependent on the concentration and composition of the phospholipid vesicles. Optimal acceleration (230-fold) of factor Va inactivation was observed with 10 μM phospholipid vesicles composed of 20 mole% dioleoylphosphatidylserine (DOPS) and 80 mole% dioleoylphosphatidylcholine (DOPC). At higher vesicle concentrations and at higher mole fractions DOPS some inhibition of APC-catalyzed factor Va inactivation was observed. Membranes that contained anionic phospholipids other than phosphatidylserine also promoted factor Va inactivation. The ability of different anionic lipids to enhance factor Va inactivation increased in the order phosphatidylethanolamine < oleic acid < phosphatidic acid < phosphatidylglycerol < phosphatidylmethanol < phosphatidylserine. APC-catalyzed factor Va inactivation in the presence of phospholipid vesicles could be saturated with respect to factor Va and the reaction obeyed Michaelis-Menten kinetics. Both the K_m for factor Va and the $V_{\max}$ of factor Va inactivation were a function of the phospholipid concentration. The K_m increased from 1 nM at 2.5 μM phospholipid (DOPS/DOPC; 20/80, mol/mol) to 65 nM at 250 μM phospholipid. The $V_{\max}$ increased from 20 nM factor Va inactivated/min/nM APC at 2.5 μM phospholipid to 62 nM factor Va inactivated/min/nM APC at 10 μM phospholipid and remained constant at higher phospholipid concentrations. Protein S appeared to be a rather poor stimulator of APC-catalyzed factor Va inactivation. Protein S-dependent rate

enhancements were only observed in reaction mixtures that contained negatively charged phospholipid vesicles. Independent of the concentration and the lipid composition of the vesicles, protein S caused a 2-fold stimulation of APC-catalyzed factor Va inactivation. This suggests that, in the human system, enhancement of APC binding to phospholipid vesicles by protein S is of minor importance. Considering that protein S is a physiologically essential antithrombotic agent, it is likely that other factors or phenomena contribute to the *in vivo* antithrombotic action of protein S.

INTRODUCTION

Protein C and protein S are vitamin K-dependent plasma proteins that play an important role in the feedback regulation of thrombin generation (Esmon, 1989). The physiological importance of protein C and protein S is demonstrated by patients who are deficient in one of these proteins and who are suffering from recurrent thrombosis (Griffin *et al.*, 1981; Branson *et al.*, 1983; Schwarz *et al.*, 1984; Seligsohn *et al.*, 1984; Comp *et al.*, 1984; Griffin, 1988). Protein C circulates in the blood as the zymogen of a proteolytic enzyme which is activated by the thrombin/thrombomodulin complex (Esmon and Owen, 1981). Activated protein C (APC) is a 57 kD serine protease with specific proteolytic activity toward factor Va and factor VIIIa (Walker *et al.*, 1979; Vehar and Davie, 1980; Marlar *et al.*, 1982; Fulcher *et al.*, 1984). Inactivation of factor Va is accompanied by limited proteolysis of the factor Va heavy chain (Suzuki *et al.*, 1983; Odegaard and Mann 1987, Van de Waart *et al.*, 1984). The activity of factor VIIIa is also lost upon cleavage of its heavy chain by APC (Vehar and Davie, 1980; Fulcher *et al.*, 1984).

Optimal expression of the anticoagulant activity of APC requires the presence of the protein cofactor, protein S, and negatively charged phospholipid membranes and calcium ions. Protein S was first isolated by DiScipio *et al.*, (1977) as a new vitamin K-dependent protein which was later proposed to be a cofactor for APC (Walker 1980). The precise mechanisms of action of protein S in the APC reaction or as an antithrombotic factor are not yet clear. Proposed functions for protein S include: 1) promotion of APC binding to phospholipid membranes (Walker, 1981) and bovine platelets (Harris and Esmon, 1985) and 2) abrogation of the protection of factor Va by factor Xa from APC-catalyzed inactivation (Solymoss *et al.*, 1988).

Negatively charged membranes are important in coagulation reactions by providing a surface that promotes the formation of functional enzyme-substrate complexes which considerably enhances the rates of coagulation factor activation (Rosing and Tans, 1988; Mann *et al.*, Blood 1990). The inactivation of factor Va by APC was also reported to be accelerated by phospholipids which indicates that this reaction is also a membrane-associated process (Walker *et al.*, 1979; Marlar *et al.*, 1982; Walker, 1981; Van de Waart, 1984; Solymoss *et al.*, 1988).

The present study concerns a detailed kinetic analysis of APC-catalyzed factor Va inactivation. We have determined the apparent second order rate constants of factor Va inactivation both in the absence and presence of protein S and varying concentrations of phospholipid vesicles of different lipid composition. The kinetic

parameters (K_m and V_{max}) of factor Va inactivation by APC were measured as a function of the phospholipid concentration in order to get more information on the mode of interaction of the components of the protein C pathway.

EXPERIMENTAL PROCEDURES

Materials - Bovine serum albumin, chicken egg albumin (ovalbumin), oleic acid, dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), dioleoyl-*sn*-glycero-3-phosphomethanol (DOPM), egg-yolk PC, brain PS, HEPES, MES and Tris were purchased from Sigma Chemical Co., St. Louis, MO, USA. The chromogenic substrates S2337, S2238 and S2366 and the thrombin inhibitor I-2581 were obtained from AB Kabi Diagnostica, Stockholm, Sweden. PPACK was from Calbiochem, La Jolla Ca. p-NPGB was purchased from Nutritional Biochemicals

Proteins - Proteins used in this study were purified from fresh frozen human plasma. Prothrombin and factor X were purified according to DiScipio *et al.*, (1977). α -thrombin was isolated from prothrombin activation mixtures in which purified prothrombin (20 mg) was activated at 37 °C in 10 ml of a buffer solution containing 50 mM Tris (pH 7.5 at 37 °C), 100 mM NaCl, 2 mM CaCl₂, 50 μ M phospholipid vesicles (brain PS/egg-yolk PC; 20/80, mol/mol), 2.5 nM factor Xa, 40 nM factor Va and 20 μ M I-2581. The reversible thrombin inhibitor I-2581 was present to prevent autocatalytic degradation of α -thrombin. Prothrombin activation was complete within 10 min after which the α -thrombin was purified from the reaction mixture as described by Pletcher and Nelsestuen (1982). Human protein C was prepared, activated and assayed as previously described by Gruber *et al.*, (1989). APC was >90% activated, as judged by SDS-polyacrylamide gel electrophoresis. The anticoagulant activity of APC (250 U/mg) was equivalent to that of purified plasma protein C activated with Protac (Gruber *et al.*, 1989). Human protein S was obtained from Enzyme Research Laboratories, South Bend, IN, USA. A reference protein S was purified following barium adsorption of normal plasma as described (Dahlback, 1983). Factor Xa was obtained from purified factor X after activation with RVV-X and was isolated from the activation mixture by affinity chromatography on soybean trypsin inhibitor-Sepharose as described by Bock *et al.*, (1989). Bovine factor Xa, which was used in the factor Va assay (see below), was purified as described by Fujikawa *et al.*, (1972). RVV-X was purified from the crude venom of Russell's viper by the method of Schiffmann *et al.*, (1969).

Factor V was purified essentially as described by Dahlbäck (1980) and by Suzuki *et al.*, (1982). To fresh frozen human plasma the irreversible thrombin inhibitor PPACK was added to a final concentration of 2 μ M. All buffer solutions used in the isolation procedure contained 250 nM PPACK. The final purification step (chromatography on AcA-34) was performed with a buffer solution that did not

contain PPACK. The presence of PPACK was necessary to obtain reproducible preparations of homogeneous and single chain factor V (>95%). Factor Va was prepared by activation of factor V with thrombin. Factor V (0.3 mg/ml) was incubated for 30 min with 30 nM thrombin in 50 mM Tris (pH 7.9 at 20 °C) 175 mM NaCl, 20% (v/v) glycerol and 5 mM CaCl₂. After activation, 30 nM PPACK was added in order to inhibit thrombin. The small amount of remaining PPACK did not interfere with the reactions carried out with the factor Va preparations. Factor Va was subsequently diluted 1/10 in 25 mM HEPES (pH 7.5 at room temperature), 126 mM NaCl, 2.68 mM KCl, 25 mM glucose and 5 mg/ml BSA and stored at -80 °C. Factor Va was stable for several months at -80 °C and lost about 10% of its activity when kept on ice for 6 hours.

Protein preparations were homogeneous and >95% pure as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate according to Laemmli (1970).

Protein concentrations - Protein concentrations were routinely determined according to Lowry *et al.*, (1951) using BSA as a standard. Molar concentrations of thrombin and factor Xa were determined by active site titration with p-NPGB (Chase and Shaw, 1969; Smith, 1973). The APC concentration was determined with S2366 using kinetic parameters reported by Sala *et al.*, (1984). Concentrations of factor Va were determined kinetically as described for bovine factor Va by Lindhout *et al.*, (1982).

Phospholipid and phospholipid vesicle preparations - 1,2-dioleoyl-sn-3-phosphoserine (DOPS), 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG), 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (PE) were prepared from 1,2-dioleoyl-sn-glycero-phosphocholine (DOPC) by enzymatic synthesis as described by Comfurius and Zwaal (1977).

Vesicles of varying composition were made by mixing the desired quantities of lipids, dissolved in CHCl₃/CH₃OH (1/1,v/v), in a small glass test tube. After drying under mild N₂ flow, 1 ml buffer containing 25 mM HEPES (pH 7.5), 126 mM NaCl, 2.68 mM KCl and 5 mM glucose was added. After vigorous vortexing for 1 minute the formed liposomes were sonicated for 10 minutes at 4 °C using a MSE Mark II 150-W ultrasonic disintegrator set at 8 µm peak to peak amplitude. Vesicles made of 20 mole % oleic acid and 80 mole % DOPC were vortexed and sonicated at 60 °C and kept at room temperature prior to use.

The phospholipid vesicles used in the factor Va assay were prepared from a mixture of 10 mole % brain-PS and 90 mole % egg PC and made in a buffer

containing 50 mM Tris-HCl (pH 7.9 at 20°C) and 175 mM NaCl. These vesicles were stored at -80 °C prior to use.

Factor Va assay - Factor Va was determined via its cofactor activity in the activation of prothrombin by factor Xa. For convenience, bovine factor Xa was used in the assay. Amounts of factor Xa, phospholipid vesicles and prothrombin present in the assay were such that the rate of prothrombin activation was linearly dependent on factor Va, was constant in time, and was not influenced by small variations of the factor Xa, phospholipid and prothrombin concentrations. In a typical experiment factor Va was quantitated as follows: to 230 μ l of a buffer solution (prewarmed at 37 °C) containing prothrombin and phospholipid, 10 μ l bovine factor Xa was added and after 15 s prothrombin activation was started with the addition of a 10 μ l aliquot containing factor Va. Final concentrations reached in the reaction mixture were: 50 mM Tris (pH 7.9 at room temp), 175 mM NaCl, 2 mM CaCl_2 , 5 nM factor Xa, 20 μ M phospholipid vesicles (10 mole% brain PS/90 mole % egg-yolk PC), 0.5 μ M prothrombin, 0.5 mg/ml ovalbumin and the factor Va from the aliquot. Prothrombin activation in the assay mixture was determined by measuring the amount of thrombin formed after 1 min using the chromogenic substrate S2238 (Rosing *et al.*, 1980). The amount of thrombin generated in this assay was linearly dependent on factor Va (up to some 60 pM factor Va present in the assay mixture) and from a calibration curve made with known amounts of factor Va the amount of factor Va present in the reaction mixture could be calculated.

Inactivation of factor Va by activated protein C - The rate of factor Va inactivation was determined in reaction mixtures of 350 μ l containing 10 mM HEPES (pH 7.5), 136 mM NaCl, 2.68 mM KCl, 5 mM glucose, 0.5% BSA and phospholipid vesicles, protein S and factor Va at the desired concentration. Incubations were carried out in flat-bottom polystyrene tubes with a diameter of 0.9 cm. Before the addition of APC, three 10 μ l aliquots were withdrawn to determine factor Va (see below). Inactivation of factor Va was started by adding APC to the reaction mixture. The decrease in factor Va activity was monitored in 10 μ l aliquots taken from the reaction mixture each 30 seconds over a period of 3.5 min. From the progressive decline in factor Va activity the rate of factor Va inactivation, expressed as nM factor Va inactivated (V_i) per min per nM APC was calculated. The amount of APC added to the reaction mixtures was chosen such that the rate of factor Va inactivation was linear in time (less than 20% factor Va became inactivated) and proportional to the amount of APC present.

Figure 1. The effect of Ca^{2+} ions on the initial rate of APC-catalyzed factor Va inactivation in free solution. Factor Va (1 nM) was inactivated by 2 nM APC at 37 °C in a buffer containing 25 mM HEPES (pH 7.5), 126 mM NaCl, 2.68 mM KCl, 5 mM glucose, 5 mg/ml BSA and concentrations of CaCl_2 indicated in the figure. Initial rates of factor Va inactivation were determined as described under experimental procedures and the inactivation rates were expressed as pM factor Va inactivated (pM Vi) per min per nM APC. If necessary the rates of factor Va inactivation were corrected for the spontaneous loss of factor Va activity occurring in the absence of APC.

Figure 2. APC-catalyzed factor Va inactivation in free solution as a function of the concentrations factor Va and APC. Factor Va was inactivated by APC at 37 °C in a buffer containing 25 mM HEPES (pH 7.5), 126 mM NaCl, 2.68 mM KCl, 5 mM glucose, 3 mM CaCl_2 and 5 mg/ml BSA at (A) concentrations of factor Va indicated in the figure and 9.6 nM APC or (B) 1 nM factor Va and concentrations of APC indicated in the figure. Initial rates of factor Va inactivation were determined as described under experimental procedures and the inactivation rates were expressed as nM factor Va inactivated per min (nM Vi/min).

RESULTS

Factor Va inactivation by APC in free solution - Limited information is available on the quantitative aspects of APC-catalyzed factor Va inactivation in free solution. Initially, it was reported that bovine factor Va inactivation by bovine APC was only observed in the presence of phospholipid (Kisiel *et al.*, 1977), whereas later studies showed that there was no absolute phospholipid requirement (Walker *et al.*, 1979). In order to appreciate the stimulatory effect of phospholipids we decided to start our investigations with a detailed kinetic study of the inactivation of human factor Va by human APC in free solution. This reaction appeared to require Ca^{2+} ions since in their absence no APC-dependent factor Va inactivation was observed. However, it should be mentioned that measurement of the inactivation rate without Ca^{2+} ions proved to be very difficult. Under these conditions factor Va loses its activity due to dissociation of the heavy and light chains (Esmon, 1979). Addition of APC to reaction mixtures without Ca^{2+} ions did, however, not result in acceleration of the spontaneous rate of factor Va inactivation. In the presence of Ca^{2+} ions spontaneous inactivation was greatly reduced and proceeded at a rate negligible to the APC-catalyzed reaction. Upon variation of the Ca^{2+} ion concentration maximal rates of APC-dependent factor Va inactivation were obtained at 2 mM CaCl_2 (Fig. 1). A further increase of the Ca^{2+} ion concentration resulted in a gradual inhibition of factor Va inactivation.

The inactivation rate at optimal CaCl_2 concentrations appeared to be linearly dependent on the concentration of factor Va (0-78 nM) present in the reaction mixture (Fig. 2A). This shows that we were unable to saturate APC with its substrate factor Va and that under these conditions the K_m for factor Va is > 78 nM. The fact that we were not able to perform experiments at higher factor Va concentrations prohibited the use of Michaelis-Menten kinetics in the kinetic analysis of factor Va inactivation in free solution. Since the rate of factor Va inactivation was also linearly dependent on the concentration of APC (Fig. 2B), it was possible, however, to calculate the second order rate constant of the reaction. At 2 mM CaCl_2 factor Va was inactivated with a second order rate constant of $6.1 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$. This rate constant was not affected by the presence of protein S, which shows that protein S requires phospholipids to exert its cofactor function (*cf.* Walker, 1980).

Figure 3. The effect of Ca^{2+} ions on the initial rate of APC-catalyzed factor Va inactivation in the presence of phospholipids. Factor Va (1 nM) was inactivated by 15 pM APC in a buffer containing 25 mM HEPES (pH 7.5), 126 mM NaCl, 2.68 mM KCl, 5 mM glucose, 25 μM phospholipid vesicles (20 mole% DOPS and 80 mole % DOPC), 5 mg/ml BSA, without protein S ($\blacklozenge$) or with 70 nM protein S ($\bullet$). The concentrations of CaCl_2 present are indicated in the figure. Initial rates of factor Va inactivation were determined as described under experimental procedures and the inactivation rates were expressed as nM factor Va inactivated per min per nM APC (nM Vi/min/nM APC).

Figure 4. Inactivation of factor Va by APC at different phospholipid concentrations. Factor Va (1 nM) was inactivated by 10 pM APC in a buffer containing 25 mM HEPES (pH 7.5), 126 mM NaCl, 2.68 mM KCl, 5 mM glucose, 3 mM CaCl_2 , 5 mg/ml BSA, with ($\bullet$) or without ($\blacklozenge$) 70 nM protein S and concentrations of phospholipid indicated in the figure. The phospholipid vesicles contained 20 mole% DOPS and 80 mole% DOPC. Initial rates of factor Va inactivation were determined as described under experimental procedures and the inactivation rates were expressed as nM factor Va inactivated per min per nM APC (nM Vi/min/nM APC).

Factor Va inactivation by APC in the presence of phospholipids - APC-catalyzed factor Va inactivation has been reported to be considerably stimulated by the presence of negatively-charged membranes (Walker *et al.*, 1979). Membrane-dependent factor Va inactivation also requires Ca^{2+} ions (Fig. 3). When rates of factor Va inactivation were determined as a function of the Ca^{2+} ion concentration in the presence of 25 μM small unilamellar phospholipid vesicles composed of 20 mole% DOPS and 80 mole% DOPC, an optimum was observed at 2 mM CaCl_2 and at higher Ca^{2+} ion concentrations inhibition occurred. The presence of saturating amounts of protein S caused a 1.5 to 3-fold stimulation of the rate of factor Va inactivation at all Ca^{2+} ion concentrations tested. Although the shape of the Ca^{2+} ion titration curves obtained in the presence of phospholipids and in free solution were similar it is likely that the mode of action of Ca^{2+} ions in the two reaction systems is different. It is generally accepted that phospholipid-dependent coagulation factor activation reactions require Ca^{2+} ions for the binding of vitamin K-dependent coagulation factors (*e.g.* APC and protein S) to the membrane surface (Nelsestuen *et al.*, 1978), whereas the Ca^{2+} ion requirement for the reaction in free solution is not yet understood.

Variation of the concentration of phospholipid vesicles (DOPS/DOPC; 20/80; mol/mol) showed that both with and without protein S an optimal rate of factor Va inactivation by APC was obtained at 10 μM phospholipid and that some inhibition occurred at higher phospholipid concentrations (Fig. 4). The presence of protein S accelerated factor Va inactivation approximately 1.5-fold independent of the phospholipid concentration present in the reaction mixture.

APC-catalyzed factor Va inactivation appeared to be dependent on the amount of negatively charged phospholipid (DOPS) present in the phospholipid vesicles (Fig. 5). When the mole percentage DOPS in DOPC vesicles was varied between 0 and 50 mole% optimal rates of factor Va inactivation were obtained at 20 mole % DOPS both in the absence and presence of protein S. A gradual inhibition was observed at mole percentages of DOPS > 20%.

It was found that the stimulation of factor Va inactivation by phospholipids was not confined to membranes containing DOPS as anionic lipid. Phospholipid vesicles composed of DOPC with DOPA, DOPG or DOPM were also able to accelerate APC-catalyzed factor Va inactivation (Table 1). The activity of negatively charged membranes was not strictly dependent on the presence of a phosphate group since lipids which only contain a carboxyl moiety (oleic acid) also promoted factor Va inactivation.

TABLE 1 Factor Va inactivation by activated protein C (APC) on membranes containing different anionic phospholipids. Factor Va (1 nM) was inactivated by APC at 37°C in a reaction mixture containing 25 mM HEPES (pH 7.5), 126 mM NaCl, 2.68 mM KCl, 3 mM CaCl₂, 5 mM glucose, 5 mg/ml BSA and 100 µM phospholipid vesicles. Phospholipid vesicles were prepared from mixtures of 20 mole% anionic lipid and 80 mole% DOPC. Factor Va inactivation was started with 10 - 300 pM APC, depending on the reaction rate. The inactivation rate was expressed as nM factor Va inactivated per minute per nM APC.

Membrane composition	Rate of FVa inactivation (nM Vi/nM APC/min)
No phospholipid	0.037
DOPC (100%)	0.034
DOPE/DOPC (20/80, mol/mol)	0.12
Oleic acid/DOPC (20/80, mol/mol)	0.34
DOPA/DOPC (20/80, mol/mol)	1.50
DOPG/DOPC (20/80, mol/mol)	2.16
DOPS/DOPC (20/80, mol/mol)	4.70
DOPM/DOPC (20/80, mol/mol)	4.71

TABLE 2 The effect of accessory components on rate constants of APC-catalyzed factor Va inactivation. The rate constants were calculated from Figures 1 and 4. Phospholipid is 25 µM DOPS/DOPC (20/80, mol/mol).

Factor Va inactivator inactivation	Rate constant of FVa ($\times 10^5 \text{ M}^{-1}\text{s}^{-1}$)
APC	<0.1
APC + Ca ²⁺	6.1
APC + Ca ²⁺ + Protein S	5.4
APC + PL	<0.1
APC + Ca ²⁺ + PL	1400
APC + Ca ²⁺ + PL + Protein S	1900

Figure 5. Inactivation of factor Va by APC in the presence of phospholipid vesicles containing varying amounts of phosphatidylserine. Factor Va (1 nM) was inactivated by 10 pM APC at 37 °C in a buffer containing 25 mM HEPES (pH 7.5), 126 mM NaCl, 2.68 mM KCl, 5 mM glucose, 3 mM CaCl_2 , 5 mg/ml BSA, with (●) or without (◆) 70 nM protein S and 25 μM phospholipid vesicles containing mole percentages DOPS indicated in the figure, supplemented with DOPC to 100 mole%. Initial rates of factor Va inactivation were determined as described under experimental procedures and the inactivation rates were expressed as nM factor Va inactivated per min per nM APC (nM V_i /min/nM APC).

In order to quantitate the stimulatory effect of phospholipids we have calculated the apparent second order rate constants of factor Va inactivation at optimal Ca^{2+} ion and phospholipid concentrations and mole fraction DOPS and compared them with the rate constants obtained for the reaction in free solution (Table 2). These data indicate that phospholipids cause a more than 230-fold increase of the rate of APC-catalyzed factor Va inactivation. The addition of protein S did not affect the reaction in free solution and caused marginal stimulation (1.4-fold) of factor Va inactivation in the presence of phospholipid.

Kinetic parameters for APC-catalyzed factor Va inactivation in the presence of phospholipids - In contrast to the reaction in free solution (see above) phospholipid-dependent factor Va inactivation by APC could be saturated with respect to the factor Va concentration (Fig. 6A). Thus, it appears that the reaction obeyed Michaelis-Menten kinetics and, their kinetic parameters were obtained from a Lineweaver-Burk plot of the data (Fig. 6B). Using the statistical approach of Eisenthal and Cornish-Bowden (1974) a K_m for factor Va of 11.5 nM and a $V_{\max}$ of

Figure 6. Substrate concentration dependence of APC-catalyzed factor Va inactivation in the presence of phospholipid. Factor Va, at concentrations indicated in the figure, was inactivated by 10–45 pM APC (dependent on the rate of inactivation) at 37 °C in a buffer containing 25 mM HEPES (pH 7.5), 126 mM NaCl, 2.68 mM KCl, 5 mM glucose, 3 mM CaCl₂, 5 mg/ml BSA and 50 μM phospholipid vesicles (20 mole% DOPS and 80 mole% DOPC). Initial rates of factor Va inactivation were determined as described under experimental procedures and the inactivation rates were expressed as nM factor Va inactivated per min per nM APC (nM Vi/min/nM APC). (A) The rate of factor Va inactivation as a function of the factor Va concentration. (B) Lineweaver-Burk plot of the same data.

Figure 7. The kinetic parameters of APC-catalyzed factor Va inactivation as a function of phospholipid concentration. Varying concentrations of factor Va were inactivated by 10–45 pM APC (dependent on the rate of inactivation) at 37 °C in a buffer containing 25 mM HEPES (pH 7.5), 126 mM NaCl, 2.68 mM KCl, 5 mM glucose, 3 mM CaCl₂, 5 mg/ml BSA and concentrations of phospholipid vesicles (20 mole% DOPS and 80 mole% DOPC) indicated in the figure. The kinetic parameters of APC-catalyzed factor Va inactivation were obtained from Lineweaver-Burk plots (*cf.* figure 6). (A) The K_m for factor Va as a function of phospholipid concentration. (B) The V_{max} of factor Va inactivation (nM Vi/min/nM APC) as a function of phospholipid concentration.

61.9 nM Va/min/nM APC were obtained for the inactivation of factor Va by APC on 50 μ M vesicles (DOPS/DOPC; 20/80, mol/mol). Since protein S had such a small effect (i.e. a 1.5 to 2-fold rate enhancement) on factor Va inactivation we did not attempt to correlate the stimulatory effect of protein S with changes of the kinetic parameters.

Both kinetic parameters appeared to be a function of the phospholipid concentration present in the reaction mixture. The $V_{\max}$ increased from 20 to 62 nM Vi/min/nM APC between 2.5 and 250 μ M phospholipid and approached a constant value at higher phospholipid concentrations (Fig. 7A). The K_m for factor Va was linearly dependent on the phospholipid concentration and varied between 1 nM at 2.5 μ M phospholipid and 65 nM at 250 μ M phospholipid (Fig. 7B). Similar effects of the phospholipid concentration on kinetic parameters have been observed for prothrombin and factor X activation (Rosing *et al.*, 1980; van Dieijen *et al.*, 1981).

DISCUSSION

In this paper we have analyzed the effects of Ca^{2+} ions, phospholipids and protein S on the kinetic parameters of APC-catalyzed factor Va inactivation using purified human proteins. In agreement with earlier reports (Walker *et al.*, 1979) we observed that factor Va inactivation by APC is not strictly dependent on the presence of phospholipids and protein S. The reaction in free solution does, however, require the presence of Ca^{2+} ions, and at the optimal Ca^{2+} ion concentration (2mM), the reaction proceeds with a second order rate constant of $6.1 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$. Since the inactivation rates were linearly dependent on the amount of factor Va present (0-78 nM) we conclude that the reaction in free solution has a K_m for factor Va $> 78 \text{ nM}$. We have as yet no explanation for the calcium ion requirement of APC-catalyzed factor Va inactivation in the absence of phospholipids. Calcium ions may act either via factor Va or via APC or both since both proteins have the ability to interact with Ca^{2+} ions (Guinto and Esmon, 1982; Amphlett *et al.*, 1981). Factor Va is composed of an equimolar complex of a heavy and light chain that are held together by a single Ca^{2+} ion (Guinto and Esmon, 1982). Since rates of APC-catalyzed factor Va inactivation were corrected for spontaneous loss of factor Va activity in the absence of calcium ions it is most likely that the site of Ca^{2+} ion involvement is on the enzyme. Bovine APC contains several Ca^{2+} binding sites associated with its Gla domain (Amphlett *et al.*, 1981) and a Gla-independent high affinity calcium-binding site (Johnson *et al.*, 1983). Both types of binding sites may be involved in Ca^{2+} -induced transformation of APC into a functionally active enzyme. In this respect it should be mentioned that both the Gla-dependent and Gla-independent Ca^{2+} -binding sites have to be occupied with Ca^{2+} ions to obtain optimal rates of activation of the protein C zymogen by the thrombin-thrombomodulin complex (Esmon *et al.*, 1982).

APC-catalyzed factor Va inactivation is greatly accelerated by negatively charged phospholipid vesicles. This stimulation appeared to be dependent on the phospholipid concentration and composition. Optimal rates of factor Va inactivation were obtained in the presence of $10 \mu\text{M}$ phospholipid vesicles composed of 20 mole % DOPS and 80 mole % DOPC. Under these reaction conditions APC-catalyzed factor Va inactivation was characterized by an apparent second order rate constant of $1.4 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ which is about 230-fold higher than the rate constant in free solution. To analyze the implications of such rate constants for the *in vivo* action of

APC we have calculated rates of factor Va inactivation under conditions at which 1% of the amounts of protein C and factor V present in plasma are present in an activated form. Assuming that the plasma factor V concentration is 23 nM (Tracy *et al.*, 1982) and the protein C concentration is 65 nM (Griffin *et al.*, 1982), one can calculate that, under the conditions described above, the times required to inactivate 50% of the circulating factor Va are 29 and 0.13 min in the absence and presence of optimal phospholipid, respectively.

The stimulatory effect of phospholipids on APC-catalyzed factor Va inactivation exhibits a clear optimum both with respect to the phospholipid concentration and composition. At low phospholipid concentrations ($< 10 \mu\text{M}$) and low mole percentages DOPS (< 20 mole%) suboptimal rates of factor Va inactivation were obtained whereas at high phospholipid concentrations and high mole fractions DOPS some inhibition occurred. These phenomena are presumably a reflection of the mechanism by which the phospholipid membranes stimulate APC-catalyzed factor Va inactivation (see below). Membranes that were solely composed of the neutral phospholipid phosphatidylcholine were not able to accelerate APC-catalyzed factor Va inactivation. In order to stimulate factor Va inactivation, membranes have to carry a negative surface charge, *i.e.* contain anionic phospholipids. The ability to stimulate factor Va inactivation is not strictly confined to phosphatidylserine-containing membranes since membranes with other anionic phospholipids also considerably enhanced factor Va inactivation (Table 1). It is even not necessary that the membrane derives its negative charge from a phosphate group since membranes with oleic acid, which are negatively charged because of the presence of carboxyl groups, also stimulate APC-catalyzed factor Va inactivation.

The acceleration of APC-catalyzed factor Va inactivation by negatively charged membranes is presumably a reflection of the fact that both APC (Nelsestuen *et al.*, 1978) and factor Va (Bloom *et al.*, 1979) bind to such membranes. This allows the inactivation of membrane-bound factor Va by membrane-bound APC. Catalysis of reactions at a lipid-water interface is a characteristic feature of the coagulation cascade (Rosing and Tans, 1988; Mann *et al.*, 1990). In such cases the presence of membranes profoundly changes the kinetic parameters of coagulation factor activations and inactivations. The K_m for the substrate is greatly decreased in the presence of phospholipid membranes and appears to be a function of the phospholipid concentration present in the reaction medium. When the concentration of phospholipid is increased there is a parallel increase of the K_m which is explained by the fact that the membrane-bound substrate molecules are diluted or bind to non-

functional sites due to the increased amount of surface. Also the $V_{\max}$ of phospholipid-dependent reactions often appears to be a function of the phospholipid concentration. In general the $V_{\max}$ increases with the amount of phospholipid present and reaches a maximum at high phospholipid concentrations. The lipid dependency of $V_{\max}$ is a reflection of the binding of the enzyme to the membrane surface which increases at increasing surface concentration until at high surface concentration all enzyme is bound and participates in coagulation enzymatic reactions. The above described effects of membrane concentrations on K_m and $V_{\max}$ are also observed for APC-catalyzed factor Va inactivation (*cf.* fig. 7) which strongly suggests that in the presence of phospholipid surface-bound factor Va is inactivated by surface-bound APC.

It is well established that protein S, a vitamin K-dependent protein, is required for the optimal expression of the anticoagulant activity of APC. The precise mechanisms of action of protein S as an antithrombotic or anticoagulant remains to be established. Original suggestions that the major function of protein S was to promote the binding of APC to phospholipids (Walker, 1981) and platelets (Harris and Esmon, 1985) were mainly based on experiments with bovine proteins. In studies with purified human proteins and human platelets, protein S appeared to be a rather poor stimulator of APC-catalyzed factor Va inactivation (Solymoss *et al.*, 1988; Tans *et al.*, 1991). In reaction systems containing purified human factor Va, APC and phospholipid vesicles or platelets, protein S increased the rate of factor Va inactivation 1.5 to 2-fold. It was also shown that factor Xa protects factor Va from inactivation by APC, an effect that is nullified by protein S. Thus, when factor Xa is present in inactivation mixtures, the addition of protein S causes a 5 to 10-fold acceleration of APC-catalyzed factor Va inactivation.

The experiments reported in this paper also indicate clearly that in the human system the promotion of APC binding to membranes by protein S is of minor importance. We have shown that protein S stimulates APC-catalyzed factor Va inactivation approximately 2-fold, independent of the phospholipid concentration or composition. In a model in which protein S promotes the binding of APC to anticoagulant membranes one would expect that protein S exerts its stimulatory effect preferentially at low phospholipid concentrations and on membranes with low amounts of phosphatidylserine, *i.e.* at conditions under which the rate of factor Va inactivation would be limited by the binding of APC to the membrane. The lack of such preferential stimulation (*cf.* Figs. 4,5) supports the alternative theories that protein S abrogates the ability of factor Xa to protect factor Va from inactivation

by APC (Solymoss *et al.*, 1988), that other proteins (e.g. a plasma protein S-binding protein) are required for full expression of protein S anticoagulant activity (Walker, 1986), and/or that protein S possesses as yet undescribed anticoagulant properties.

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Functional Properties of Human Factor Va Lacking the Asp⁶⁸³-Arg⁷⁰⁹ Domain of the Heavy Chain

ABSTRACT

A protease, purified from the venom of the elapid snake *Naja naja oxiana*, converts human blood coagulation factor Va into a molecule (designated factor Va_(NO)) with greatly reduced cofactor activity. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate revealed that the venom protease cleaves a small peptide from the heavy chain of factor Va and reduced its apparent MW from 105,000 to 101,000. Amino acid sequence analysis indicated that the venom enzyme cleaved the peptide bond between His⁶⁸² and Asp⁶⁸³ thus removing 27 amino acids from the carboxyterminal part of the heavy chain. The cofactor activities of factor Va and Va_(NO) were compared by measuring their abilities to support factor Xa-catalyzed prothrombin activation in the presence of phospholipids and calcium ions. Both factor Va molecules stimulated the binding of factor Xa to negatively charged phospholipids. However, the amounts of factor Va required for half maximal incorporation of factor Xa into the membrane-bound factor Xa-Va complex were much lower for native factor Va (0.25 nM) than for factor Va_(NO) (2.01 nM). At saturating concentrations factor Va or Va_(NO) both forms of factor Va yielded similar values for the k_{cat} of prothrombin activation (114 s⁻¹ for factor Va and 128 s⁻¹ for factor Va_(NO)). The K_m 's for prothrombin determined under these conditions were 0.24 μM and 0.83 μM for prothrombinase complexes with native factor Va and factor Va_(NO), respectively. Direct binding studies revealed that factor Va and Va_(NO) bind with equal affinity to phospholipids. These data indicate that factor Va_(NO) is impaired in its ability to interact with factor Xa and prothrombin. Together with the structural data this implies that the aminoterminal Asp⁶⁸³-Arg⁷⁰⁹ domain of the heavy chain is required for optimal interaction of factor Va with factor Xa and prothrombin.

INTRODUCTION

Blood coagulation factor V is a single chain glycoprotein with a molecular weight of 330,000Da (Dahlbäck, 1980; Kane and Majerus, 1981) that contains three different types of protein domains (A, B and C) arranged in the order A1-A2-B-A3-C1-C2 (Jenny *et al.*, 1987; Cripe *et al.*, 1992). Activation of human factor V by thrombin results in the removal of the major part of the B domain and the generation of factor Va, a molecule that consists of a heavy chain (containing the A1, A2 and a small piece of the B domain) and a light chain (containing the A3, C1 and C2 domains) that are associated via a single Ca^{2+} ion (Esmon, 1979; Guinto and Esmon, 1982).

Factor Va is a non-enzymatic protein cofactor which together with the serine protease factor Xa, Ca^{2+} ions and a procoagulant membrane surface forms the prothrombinase complex. Depending on the reaction conditions the presence of factor Va causes a 10^3 - 10^6 -fold stimulation of the rate of prothrombin activation (Nesheim *et al.*, 1979; Rosing *et al.*, 1980). This rate enhancement appears to be due to 1) an increase of the catalytic activity (k_{cat}) of the enzyme factor Xa (Nesheim *et al.*, 1979; Rosing *et al.*, 1980), 2) stimulation of the binding of factor Xa to phospholipid membranes (Nesheim *et al.*, 1979; Lindhout *et al.*, 1982; van Rijn *et al.*, 1984) and 3) an increased interaction of the substrate prothrombin with the prothrombinase complex (van Rijn *et al.*, 1984).

Snake venom proteins have been shown to be helpful tools in studying structure-function relationships of blood coagulation factors. Recently, we have purified a protease from the venom of *Naja naja oxiana* (Gerads *et al.*, 1992) which is able to activate both human and bovine factor V. However, the factor Va molecule generated by this venom activator exhibited a much lower cofactor activity than thrombin-activated factor V. Actually, the venom protease can also be regarded as a factor Va inactivator since incubation of thrombin-activated human factor Va with the venom protease resulted in 80-90% loss of cofactor activity.

In the present paper we describe the structural and functional properties of the factor Va derivative obtained after incubation of factor Va with the venom protease. It is shown that the protease cleaves a 27 amino acid peptide ($\text{Asp}^{683}\text{-Arg}^{709}$) from the carboxyterminal part of the heavy chain of factor Va. The corresponding loss of cofactor activity appears to be due to impaired interaction of venom-treated factor Va with prothrombin and factor Xa.

EXPERIMENTAL PROCEDURES

Materials - Hepes, Tris, bovine serum albumin, chicken egg albumin (ovalbumin), soybean trypsin inhibitor (type IS), bovine brain PS, egg-yolk PC and Russell's viper venom were purchased from Sigma Chemical Co., USA. DOPC and DOPS were obtained from Avanti Polar Lipids, Pelham, AL., USA. The chromogenic substrates S2238, S2337 and the thrombin inhibitor I2581 were supplied by Chromogenix, Mölndal, Sweden. PPACK was obtained from Calbiochem and p-NPGB was from Nutritional Biochemicals. FPLC equipment and column materials used for protein purification were purchased from Pharmacia, Uppsala, Sweden.

Proteins - Human coagulation factors used in this study were purified from fresh frozen plasma. Human prothrombin and factor X were purified according to DiScipio *et al.* (1977). Human thrombin was prepared from prothrombin activation mixtures as described by Pletcher and Nelsestuen (1982). Human factor Xa was obtained from purified factor X after activation with RVV-X and isolation from the activation mixture by affinity chromatography on soybean trypsin inhibitor-Sepharose (Boch *et al.*, 1989). RVV-X was purified from Russell's viper venom according to Schiffman *et al.* (1969). Human factor V was purified essentially as described by Dahlbäck (1980) with minor modifications (Tans *et al.*, 1991b). Factor Va was obtained after activation of factor V with thrombin and separated from activation peptides on a prothrombin-CL4B-Sepharose column (Guinto and Esmon, 1984) and stored in a buffer containing 25 mM Hepes (pH 7.5), 100 mM NH_4Cl , 5 mM CaCl_2 and 5 mg/ml BSA. The purified coagulation factors were stored at -80°C . Protein preparations were $>95\%$ pure as judged by SDS-PAGE according to Laemmli (1970).

Protein Concentrations - Protein concentrations were determined according to Lowry *et al.* (1951). Molar thrombin and factor Xa concentrations were determined by active site titration with p-NPGB (Chase and Shaw, 1969; Smith, 1973). Prothrombin concentrations were determined after complete activation of prothrombin with *Echis carinatus* venom and quantitation of thrombin with p-NPGB. Factor V concentrations were estimated from the absorbance at 280 nm using an $A_{280}^{1\%}$ of 8.9 (Kane and Majerus, 1981). Factor Va was quantitated as described below.

Amino-terminal sequence analysis - 200 μl samples containing 6 μg factor Va

or 6 μg factor Va treated with 0.06 μg venom protease or 0.06 μg venom protease were adsorbed onto Prospin filters and the filters were washed with methanol/ H_2O (20/80). The samples were subsequently subjected to automated amino-terminal sequencing on an Applied Biosystems Model 476A Pulse Liquid Sequencer.

Phospholipid vesicle preparations - Appropriate quantities of phospholipids dissolved in chloroform/methanol (1/1, v/v) were mixed in a small glass tube. After drying under a mild flow of N_2 the phospholipids were suspended in 2 ml of a buffer containing 25 mM Hepes (pH 7.5) and 175 mM NaCl. The phospholipid suspension was vigorously vortexed for 1 min and subsequently sonicated for 10 min at 0°C using a MSE Mark II 150-W ultrasonic disintegrator set at 8 μm peak to peak amplitude. Phospholipid concentrations were determined by phosphate analysis (Böttcher et al., 1961).

Factor Va assay - Factor Va was quantitated by determining the rate of factor Xa-catalyzed prothrombin activation in reaction mixtures that contained a limiting amount of factor Va and saturating concentrations phospholipid vesicles (50 μM PS/PC, 10/90, M/M), factor Xa (5 nM) and prothrombin (0.5 μM) (Tans et al., 1991b). The molar factor Va concentration in the assay mixture was calculated from the rate of prothrombin activation using a turnover number of 6000 moles prothrombin activated per min per mole of factor XaVa complex (Tans et al., 1991a). Molar factor $\text{Va}_{(\text{NO})}$ concentrations were calculated from and taken to be identical to the factor Va concentration initially present.

The assay conditions were modified in experiments in which differences in cofactor activity between factor Va and $\text{Va}_{(\text{NO})}$ were monitored. For optimal display of functional differences between factor Va and $\text{Va}_{(\text{NO})}$, the factor Va assay was performed at suboptimal factor Xa (0.5 nM), phospholipid (5 μM PS/PC, 5/95, M/M) and prothrombin (0.25 μM) concentrations (see legend to Figure 1).

Kinetic data analysis - The formation of a membrane-bound factor XaVa complex was determined by measuring rates of prothrombin activation in the presence of phospholipid vesicles at a fixed (limiting) concentration of factor Xa and varying amounts of factor Va or *vice versa*. Factor Xa, factor Va and phospholipid vesicles were preincubated for 5 minutes at 37°C in 25 mM Hepes (pH 7.5), 175 mM NaCl, 2 mM CaCl_2 and 5 mg/ml BSA. Prothrombin activation was started by the addition of prothrombin (preincubated at 37°C in the same buffer). Rates of prothrombin activation were determined with the chromogenic substrate S2238 (Rosing et al., 1980). The apparent K_d for dissociation of the membrane-bound factor Xa-Va complex ($K_{1/2\text{Va}}$) and the prothrombin-converting activity of this

complex at $[\text{factor Va}] \rightarrow \infty$ ($V_{\text{Va} \rightarrow \infty}$) were obtained from a plots of the rate of prothrombin activation as function of the factor Va or Xa concentration that were fitted to the equation for a single site binding isotherm (hyperbola) via non-linear least squares analysis.

The kinetic parameters (K_m and V_{max}) for factor Xa-catalyzed prothrombin activation were determined by measuring the rate of thrombin formation at varying prothrombin concentrations in the presence of a fixed phospholipid concentration, a limiting amount of factor Xa and a saturating concentration factor Va or $\text{Va}_{(\text{NO})}$ as described in the previous paragraph. The kinetic parameters were obtained by fitting the data to the Michaelis-Menten equation using non-linear least squares analysis.

Binding Studies - Binding parameters for factor Va-membrane association were determined on planar phospholipid bilayers. The free factor Va concentrations were determined with the functional assay described above and the concentrations of membrane-bound factor Va were determined by ellipsometry (Cuypers *et al.*, 1983). Amounts of phospholipid-bound factor Va were plotted as function of the free factor Va concentration and the binding parameters (K_d and number of membrane binding sites) were obtained by non-linear least squares analysis of the data to the equation for a single site binding isotherm.

TABLE 1. Amino-terminal sequences of factor Va before and after incubation with the venom protease from *Naja naja oxiana*. Human factor Va (30 µg/ml) was incubated for 1 hour at 37 °C in 25 mM Hepes (pH 7.5), 175 mM NaCl, 2 mM CaCl₂ either in the absence or presence *Naja naja* venom protease (0.3 µg/ml). Both factor Va samples were subjected to amino-terminal sequencing as described under EXPERIMENTAL PROCEDURES.

Cycle no.	Factor Va			Factor Va + <i>Naja oxiana</i>		
	Residue (pmol)	FV-residues		Residue (pmol)	New NH ₂ -terminus	FV-residues
		1-8 ^a	1546-1553 ^a			683-690 ^a
1	A (31.1) S (14.4) D (22.9)	A	S	A (30.5) S (15.4) D (68.0)	D	D
2	Q (29.6) N (15.6)	Q	N	Q (39.6) N (18.7) R (10.6)	R	R
3	L (17.4) N (20.3)	L	N	L (22.7) N (19.7)	L	L
4	R (19.1) G (16.6)	R	G	R (22.6) G (16.5) E (13.4)	E	E
5	Q (16.1) N (19.1)	Q	N	Q (19.7) N (17.5) P (15.5)	P	P
6	F (13.4)	F	R	F (14.6) E (19.0)	E	E
7	Y (14.0)	Y	R	Y (12.5)	--	D
8	V (14.0)	V	N	V (16.0) E (19.8)	E	E

^aFrom Jenny *et al.*, 1987.

RESULTS

Partial inactivation of factor Va by a protease purified from the venom of Naja naja oxiana - We recently purified an enzyme from the venom of *Naja naja oxiana* which converts thrombin-activated factor V (factor Va) into a molecule with reduced cofactor activity (Gerads *et al.*, 1992). The effects of the venom protease on the structure and function of human factor Va were monitored with a functional assay (Figure 1A) and with SDS-PAGE analysis (Figure 1B). Factor Va activity was determined via its ability to act as a cofactor in factor Xa-catalyzed prothrombin activation. To optimize the detection of functional differences between factor Va and venom-treated factor Va (further designated as factor Va_(NO)) the activity assay was performed at suboptimal factor Xa, prothrombin and phospholipid concentrations (Figure 1, see also under EXPERIMENTAL PROCEDURES). Incubation of factor Va with venom protease resulted in a rapid decrease of cofactor function until the activity reached a plateau at approximately 15% of the activity of native factor Va (Figure 1A). SDS-PAGE analysis of factor Va before (Figure 1B, left lane) and after incubation with venom protease (Figure 1B, right lane) shows that the heavy chain of factor Va_(NO) had a slightly increased electrophoretic mobility indicating the loss of a small peptide (MW ~ 4kD) from the heavy chain.

Amino-terminal sequencing of factor Va and factor Va_(NO) -To gain more insight in the localization of the cleavage site in the factor Va molecule we subjected factor Va to aminoterminal sequencing before and after complete reaction with *Naja naja oxiana* venom protease (Table 1). The sequence data obtained for native factor Va indicated the presence of two polypeptide chains with aminoterminal sequences that are in agreement with the aminoterminal sequence reported for the heavy and light chain of factor Va (Jenny *et al.*, 1987).

After complete reaction of factor Va with venom protease an additional third aminoterminal (present in about equal amounts) could be deduced from the sequence data. This new aminoterminal does not originate from the venom enzyme since the amounts of venom protein present in the reaction mixture were too low to be detectable in the sequence analysis. Thus we conclude that the newly formed aminoterminal sequence (D-R-L-E-P-E-?-E or Asp-Arg-Leu-Glu-Pro-Glu-?-Glu) originates from the polypeptide fragment that is removed from the heavy chain during incubation with the venom protease. This sequence yields a unique match with the published sequence of factor V(Va) at Asp⁶⁸³ through Glu⁶⁹⁰ (Jenny *et al.*,

Figure 1. Effect of the *Naja naja oxiana* venom protease on human factor Va. Purified human factor Va (200 µg/ml) was incubated with purified *Naja naja oxiana* venom protease (2 µg/ml) in 250 µl 25 mM Hepes (pH 7.5), 175 mM NaCl and 5 mM CaCl₂ at 37 °C. **A)** At the indicated time points factor Va activity was determined in a reaction mixture containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 2 mM CaCl₂, 5 mg/ml bovine serum albumin, 0.5 nM factor Xa, 0.25 µM prothrombin, 5 µM brain phosphatidylserine/egg yolk phosphatidylcholine (5/95; M/M). Further details of the assay procedure are described under EXPERIMENTAL PROCEDURES. **B)** Before addition of venom protease (lane identified as Va) and after complete reaction (50 min, lane Va_(NO)) 25 ul aliquots were taken for gel electrophoretic analysis on a 7.5 % polyacrylamide slabgel (5% stacking gel) in the presence of sodium dodecyl sulfate and 5% 2-mercaptoethanol (Laemmli, 1970). The positions of the Va heavy chain (HC) and the light chain doublet (LC) are indicated together with the positions of molecular weight markers run on a separate lane on the gel.

1987). We conclude, therefore, that incubation of factor Va with the purified protease from *Naja naja oxiana* venom resulted in cleavage of the peptide bond between His⁶⁸²-Asp⁶⁸³. Due to this cleavage a peptide of 27 amino acids (with a calculated MW of about 3140) is removed from the carboxyl terminal part of the heavy chain of factor Va. This is in reasonable agreement with the change in apparent MW of the factor Va heavy chain observed in the SDS gels after reaction of factor Va with venom protease (*cf.* Figure 1B).

Functional properties of Naja naja oxiana-treated factor Va (Va_(NO)) - Optimal cofactor activity of factor Va in prothrombin activation requires interaction

Figure 2. Binding of factor Va and $Va_{(NO)}$ to planar phospholipid bilayers. Binding of factor Va (●) and factor $Va_{(NO)}$ (○) to planar phospholipid bilayers (DOPS/DOPC, 20/80, M/M) was determined by ellipsometry (Cuypers *et al.*, 1983) in a buffer containing 25 mM Hepes (pH 7.5 at 37 °C), 175 mM NaCl, 2 mM $CaCl_2$ and 5 mg/ml BSA. The solid lines represent hyperbolas obtained by a non-linear least squares fit of the data to a single site binding isotherm. The binding parameters describing these hyperbola are $K_{d(Va)} = 2.2$ nM and 0.34 µg factor Va bound/cm² phospholipid at saturating factor Va or $K_{d(Va(NO))} = 1.7$ nM and 0.35 µg factor $Va_{(NO)}$ bound/cm² phospholipid at saturating factor $Va_{(NO)}$.

Figure 3. Comparison of cofactor activities of factor Va and $Va_{(NO)}$. Initial rates of prothrombin activation were determined at a fixed, limiting concentration of factor Xa in 250 µl buffer containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 2 mM $CaCl_2$, 5 mg/ml BSA, 25 µM phospholipid vesicles (DOPS/DOPC; 5/95; M/M), 3 pM Xa, 1 µM prothrombin and varying amounts of factor Va (■) or $Va_{(NO)}$ (○) as described in the EXPERIMENTAL PROCEDURES. A) Rate of prothrombin activation as a function of factor Va or $Va_{(NO)}$ concentration. The solid lines represent hyperbola obtained after fitting the data with $K_{1/2Va} = 0.25$ nM and $V_{Va \rightarrow \infty} = 21.7$ nM Ila/min (■) and with $K_{1/2Va(NO)} = 2.01$ nM and $V_{Va(NO) \rightarrow \infty} = 16.9$ nM Ila/min (○). B) Double reciprocal plot of the same data.

TABLE 2. Comparison of functional properties of factor Va and $Va_{(NO)}$. This table summarizes the different properties of factor Va and $Va_{(NO)}$ obtained from the data presented in Figures 2-4. For details see text and the legends to the figures.

	Factor Va	Factor $Va_{(NO)}$
$K_{d\text{ PL}}$	2.7 nM	1.7 nM
$K_{d\text{ XaVa}}^a)$	0.25 nM	2.01 nM
$K_{d\text{ XaVa}}^b)$	0.24 nM	2.27 nM
K_m	0.24 μM	0.83 μM
k_{cat}	114 s^{-1}	128 s^{-1}

$K_{d\text{ PL}}$, dissociation constant for membrane-factor Va or membrane-factor $Va_{(NO)}$ complex (cf. Figure 2); $K_{d\text{ XaVa}}^a)$, apparent dissociation constant for the membrane-bound factor XaVa or $XaVa_{(NO)}$ complex determined at limiting factor Xa^{a)} or limiting factor $Va/Va_{(NO)}$ ^{b)} concentrations (cf. Figure 3); K_m and k_{cat} , kinetic parameters of prothrombin activation by the factor XaVa or factor $XaVa_{(NO)}$ complex (cf. Figure 4).

Figure 4. Prothrombin activation by $XaVa$ and $XaVa_{(NO)}$ as a function of prothrombin concentration. Initial rates of prothrombin activation were determined as described in the EXPERIMENTAL PROCEDURES at a fixed, limiting concentration of factor Xa in a reaction mixture containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 2 mM CaCl_2 , 5 mg/ml BSA, 25 μM phospholipid vesicles (DOPS/DOPC; 5/95; M/M), 3 pM Xa, saturating amounts of Va (5 nM, $\blacksquare$) or $Va_{(NO)}$ (35 nM, $\triangle$) and varying amounts of prothrombin. A) Initial rate of activation as a function of prothrombin concentration. The solid lines represent hyperbola with $K_m = 0.24 \mu\text{M}$ and $V_{\text{max}} = 6860$ mole IIa/min/mole factor $XaVa$ ($\blacksquare$) and with $K_m = 0.83$ and $V_{\text{max}} = 7685$ mole IIa/min/mole factor $XaVa_{(NO)}$ ($\triangle$) which were obtained by fitting the data to the Michaelis-Menten equation using non-linear least squares analysis. B) Lineweaver-Burk plots of the same data.

of factor Va with procoagulant membranes, factor Xa and prothrombin. To examine which of these functions was impaired in *Naja naja oxiana*-treated factor Va we compared factor Va and factor Va_(NO) in direct binding studies and in a kinetic analysis of prothrombin activation.

Figure 2 shows an experiment in which we tested the ability of factor Va and Va_(NO) to bind to a planar lipid bilayer composed of 20 mole % DOPS in DOPC. Factor Va and Va_(NO) bound equally well to the membrane and half maximal binding was observed at 2.2 nM factor Va and 1.7 nM factor Va_(NO). At saturating concentrations factor Va and Va_(NO) the amounts of bound protein were 0.34 μg factor Va/cm² phospholipid and 0.35 μg factor Va_(NO)/cm² phospholipid, respectively. Considering that 1 cm² of a phospholipid bilayer contains 0.4 μg phospholipid and that the molecular weights of factor Va and the phospholipid used are ~180,000 and ~800 Da, respectively, it can be calculated that the phospholipid to factor Va ratio (mol/mol) at saturation is 265 for factor Va and 257 for factor Va_(NO). Thus, it appears that the cleavage in the factor Va heavy chain by *Naja naja oxiana* did not result in a change in lipid binding properties of the factor Va molecule. These data support the concept that it is the light chain region of factor Va that is actually responsible for lipid binding (van de Waart *et al.*, 1983; Higgins and Mann, 1983; Pusey and Nelsestuen, 1984; Rosing *et al.*, 1993).

To compare the ability of factor Va and Va_(NO) to assemble into a membrane-bound prothrombinase complex we determined initial steady state rates of prothrombin activation at a limited amount of factor Xa as a function of the factor Va or factor Va_(NO) concentration (Figure 3A). Low amounts of factor Va were required for full expression of prothrombinase activity whereas much higher amounts of Va_(NO) were needed in order to obtain similar activation rates. Half-maximal prothrombinase complex formation was observed at 0.25 nM and 2.01 nM factor Va and factor Va_(NO), respectively. The double reciprocal plot shown in Figure 3B shows that the intercepts at the Y-axis were the same for both factor Va and Va_(NO) indicating that equal rates of prothrombin activation were obtained at saturating concentrations factor Va and Va_(NO). Thus, once formed both the factor XaVa and factor XaVa_(NO) complexes are equally capable of activating prothrombin.

With respect to the different cofactor activities of factor Va and Va_(NO), the possibility had to be ruled out that factor Va_(NO) lacks functional activity and that its cofactor activity is due to a small (~15%) remaining amount of factor Va. This possibility is excluded by the observations that a) titrations of fixed limiting amounts of factor Va or Va_(NO) with factor Xa yield K_d XaVa of 0.24 nM and 2.27 nM for

factor XaVa and XaVa_(NO) complexes, respectively (Table 2) and that b) a functional factor Va assay performed at saturating factor Xa (5 nM), prothrombin (3 μ M) and phospholipid (50 μ M DOPS/DOPC, 20/80, M/M) yield identical activities for factor Va and factor Va_(NO) (data not shown).

We also determined the kinetic parameters of prothrombin activation at a fixed factor Xa and phospholipid concentration and at saturating concentrations of factor Va or Va_(NO). Figure 4A shows the rates of thrombin formation obtained for the factor XaVa and factor XaVa_(NO) complexes as a function of prothrombin concentration. From the Lineweaver-Burk plots shown in Figure 4B it can be concluded that the reaction adhered to overall Michaelis-Menten kinetics. With factor XaVa the reaction was characterized by a K_m for prothrombin of 0.24 μ M and a V_{max} of 6860 mol prothrombin activated/min/mole factor Xa. This is in reasonable agreement with data reported earlier (Tans *et al.*, 1991a; Rosing *et al.*, 1993). In the case of the factor XaVa_(NO) complex the K_m was less favourable (0.83 μ M) whereas V_{max} was slightly higher (7685 mol prothrombin activated/min/mole factor Xa).

DISCUSSION

Factor Va exerts multiple effects in factor Xa-catalyzed prothrombin activation. The cofactor strongly increases the catalytic turnover (k_{cat}) of the enzyme factor Xa and promotes the assembly of productive enzyme-substrate complexes at the procoagulant membrane by stimulating the binding of factor Xa and prothrombin to the membrane. Thus, it appears that factor Va is a multifunctional protein that promotes prothrombin activation via interaction with phospholipids, factor Xa and prothrombin. The phospholipid binding site of factor Va appears to be located on the light chain (van de Waart *et al.*, 1983; Higgins and Mann, 1983; Pusey and Nelsestuen, 1984; Rosing *et al.*, 1993), prothrombin has been reported to interact with the heavy chain (Guinto and Esmon, 1984), whereas both the heavy and light chain of factor Va have been implicated to interact with factor Xa (Guinto and Esmon, 1984; Tucker *et al.*, 1983; Annamalai *et al.*, 1987).

The data presented in this paper give additional insight into the domains of the Va molecule that are involved in the interaction with factor Xa and prothrombin. Using a protease purified from the venom of *Naja naja oxiana* we obtained a factor Va molecule (factor Va_(NO)) that lacks 27 amino acid residues from the carboxylterminus of the heavy chain and that is further characterized by a greatly reduced cofactor activity.

Table 2 summarizes the functional properties of factor Va and factor Va (Va_(NO)). Both factor Va and Va_(NO) bind equally well to negatively charged phospholipids. This is in agreement with earlier reports which indicate that the heavy chain of factor Va is not involved in membrane binding. The apparent K_d for XaVa complex formation at the phospholipid membrane is, however, about an order of magnitude less favourable in the truncated factor Va_(NO) molecule (Table 2, Figure 3). This strongly suggests that the carboxyterminal residues of the heavy chain are important for the interaction of factor Va with factor Xa. Once formed, the factor XaVa_(NO) complex also has somewhat less favourable kinetic parameters for prothrombin activation as evidenced by the increase in K_m for prothrombin (Table 2, Figure 4). The ability of factor Va to increase the enzymatic turnover (k_{cat}) of prothrombin activation is not affected in the factor Va_(NO) molecule (Table 2). Thus, the final 27 carboxylterminal residues of the factor Va heavy chain do not play a role in the increase of k_{cat} of prothrombin activation but these residues are apparently involved in complex formation of factor Va with both factor Xa and prothrombin.

The fact that the carboxylterminus of the factor Va heavy chain plays a role in protein-protein interactions between factor Va and other proteins can be appreciated on basis of the primary sequence of factor V(Va). The carboxyterminus of the heavy chain of factor Va is highly acidic (Jenny *et al.*, 1987) and can, therefore, be expected to be at the surface of the molecule and to be readily available for interaction with other molecules. In this respect it is interesting to note that Kalafatis *et al.* (1990) recently reported that the heavy chain of bovine factor Va can be phosphorylated at Ser⁶⁹⁰ and that this phosphorylation renders the molecule more susceptible to inactivation by activated protein C. No data were given, however, on the effect of phosphorylation on the interaction of factor Va with factor Xa or prothrombin. Whether such phosphorylation can also occur in the human molecule is as yet also unknown. The literature data emphasise, however, that this region of the factor Va heavy chain is accessible for interaction with other macromolecules and plays an important role in the expression and regulation of factor Va cofactor activity.

The cleavage site at which the protease purified from *Naja naja oxiana* venom cleaves factor Va (His⁶⁸²-Asp⁶⁸³) is an unusual target site for proteolytic enzymes. There is, however, a report on an abnormal fibrinogen in which Arg¹⁶ of the A α -chain is replaced by His¹⁶ and which appears to be cleaved quantitatively and selectively by thrombin after this histidine residue (Henschen and McDonagh, 1986). To our knowledge no other proteases have been identified which specifically cleave after a histidine residue. It is interesting, therefore, to further study this snake venom protease in order to gain more insight in the molecular basis for its proteolytic specificity.

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Characterization of two forms of human factor Va with different cofactor activities

ABSTRACT

Factor Va is an essential cofactor in factor Xa-catalyzed prothrombin activation. Purified human factor Va appears to consist of a heavy chain (MW $\approx$ 105,000Da) and a light chain doublet with MWs of $\approx$ 74,000Da and $\approx$ 71,000Da, respectively. We separated factor Va by chromatography on a Mono S column into two fractions, designated factor Va₁ and factor Va₂. Factor Va₁ contains the light chain with a MW of $\approx$ 74,000Da and factor Va₂ exclusively contains the light chain with a MW of $\approx$ 71,000Da. The two forms of factor Va express different cofactor activities when prothrombin is activated at low phospholipid concentrations or on membranes containing low amounts of phosphatidylserine (PS) in phosphatidylcholine (PC). Compared with factor Va₂, much higher amounts of factor Va₁ are required for factor XaVa complex formation at the membrane surface. Once incorporated into the prothrombinase complex, factors Va₁ and Va₂ are equally active in prothrombin activation. This indicates that the two forms of factor Va do not differ in their ability to promote the catalytic activity of factor Xa or to interact with prothrombin. Direct binding experiments show that the different cofactor activities are explained by a greatly impaired ability of factor Va₁ to bind to negatively charged membranes. Factor V is also separated into two protein peaks after chromatography on Mono S column. Upon incubation with thrombin, the first peak yields factor Va₁ and the second peak factor Va₂. The same two forms of factor Va were generated when freshly prepared plasma samples or platelet suspensions were treated with thrombin. This shows that the heterogeneity of the light chain domain is an intrinsic property of both plasma and platelet factor V. It is hypothesized that the heterogeneity is caused by small differences in the carboxyl-terminal C2 domain of factor V that are introduced as the result of post-ribosomal processing.

INTRODUCTION

Activated blood coagulation factor V (factor Va) plays an essential role as cofactor in the conversion of prothrombin into thrombin by the serine protease factor Xa. Factor Va accelerates factor Xa-catalyzed prothrombin activation four to five orders of magnitude by causing a 1000-fold increase of the catalytic capacity (k_{cat}) of factor Xa (Nesheim *et al.*, 1979; Rosing *et al.*, 1980) and by promoting the binding of both factor Xa and prothrombin to procoagulant membranes (Nesheim *et al.*, 1979; Lindhout *et al.*, 1982; van Rijn *et al.*, 1984).

Factor Va is generated from the pro-cofactor, factor V, which circulates in plasma as a single chain glycoprotein with a MW of about 330,000Da (Dahlbäck, 1980; Kane and Majerus, 1981). Factor V has little or no procoagulant activity (Nesheim *et al.*, 1979) and is converted into factor Va after proteolysis of specific peptide bonds which among others can be catalyzed by thrombin (Dahlbäck, 1980; Kane and Majerus, 1981; Suzuki *et al.*, 1982). Thrombin-activated factor V is a two-chain molecule (Suzuki *et al.*, 1982), that consists of a heavy chain and light chain held together by a tightly-bound calcium ion (Esmon, 1979; Guinto and Esmon, 1982). The heavy chain is derived from the amino-terminal region of factor V and has a MW of 105,000Da. The light chain originates from the carboxyl-terminal region and appears on SDS-polyacrylamide gels as a doublet with apparent MWs of 71,000 and 74,000Da, both in case of human (Suzuki *et al.*, 1982) and bovine factor Va (Nesheim *et al.*, 1984; Odegaard and Mann, 1987).

There is as yet no good explanation for the heterogeneity of the light chain of factor Va. It is likely, however, that the structural basis for the light chain doublet has to be sought in its carboxyl-terminal region since large parts of the amino-terminal region of the light chain (with MW up to 62,000Da) appear as single bands after SDS-gelelectrophoretic analysis (Odegaard and Mann, 1987; Ortel *et al.*, 1992).

In this paper we show that the heterogeneity of the carboxyl-terminal domain of the light chain region of factor Va is already observed in factor V present in fresh plasma and platelet samples. We further report the separation of factor Va into two fractions that are homogeneous with respect to their light chain composition. The factor Va preparation with the 71,000Da light chain is characterized by a more efficient incorporation in the prothrombinase complex than the factor Va that contains the light chain with MW = 74,000Da. This difference in cofactor function appears to be due to different binding affinities for procoagulant membranes.

EXPERIMENTAL PROCEDURES

Materials - Bovine serum albumin, chicken egg albumin (ovalbumin), soybean trypsin inhibitor (type IS), bovine brain PS, egg-yolk PC, Russell's viper venom, Hepes and Tris were purchased from Sigma Chemical Co., St Louis, MO., USA. DOPC and DOPS were obtained from Avanti Polar Lipids, Pelham, AL., USA. S2238, S2337 and I2581 were supplied by AB Kabi Diagnostica, Stockholm, Sweden. PPACK was obtained from Calbiochem and p-NPGB was from Nutritional Biochemicals. Column materials and FPLC equipment used for protein purification were purchased from Pharmacia, Uppsala, Sweden.

Proteins- The human coagulation factors used in this study were purified from fresh frozen plasma. Human prothrombin and factor X were purified according to DiScipio *et al.* (1977). Human thrombin was prepared from prothrombin activation mixtures by the method of Pletcher and Nelsestuen (1982). Human factor Xa was obtained from purified factor X after activation with RVV-X and isolation from the activation mixture by affinity chromatography on soybean trypsin inhibitor-Sepharose (Bock *et al.*, 1989). RVV-X was purified from Russell's viper venom according to Schiffman *et al.* (1969). Human factor V was purified essentially as described by Dahlbäck (1980) with minor modifications (Tans *et al.*, 1991). Factor Va was prepared by incubating factor V (0.3 mg/ml) for 20 min with 30 nM thrombin in a buffer containing 10 mM Hepes (pH 7.5), 50 mM NaCl and 5 mM CaCl_2 . After activation 60 nM PPACK was added to inhibit thrombin. The factor Va preparation was subsequently applied to a 1 ml prothrombin-CL4B-Sepharose column and eluted with a linear gradient of 50-500 mM NH_4Cl in 10 mM Hepes (pH 7.5). Factor Va eluted at 260 mM NH_4Cl . The factor Va containing fractions were pooled and diluted in a buffer containing 25 mM Hepes (pH 7.5), 100 mM NH_4Cl , 5 mM CaCl_2 and 5 mg/ml BSA. Prothrombin, factor Xa, factor V and factor Va were stored at -80°C . Protein preparations were homogeneous and >95% pure as judged by SDS-PAGE according to Laemmli (1970).

Separation of the Two Forms of Factor Va (Factor Va₁ and Factor Va₂) - The factor Va obtained from the prothrombin-CL4B-Sepharose column (see previous paragraph) was further subjected to fast protein liquid chromatography (FPLC) on a Mono S column (HR 5/5) at room temperature. The flow rate during the whole purification procedure was 0.5 ml/min. After application of 0.6 mg factor Va the column was washed with 15 ml of a buffer containing 25 mM Hepes (pH 7.5), 50 mM NH_4Cl and 5 mM CaCl_2 . No factor Va activity eluted during the application

and wash procedure. The Mono-S column was then developed with 15 ml of a linear gradient (50 mM - 1000 mM NH_4Cl) in the same buffer. Factor Va activity eluted from the column in two well-separated protein peaks at 450 mM and 750 mM NH_4Cl , respectively. The first factor Va peak contained the light chain with MW = 74,000Da (factor Va₁) and the second peak consisted of factor Va with a light chain with MW = 71,000Da (factor Va₂). The two factor Va containing peaks were pooled separately and contained pure and homogeneous factor Va as judged by SDS-PAGE according to Schagger and von Jagow (1987). In a large number of factor Va preparations isolated from different batches of human plasma the amount of protein present in peak 1 was about half of that present in peak 2.

Separation and identification of different factor V forms present in purified factor V preparations and in plasma and platelet samples was accomplished by the same procedure and is described in the legends to the figures.

Gel Electrophoretic Techniques - Factor Va preparations and the activation patterns of factor V activated with thrombin were analysed by SDS-PAGE in the presence of Tricine according to Schagger and von Jagow (1987). This electrophoretic technique has the advantage that the separation of protein bands in the gel is hardly affected by the ionic strength of the samples.

Protein Concentrations - Protein concentrations were determined according to Lowry *et al.* (1951). Molar thrombin and factor Xa concentrations were determined by active site titration with p-NPGB (Chase and Shaw, 1969; Smith, 1973). Prothrombin concentrations were determined after complete activation of prothrombin with *Echis carinatus* venom and quantitation of thrombin with p-NPGB. Factor V concentrations were estimated from the absorbance at 280 nm using an $A_{280}^{1\%}$ of 8.9 (Kane and Majerus, 1981). Factor Va concentrations were determined as described by Lindhout *et al.* (1982).

Phospholipid vesicle preparations - Appropriate quantities of phospholipids dissolved in $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1/1, v/v) were mixed in a glass tube and dried under a mild flow of N_2 . The phospholipids were suspended in 2 ml buffer (25 mM Hepes, pH 7.5, 175 mM NaCl) and vigorously vortexed for 1 min. The phospholipid suspension was subsequently sonicated for 10 min at 0 °C using a MSE Mark II 150-W ultrasonic disintegrator set at 8 μm peak to peak amplitude. Phospholipid concentrations were determined by phosphate analysis (Bottcher *et al.*, 1961).

Factor Va assay - Factor Va was quantitated by measuring its cofactor activity in factor Xa-catalyzed prothrombin activation at saturating concentrations of factor Xa, phospholipid vesicles and prothrombin and at a limiting amount of factor Va (Tans *et al.*, 1991). The factor Va concentration in the assay mixture was

calculated from the rate of prothrombin activation using a turnover number of 6000 moles prothrombin activated per min per mole of factor XaVa complex (Tans *et al.*, 1991).

It should be emphasized that the quantitation of the two forms of factor Va (factor Va₁ and factor Va₂) that are studied in this paper is correct despite the fact that there are reaction conditions at which they express different cofactor activities in prothrombin activation. This is inherent to the fact that the high concentrations of factor Xa, prothrombin and phospholipids used in the assay system nullify the differences in cofactor activities (see Results, Table 1)

Kinetic data analysis - Complex formation between membrane-bound factor Xa and factor Va was determined by measuring the rate of prothrombin activation in the presence of phospholipid vesicles at a fixed limiting concentration of factor Xa and varying amounts of factor Va. Factor Va, factor Xa and phospholipid vesicles were preincubated for 5 minutes at 37 °C in 25 mM Hepes (pH 7.5), 175 mM NaCl, 2 mM CaCl₂ and 5 mg/ml BSA. Activation was started by addition of prothrombin (preincubated at 37 °C in the same buffer). Rates of prothrombin activation were determined with the chromogenic substrate S2238 (Rosing *et al.*, 1980). The apparent K_d for dissociation of the membrane-bound factor XaVa complex ($K_{1/2Va}$) and the prothrombin-converting activity of this complex at [factor Va] $\rightarrow \infty$ ($V_{Va \rightarrow \infty}$) were obtained from a plot of the rate of prothrombin activation as function of the factor Va concentration that was fitted to the equation for a single site binding isotherm (hyperbola) in the computerprogram "Enzfitter".

The kinetic parameters of factor Xa-catalyzed prothrombin activation (K_m for prothrombin and V_{max} of prothrombin activation) were determined by measuring the rate of thrombin formation at varying prothrombin concentrations in the presence of a fixed phospholipid concentration, a limiting amount of factor Xa and a saturating concentration factor Va under conditions described in the previous paragraph. The kinetic parameters were obtained by fitting the data to the Michaelis-Menten equation using the Enzfitter computer program.

Binding Studies - Binding parameters for factor Va-membrane association were determined on planar phospholipid bilayers. The concentrations of membrane-bound factor Va were determined by ellipsometry (Cuypers *et al.*, 1983) and the free factor Va concentrations were determined with the functional assay described above. Amounts of phospholipid-bound factor Va were plotted as function of the free factor Va concentration and the binding parameters (K_d and number of membrane binding sites) were obtained by fitting the data to the equation for a single site binding isotherm in the computerprogram "Enzfitter".

Figure 1. Gelelectrophoretic analysis of factor V and Va preparations. Factor V, thrombin-activated factor V, and factor Va₁ and Factor Va₂ that eluted from the mono-S column were subjected to SDS-PAGE on slabgels containing 7.5 % acrylamide (Schägger and von Jachow, 1987). 20 μ l of a factor V or Va-containing sample (0.6-5 μ g protein) was mixed with 5 μ l electrophoresis sample buffer and applied to the gel. Lane 1, factor V; lane 2, thrombin-activated factor V (factor Va); fraction 79-81 = factor Va₁; fraction 87-90 = factor Va₂. Gels were stained with Coomassie brilliant blue R-250.

Figure 2. Chromatography of factor Va on mono-S. Factor Va obtained from prothrombin CL 4B-Sepharose was diluted to 0.6 mg/20 ml in a buffer containing 25 mM HEPES pH 7.5, 50 mM NH₄Cl and 5 mM CaCl₂ and was applied to a mono-S column (HR 5/5) that was equilibrated with the same buffer. After application of factor Va the column was washed with 15 ml buffer. Factor Va was subsequently eluted from the column with 15 ml of a linear gradient (50 to 1000 mM NH₄Cl) in 25 mM HEPES (pH 7.5), 5 mM CaCl₂. Fractions (0.5 ml) were collected and assayed for factor Va activity (●) as described under the EXPERIMENTAL PROCEDURES. Fractions from peak 1 (79-81) and peak 2 (87-90) were subjected to SDS-PAGE (figure 1).

RESULTS

Separation of two forms of Factor Va by FPLC on a MonoS column -

Purified human factor V was converted into factor Va with thrombin. Factor Va was subsequently separated from the activation fragments by chromatography on a prothrombin-Sepharose column (*cf.* Guinto and Esmon, 1984). SDS-gelelectrophoretic analysis showed that this procedure yielded a pure factor Va preparation that consisted of a heavy chain with a MW of 105,000Da and the characteristic light chain doublet migrating in the 71,000-74,000 molecular weight region (Figure 1, lane 2).

Although anion exchangers are usually employed in the purification of factor V and Va it appears that the light chain of factor Va is a basic protein with a high affinity for cation exchangers (Lindhout *et al.*, 1982; Odegaard and Mann, 1987). Figure 2 shows that factor Va also bound to a Mono-S column. Subsequent elution with a NH_4Cl gradient yielded two protein peaks with factor Va activity. SDS-PAGE analysis showed that the first peak consisted of factor Va molecules with a light chain of MW = 74,000Da (Figure 1, fraction 79-81) and that the factor Va that eluted in the second peak contained the light chain with a MW = 71,000Da (Figure 1, fraction 87-90). In the text that follows we will designate the factor Va present in the first peak, factor Va_1 , while the factor Va present in the second peak will be called factor Va_2 .

Separation of Two Forms of Factor V on a Mono-S Column -

Non-activated human factor V showed a chromatographic behaviour that strongly resembled factor Va. Factor V also bound to the Mono S column and eluted as a double peak when the column was developed with a NH_4Cl gradient (Figure 3A). Incubation of the protein fractions with thrombin yielded two factor Va activity peaks which, as shown by SDS-PAGE analysis (Figure 3B), were highly enriched in the 74,000Da and 71,000Da light chains, respectively. This experiment indicates that the light chain doublet present on SDS gels of factor Va preparations does not result from additional peptide bond cleavages that occur during the activation of factor V by thrombin but shows that the heterogeneity in the light chain domain is already present in factor V.

Structural Properties of Plasma and Platelet Factor V Present in Freshly

Collected Blood Samples - The experiments presented in the previous paragraph strongly suggest that purified factor V preparations already contain two forms of

Figure 3. (A) Chromatography of factor V on monoS. 5 ml of purified factor V (5 mg) in 10 mM HEPES (pH 7.5), 50 mM NaCl was applied to a Mono S column (HR 5/5). The column was washed with 15 ml of a buffer containing 25 mM HEPES (pH 7.5), 50 mM NH_4Cl and 5 mM $CaCl_2$ and subsequently developed with 30 ml of a linear gradient from 50 to 1000 mM NH_4Cl in 25 mM HEPES (pH 7.5) and 5 mM $CaCl_2$. Samples from the column fractions were incubated for 1 hour at 37 °C with thrombin (final concentration 25 nM) and the factor Va generated ($\bullet$) was assayed as described under EXPERIMENTAL PROCEDURES. (B) Gelelectrophoretic analysis of factor Va generated in the factor V fractions that eluted from the monoS column. Aliquots (50 μl) from the column fractions (64, 66, 72, 76, 78, and 82) were activated with thrombin (Figure 3A) and subsequently mixed with 12.5 μl gelelectrophoresis sample buffer (Schägger and von Jachow, 1987). 20 μl of the gelsamples were then applied to a 7.5% slabgel and subjected to SDS-PAGE (Schägger and von Jachow, 1987). Sm is a lane with starting material (factor V) activated with thrombin, MW is a lane with molecular weight markers. The proteins on the gel were stained with Coomassie brilliant blue R-250.

factor V. However, this does not necessarily mean that there are also two forms of factor V circulating in plasma, since it is possible that the heterogeneity of purified factor V preparations results from reactions that occurred during the purification procedure. In order to exclude this possibility we performed the following experiment. Freshly collected blood samples were subjected to two centrifugation steps which successively removed the erythrocytes and blood platelets and yielded platelet-poor plasma. The platelet pellet was resuspended in buffer and thrombin was added to both the platelet poor plasma and to the platelet suspension. The generation of factor Va activity was followed and after complete activation of factor V the plasma and platelet suspensions were diluted and applied to a MonoS column. Development of this column with a linear NH_4Cl gradient yielded both for the plasma (Figure 4) and the platelet sample (data not shown) two factor Va activity peaks that eluted between 450-500 mM and 700-750 mM NH_4Cl , respectively.

From the fact that the time interval between taking the blood samples and separating the plasma and platelet factor Va samples on the MonoS column was only 50 min and since the activity ratios of the two factor Va peaks in the plasma and platelet samples were approximately the same as those observed in purified factor V(a) preparations (the purification of which took 4 days) we conclude that the heterogeneity of factor V does not result from reactions that have occurred during the purification procedure. The presence of two forms of factor V in fresh plasma and platelet samples indicates that the heterogeneity is an intrinsic property of factor V. *

Cofactor activities of the two forms of factor Va - Expression of the cofactor activity of factor Va is dependent on direct interactions with prothrombin, factor Xa and phospholipid. In the factor Va assay (see EXPERIMENTAL PROCEDURES) the reaction conditions are chosen such that factor Va is the limiting factor and all other components are present in excess. This means that under these conditions differences in protein-protein or protein-lipid interactions between the two forms of factor Va may nullify and escape attention.

*In the factor Va assay reaction conditions were such that factor Va was present at a limiting concentration and all other components were added in excess. The phospholipid concentration and composition (100 μM PS/PC, 10/90, M/M) and the high factor Xa concentration (5 nM) ensures full incorporation of factor Va into the membrane-bound factor XaVa complex. Complete incorporation of both factor Va_1 and factor Va_2 into the prothrombinase complex is shown by the fact that rates of prothrombin activation were not further increased when the prothrombin, phospholipid and factor Xa concentrations in the assay mixture were increased. The validity of this assay for quantitating factor Va_1 and Va_2 preparations is also shown by the fact that preparations which were set at the same protein concentration on basis of A_{280} yielded the same concentrations in the functional assay.

Figure 4. Chromatography of thrombin-activated plasma on a monoS column. Platelet poor plasma derived from blood collected in citric acid was brought at 5 mM free CaCl_2 and incubated for 10 min at 37 °C with thrombin (final concentration 10 nM). The fibrin clot that was formed was removed and 1 ml of the serum was diluted 5 times with column buffer (25 mM HEPES pH 7.5, 5 mM CaCl_2) and applied to a monoS column (HR 5/5). The column was washed with 13 ml of a buffer containing 25 mM HEPES (pH 7.5), 150 mM NH_4Cl , and 5 mM CaCl_2 and the proteins bound to the column were subsequently eluted with 15 ml of a salt gradient from 50 to 1000 mM NH_4Cl in 25 mM HEPES (pH 7.5) and 5 mM CaCl_2 . Column fractions (0.5 ml) were assayed for factor Va activity (●) as described under EXPERIMENTAL PROCEDURES.

Figure 5. Comparison of cofactor activities of factor Va_1 and factor Va_2 . Rates of prothrombin activation at limiting factor Xa were determined in a reaction mixture containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 2 mM CaCl_2 , 5 mg/ml BSA, 2 μM phospholipid vesicles (DOPS/DOPC, 5/95, M/M), 3 pM factor Xa, 1 μM prothrombin and amounts of factor Va_1 (●) or factor Va_2 (▲) indicated in the figure. Further experimental conditions are described under EXPERIMENTAL PROCEDURES. (A) Rate of prothrombin activation as function of the concentration factor Va_1 or factor Va_2 . The solid lines represent hyperbola obtained after fitting the data with $K_{1/2\text{Va}_1} = 5.93$ nM and $V_{\text{Va} \rightarrow \infty} = 5.35$ nM Ila/min (●) or $K_{1/2\text{Va}_2} = 0.45$ nM and $V_{\text{Va} \rightarrow \infty} = 5.15$ nM Ila/min (▲). (B) Double reciprocal plots of the same data.

To compare the cofactor activities of the two forms of factor Va we have, therefore, performed a kinetic analysis of the stimulatory effects of the factor Va preparations on prothrombin activation under suboptimal reaction conditions *i.e.* at a limiting amount of factor Xa and at a low concentration of phospholipid vesicles containing a low mole percentage PS (2 μ M DOPS/DOPC, 5/95, M/M). Figure 5 shows that there was a rather large difference between the amounts of factor Va₁ and factor Va₂ required for the assembly of a functionally active membrane-bound factor XaVa complex. Half maximal prothrombinase complex formation was observed at 5.93 nM factor Va₁ and 0.45 nM factor Va₂, respectively. A double reciprocal plot of these data (Figure 5B) shows that the rates of prothrombin activation attained at saturating levels of factor Va were approximately the same (Table 1), which indicates that the factor XaVa₁ and XaVa₂ complexes, once they are formed, have equal catalytic activities.

The differences in factor XaVa complex formation disappeared when the same experiment was performed with phospholipid vesicles that contained a high mole percentage PS (*i.e.* 2 μ M PS/PC, 20/80 M/M). On these membranes half maximal prothrombinase complex formation required 0.047 nM factor Va₁ and 0.044 nM factor Va₂, respectively (Table 1). This experiment indicates that the different efficiencies by which factors Va₁ and Va₂ incorporate in the

Table 1. Kinetic analysis of the functional activities of Factor Va₁ and Va₂. Cofactor activities of factor Va₁ and factor Va₂ were determined by measuring rates of prothrombin activation at a limiting factor Xa concentration in a reaction mixture containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 2 mM CaCl₂, 5 mg/ml BSA, 3 pM factor Xa, 1 μ M prothrombin, varying amounts of factor Va₁ or factor Va₂ and phospholipid vesicles as indicated in the Table. Rates of prothrombin activation were plotted against the factor Va concentrations and $K_{1/2Va}$ and $V_{Va \rightarrow \infty}$ were obtained by fitting the data to a hyperbola (see Figure 5). Further experimental conditions are given under EXPERIMENTAL PROCEDURES.

Phospholipid	Factor Va ₁		Factor Va ₂	
	$K_{1/2Va_1}$ (nM)	$V_{Va \rightarrow \infty}$ (nM IIa/min)	$K_{1/2Va_2}$ (nM)	$V_{Va \rightarrow \infty}$ (nM IIa/min)
2 μ M PS/PC (5/95)	5.93	5.35	0.45	5.15
2 μ M PS/PC (20/80)	0.047	18.6	0.044	17.9
0.1 μ M PS/PC (20/80)	0.39	12.8	0.044	13.5

Table 2. Kinetic parameters of prothrombin activation. Rates of prothrombin activation at a limiting factor Xa concentration were determined in a reaction mixture containing 25 mM Hepes (pH 7.5), 175 mM NaCl, 2 mM CaCl_2 , 5 mg/ml BSA, 10 μM phospholipid vesicles (DOPS/DOPC, 5/95, M/M), 3 pM factor Xa, 25 nM factor Va_1 or factor Va_2 and varying amounts of prothrombin. Kinetic parameters (K_m and $V_{\max}$) were obtained by fitting the data to the Michaelis-Menten equation using the Enzfitter computer program. Further experimental conditions are given under EXPERIMENTAL PROCEDURES.

	K_m (μM)	$V_{\max}$ (mol/min/mol Xa)
Factor Va_1	0.32	5386
Factor Va_2	0.16	5687

Figure 6. Binding of factor Va_1 and Va_2 to planar phospholipid bilayers. Binding of factor Va_1 (●) and factor Va_2 (▲) to planar phospholipid bilayers (DOPS/DOPC, 20/80, M/M) was determined by ellipsometry (Cuypers *et al.*, 19883) in a reaction mixture containing 25 mM Hepes (pH 7.5), 150 mM NH_4Cl , 5 mM CaCl_2 and 5 mg/ml BSA. The solid lines represent hyperbolas obtained after fitting the data with $K_d \text{ Va}_1 = 24$ nM and $0.397 \mu\text{g}$ factor Va_2 bound/ cm^2 phospholipid at saturating factor Va_1 or $K_d \text{ Va}_2 = 0.53$ nM and $0.364 \mu\text{g}$ factor Va_2 bound/ cm^2 phospholipid at an infinite concentration of factor Va_2 .

prothrombinase complex are likely compensated by an increased binding affinity and an increased number of binding sites for factor Xa and Va on membranes containing 20 mole % PS (Lindhout *et al.*, 1982; van de Waart *et al.*, 1983; Nelsestuen and Broderius, 1977; Bloom *et al.*, Pusey *et al.*, 1982; Krishnaswamy and Mann, 1988). This argumentation predicts that differences between factors Va₁ and Va₂ can also be observed on membranes with 20 mole% PS, provided that the experiment is performed at a lower phospholipid concentration. Indeed titration of a limited amount of factor Xa with factor Va₁ or factor Va₂ on 0.1 μ M PS/PC (20/80, M/M) vesicles again showed a difference between the two forms of factor Va (Table 1). At this phospholipid concentration a 9-fold higher concentration of factor Va₁ was required to obtain half maximal rates of prothrombin activation (0.389 nM factor Va₁ versus 0.044 nM factor Va₂).

With respect to the different functional properties of factors Va₁ and Va₂ the possibility has to be ruled out that factor Va₁ lacks functional activity and that the cofactor activity of factor Va₁ is due to a small (~8%) contamination with factor Va₂. This possibility is excluded by the observations that a) the amounts of factor Va₁ and factor Va₂ required for factor XaVa complex formation on 2 μ M PS/PC (20/80) were the same (Table 1) and b) that equal protein amounts of factor Va₁ or factor Va₂ yield the same concentrations in the quantitative functional assay (Rosing *et al.*, 1980).

Table 2 summarizes the kinetic parameters for prothrombin activation determined at saturating concentrations of factor Va₁ or factor Va₂. Similar values were obtained for the K_m for prothrombin and the V_{max} of prothrombin activation for both forms of factor Va. This indicates that factor Va₁ and Va₂ neither differ in their interaction with prothrombin nor in their capacity to promote the catalytic activity of factor Xa, but exhibit considerable differences in their ability to assemble into a membrane-bound factor XaVa complex.

Membrane Binding Properties of Factor Va₁ and Factor Va₂ - The impaired incorporation of factor Va₁ into the membrane-bound factor XaVa complex most likely results from a decreased affinity for either phospholipid or factor Xa. To distinguish between these possibilities we have determined the affinity of both forms of factor Va for

PS-containing membranes (Figure 6). Factor Va₂ bound with high affinity to membranes composed of 20 mole% PS in PC. A hyperbolic fit of the binding data yielded a K_d of 0.53 nM and 0.364 μ g factor Va₂ bound/cm² phospholipid at saturating factor Va. Factor Va₁ had a much lower affinity for phospholipid and with

the amounts of factor Va_1 available we were unable to add sufficiently high concentrations to achieve saturation of the membrane surface (Figure 6). From a hyperbolic fit of the binding curve it can be estimated, however, that the interaction between factor Va_1 and the membrane is characterized by a K_d of about 24 nM and 0.397 μg factor Va_2 bound/ cm^2 phospholipid at saturating factor Va_1 . From the molecular weights of factor Va ($\sim 180,000$) and the phospholipid used (~ 800) and considering that 1 cm^2 of a bilayer phospholipid surface contains 0.4 μg phospholipid it can be calculated that phospholipid to factor Va ratios at saturation are 227 and 247 for factor Va_1 and factor Va_2 , respectively.

These observations show that the two forms of factor Va differ considerably in their affinity for phospholipids while the number of binding sites for factor Va_1 and factor Va_2 on the phospholipid surface are the same. The large affinity difference very likely explains the different cofactor activities of factor Va_1 and factor Va_2 in prothrombin activation.

DISCUSSION

Purified human factor Va preparations have been reported to consist of a heavy chain with a MW of $\approx 105,000$ Da and a doublet light chain with MWs of $\approx 71,000$ Da and MW $\approx 74,000$ Da, respectively (Suzuki *et al.*, 1982). The data presented in this paper show that it is possible to bind factor Va to a MonoS column and to separate two forms of factor Va by developing the column with a NH_4Cl gradient. The peak eluting at the low salt concentration consisted of factor Va (Va_1) with the $\approx 74,000$ Da light chain, whereas factor Va with a $\approx 71,000$ Da light chain (factor Va_2) eluted at a much higher salt concentration. It is likely that the factor Va bound to the MonoS column via its light chain since this subunit is considered to be a basic protein which exhibits a high affinity for cation exchangers (Lindhout *et al.*, 1982; Odegaard and Mann, 1987). The elution profile from the MonoS column is, therefore, indicative for a charge difference between the light chains of factors Va_1 and Va_2 , in the sense that the light chain of factor Va_2 is more positively charged than the light chain of factor Va_1 .

The interaction of factor Va with procoagulant membranes appears to depend on both hydrophobic interactions (Higgins and Mann, 1983; Lecompte *et al.*, 1987; Krieg *et al.*, 1987; Kalafatis *et al.*, 1990) and on electrostatic interactions between the positively charged light chain and negatively charged phospholipids (Pusey *et al.*, 1982; van de Waart *et al.*, 1983; Pusey and Nelsestuen, 1984). Considering that electrostatic interactions are important for factor Va-membrane association and that there might be a considerable charge difference between the light chains of the two factors Va forms, we hypothesized that factor Va_1 may exhibit a lower affinity for negatively charged membranes than factor Va_2 . Direct binding experiments show that this is indeed the case. Factor Va_2 binds with a high affinity ($K_d = 0.53$ nM) to PS-containing membranes (PS/PC, 20/80), whereas the affinity of factor Va_1 for such membranes is much less ($K_d \approx 24$ nM). The lipid/factor Va ratios (n) at protein saturation were approximately the same ($n=227$ for factor Va_1 and $n=247$ for factor Va_2) which shows that the membrane surface contains the same number of binding sites for both forms of factor Va. It is difficult to compare the binding parameters with literature values since the interaction of human factor Va with membranes is not well documented whereas a wide range of binding parameters ($K_d = 0.05$ -10 nM and $n = 42$ -250) is reported to describe the interaction of bovine factor Va with PC membranes that contain 25 mole % PS (Bloom *et al.*, 1979; Pusey *et al.*, 1982; van

de Waart *et al.*, 1983; Pusey and Nelsestuen, 1984; Krishnaswamy and Mann, 1988).

Factor Va plays an important role in both the assembly and expression of the catalytic activity of the prothrombinase complex on procoagulant membrane surfaces (Nesheim *et al.*, 1979; Rosing *et al.*, 1980; Lindhout *et al.*, 1982; van Rijn *et al.*, 1984). Since proper cofactor function requires the binding of factor Va to the membrane it is to be expected that the different affinities of factors Va₁ and Va₂ for negatively charged membranes will result in different efficiencies to act as a cofactor in prothrombin activation. Kinetic experiments showed that the cofactor function of factor Va₁ is indeed impaired. Much higher amounts of factor Va₁ were required for full assembly of the prothrombinase (factor XaVa) complex at the membrane surface. Once formed, the factor XaVa₁ and XaVa₂ complexes exhibit the same catalytic activity in prothrombin activation. This can be inferred from the fact that the kinetic parameters (k_{cat} and K_m) for prothrombin activation are similar (Table 2). Differences in efficiencies by which factor Va₁ and Va₂ assemble into a membrane-bound factor XaVa complex were only observed on membranes that contain low amounts PS or when prothrombin is activated at low phospholipid concentrations. This is not surprising if one considers that factor XaVa complex formation on the phospholipid surface is a reflection of the concentrations of surface-bound factor Va and factor Xa (Lindhout *et al.*, 1982; Krishnaswamy *et al.*, 1988). At high mole percentages PS and at high phospholipid concentrations differences between factor Va₁ and Va₂ will be nullified when the concentration of factor Va binding sites exceeds the K_d 's for the membrane-factor Va complexes since this condition results in virtually complete binding of both forms of factor Va.

The heterogeneity in the light chains of factor Va is not a unique property of factor Va but is already present in factor V. It appears that the purified factor V preparations and even freshly prepared plasma samples and platelet suspensions contain two forms of factor V which after activation with thrombin are converted into factor Va₁ and Va₂, respectively. This shows that the heterogeneity is an intrinsic property of factor V and is not caused by reactions occurring during the purification procedure or to additional peptide bond cleavages that take place when factor V is activated with thrombin.

Our experiments give no direct insight in the structural basis underlying the difference between the light chain regions of the two forms of factor V. However, combination of information presented in this paper with data recently reported by Ortel *et al.* (1992) strongly suggest that the observed heterogeneity originates from

the carboxyl-terminal C2 domain of the light chain region of factor V. Ortel *et al.* (1992) isolated recombinant human factor V and showed that activation with thrombin resulted in the formation of a light chain doublet. Activation of mutant molecules that lack the carboxyl-terminal C2 domain (*ie.* amino acid residues 2037-2196) yield a factor Va molecule with a single truncated light chain with a MW of $\approx$ 62,000Da. Deletion of the C2 domain caused an essentially complete loss of the ability of the mutant factor Va molecule to bind to negatively charged membranes. This indicates that the occurrence of two forms of factor Va (Va₁ and Va₂) with different affinities for procoagulant membranes results from the presence of two light chains with structural differences in the C2 domain. The fact that heterogeneity is also observed in the factor V present in freshly prepared platelet and plasma samples and in recombinant factor V (Ortel *et al.*, 1992) strongly suggests that the heterogeneity is due to differences in post-ribosomal processing or post-ribosomal modification (*e.g.* glycosylation) of the two factor V molecules. Whether the presence of two forms of factor V in plasma has a physiological function remains to be clarified.

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SUMMARY and CONCLUDING REMARKS

The purpose of the work described in this thesis was to gain more insight in some of the mechanisms of which thrombin formation is controlled during haemostasis, in particular with respect to the plasma proteins factor V (Va), activated protein C and protein S. The first chapter gives an overview of the blood coagulation mechanism and to the literature pertinent to the current hypothesis concerning the mode of action of these proteins in the regulation of prothrombin activation. Purification of factor V from human plasma is described in Chapter 2. Factor V circulates in plasma in a concentration of approximately 8 $\mu\text{g/ml}$ (~ 25 nM). Due to the lability of factor V complete inhibition of proteases during the isolation procedure is essential. The factor V purification method is in part based on the procedure developed by Dahlbäck (1980) and Suzuki *et al.* (1982). Modifications we made to this procedure resulted in decrease of the isolation time, higher yield, higher concentration of factor V and less contamination in the final preparation. Gel filtration (AcA-34 in the original procedures) is now performed with a Superdex-200 column coupled to a Pharmacia FPLC system. We also included a final ion-exchange chromatography step using a MonoS column with the FPLC system to remove final remaining contaminations. This considerably improved our factor V isolation in terms of speed, quantity, and quality compared to the preparations chromatographed on low pressure gel filtration materials. Using this procedure, factor V was purified from plasma more than 4000-fold with a yield of 20% in 55 hours.

Protein C, when activated, is an important regulator of factor Va (see Chapter 4) and VIIIa activity. Activation of protein C by non-physiological activators is of special interest since such activators can be used as tools in studies on the structure-function relationship of protein C, in the preparation of APC from protein C, and in the development of functional protein C assays. Current quantitative protein C assay methods use a protein C activator isolated from the venom of *Agkistrodon contortrix contortrix*. This reaction is strongly inhibited by calcium ions and (at least in the case of human protein C) by NaCl. This phenomenon is of special importance for the use of this activator in a quantitative protein C assay, which, because of this sensitivity, requires well-controlled ionic strength and free CaCl_2 concentration.

Chapter 3 describes the purification and characterization of a protein C activator from the venom of *Agkistrodon halys halys*. The protein C activator from *Agkistrodon halys halys* venom was purified 533-fold by ion-exchange chromatography on QAE-Sephadex A-50, affinity chromatography on aprotinin-Sepharose and MonoQ fast protein liquid chromatography (FPLC). The purified enzyme is a single chain protein with an apparent molecular weight of 36,000. Activation of human protein C results from a specific cleavage in the heavy chain of the molecule. In the case of protein C activation by its physiological activator, thrombin, this cleavage has been identified to result in the removal of a dodecapeptide of 1,400Da from the amino-terminus of the heavy chain (Kisiel, 1979). The protein C activator purified from *Agkistrodon halys halys* very likely activates human protein C via the same pathway. The protein C activator exhibits a high amidolytic activity towards the chromogenic substrates S2302 and S2238. The activity of the activator is not affected by thiolprotease or metalloprotease inhibitors. Considerable inhibition, however, is observed when the protein C activator is incubated with the serine protease inhibitors benzamidin and soybean trypsin inhibitor, and virtually complete inhibition with the active site titrant pNPGb and inhibitor PPACK. This indicates that the protein C activator from *Agkistrodon halys halys* contains an active site serine and histidine which classifies the enzyme as a serine protease. The purified protease can activate both human and bovine protein C. Activation of human protein C only occurs at an appreciable rate in a calcium-free reaction medium at low ionic strength. Ca^{2+} ions inhibited the activation of human protein C with an apparent K_i of 0.8 mM. Sodium chloride also strongly inhibits human protein C activation (with a $K_{i \text{ app}}$ of 20 mM NaCl). In contrast, activation of bovine protein C is hardly affected by variation of Ca^{2+} and NaCl concentrations in the reaction medium. The apparent K_i 's for calcium ion and NaCl inhibition of bovine protein C activation are >10 mM and 220 mM, respectively. Kinetic analysis of human protein C activation by the venom activator (in a calcium-free medium and at low ionic strength, $I = 0.05$) revealed an apparent K_m for protein C of 0.52 μM and a k_{cat} of 0.17 s^{-1} . The catalytic efficiency (k_{cat}/K_m) observed is $3.3 \cdot 10^5 \text{ M}^{-1}\text{s}^{-1}$. At higher ionic strength ($I = 0.15$) rates of human protein C activation become linear with protein C, indicating a strong increase in K_m with increasing ionic strength. Bovine protein C is activated (in the absence of Ca^{2+} ions and at $I = 0.1$) with a K_m of 0.056 μM and a k_{cat} of 0.24 s^{-1} . The catalytic efficiency of bovine protein C activation ($k_{\text{cat}}/K_m = 4.3 \cdot 10^6 \text{ M}^{-1}\text{s}^{-1}$) is 13-fold higher than of human protein C activation ($3.3 \cdot 10^5 \text{ M}^{-1}\text{s}^{-1}$). This is caused by a lower K_m of the venom activator for

bovine protein C. These data are indicative for a rather large conformational and/or structural difference between human and bovine protein C at physiological ionic strength. In addition it seems likely that, in contrast to bovine protein C, the conformation of human protein C changes considerable with changing ionic strength. Protein C activation by thrombin is also inhibited by calcium-ions but when thrombomodulin is present this reaction requires the presence of Ca^{2+} .

Factor Va cofactor activity *in vivo* is down regulated by activated protein C in concert with protein S. In Chapter 4, we have analyzed the effects of Ca^{2+} ions, phospholipids and protein S on the kinetic parameters of APC-catalyzed factor Va inactivation using purified human proteins. In agreement with earlier reports, we observed that factor Va inactivation by APC is not strictly dependent on the presence of phospholipids and protein S. The reaction in free solution requires the presence of Ca^{2+} ions, and at the optimal Ca^{2+} ion concentration (2 mM), the reaction proceeds with a second order rate constant of $6.1 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$. The inactivation reaction in free solution has a high K_m for factor Va ($>78 \text{ nM}$). APC-catalyzed factor Va inactivation is greatly accelerated by negatively charged phospholipid vesicles. The extent to which phospholipids stimulate the reaction is dependent on the phospholipid concentration and composition. Optimal rates of factor Va inactivation are obtained in the presence of $10 \text{ }\mu\text{M}$ phospholipid vesicles with high mole percentage anionic phospholipid (DOPS/DOPC; 20/80; M/M). Under these reaction conditions APC-catalyzed factor Va inactivation was characterized by an apparent second order rate constant of $1.4 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$. This is 230-fold higher than the rate constant in free solution. The stimulatory effect of phospholipids on APC-catalyzed factor Va inactivation exhibits a clear optimum both with respect to the phospholipid concentration and DOPS/DOPC composition. Membranes that are solely composed of the neutral phospholipid phosphatidylcholine are not able to accelerate APC-catalyzed factor Va inactivation. Thus, in order to stimulate factor Va inactivation, membranes have to contain anionic phospholipids. The ability to stimulate factor Va inactivation is not strictly confined to phosphatidylserine-containing membranes since membranes with other anionic phospholipids considerably enhance factor Va inactivation as well. The ability of different anionic phospholipids to enhance factor Va inactivation increased in the order phosphatidylethanolamine < oleic acid < phosphatidic acid < phosphatidylglycerol < phosphatidylmethanol < phosphatidylserine. Acceleration of APC-catalyzed factor

Va inactivation by negatively charged membranes is presumably a reflection of the fact that both APC and factor Va bind to such membranes. This allows the inactivation of membrane-bound factor Va by membrane-bound APC. The apparent K_m for factor Va is greatly decreased in the presence of phospholipid membranes and appears to be a function of the phospholipid concentration present in the reaction medium. When the concentration of phospholipid is increased there is a parallel increase of the K_m . Also the V_{max} of APC-catalyzed inactivation of factor Va appears to be a function of the phospholipid concentration. The V_{max} increases with the amount of phospholipid present and reaches a plateau at 10 μ M phospholipid. This strongly suggests that, in the presence of phospholipid, surface-bound factor Va is inactivated by surface-bound APC. In addition, the experiments presented in Chapter 4 indicate clearly that the promotion by protein S of APC binding to membranes is of minor importance in purified human system. In a model in which protein S promotes the binding of APC to phosphatidylserine containing membranes, one would expect that protein S exerts its stimulatory effect preferentially at low phospholipid concentrations and on membranes with low amounts of phosphatidylserine, *i.e.* at conditions under which the rate of factor Va inactivation would be limited by the binding of APC to the membrane. The lack of such preferential stimulation may be regarded to support the alternative hypothesis that protein S abrogates the ability of factor Xa to protect factor Va from inactivation by APC, or that other proteins (*e.g.* a plasma protein S-binding protein) are required for full expression of protein S anticoagulant activity (Walker, 1986). Furthermore it is possible that protein S exhibits other anticoagulant properties, such as the recently described APC independent interaction of protein S with factor Va (Heeb *et al.*, 1993). It is clear that the precise mechanisms of action of protein S as an antithrombotic or anticoagulant remains to be established.

Snake venom proteases have been shown to be excellent tools in studying the structure-function relationship of coagulation factors. A proteolytic enzyme capable of inactivating factor Va was previously isolated from venom of the Elapid snake *Naja naja oxiana* (NO) (Gerads *et al.*, 1992). The *Naja naja oxiana* enzyme alters factor Va into a molecule with greatly diminished cofactor activity (factor Va_(NO)). Incubation of factor Va with the venom protease results in a time-dependent loss of factor Va activity with a stable residual activity of some 10-15% after 40 minutes of incubation. In Chapter 5, we describe the structural changes in factor Va following incubation with the *Naja naja oxiana* enzyme. The resulting change in cofactor activity of factor Va_(NO) was subsequently analyzed. SDS-PAGE revealed that the

molecular weight of the heavy chain changed from 105 kD to approximately 100 kD after incubation with the venom protease. The light chain is not affected by incubation of factor Va with the *NO* protease. Direct binding studies on planar phospholipid bilayers in an ellipsometer show that factor Va and factor $Va_{(NO)}$ bound with comparable affinity to a phospholipid membrane (K_d 's of 4.2 and 2.8 nM respectively). From this experiment we conclude that the decreased cofactor activity of factor $Va_{(NO)}$ in prothrombin activation is not due to reduced binding affinity of $Va_{(NO)}$ for phospholipid membranes. N-terminal amino acid sequence analysis showed that the factor $Va_{(NO)}$ preparation contains, in addition to the known termini of the heavy and light chains of factor Va, a new amino-terminus with the amino acid sequence Asp-Arg-Leu-Glu-Pro-Glu. Matching the newly formed N-terminus in factor $Va_{(NO)}$ with the known complete amino acid sequence of factor V indicates that the venom enzyme cleaves the heavy chain between His⁶⁸²-Asp⁶⁸³, removing 27 amino acids from the carboxyterminal part of the heavy chain. The cofactor activities of factor Va and factor $Va_{(NO)}$ were compared by measuring their ability to support factor Xa-catalyzed prothrombin activation in the presence of phospholipids and calcium ions. Both factor Va and $Va_{(NO)}$ molecules stimulate the binding of factor Xa to negatively charged phospholipids. However, the amounts of factor Va and $Va_{(NO)}$ required for half maximal prothrombinase complex formation differ considerably (0.7 nM factor Va *versus* 9.42 nM factor $Va_{(NO)}$). Further kinetic analysis revealed that saturating amounts of both forms of factor Va yielded the same k_{cat} for prothrombin activation. The K_m of prothrombin determined at a saturating concentration of factor $Va_{(NO)}$ is approximately 3 times higher than in the case of saturation with factor Va (0.87 μ M *versus* 0.24 μ M). These data indicate that factor $Va_{(NO)}$ is impaired in its ability to interact with factor Xa and, to a lesser extent with prothrombin. In combination with the kinetic data this indicates that the small carboxyterminal peptide is required for optimal interaction of factor Va with prothrombin and factor Xa.

It has been known for a long time that gel electrophoresis of purified human factor Va shows a heavy chain of 105 kD and two slightly different light chains of 74 and 71 kD, respectively. Because factor Va consists of an equimolar complex of a heavy chain and a light chain, two different molecular forms of factor Va must exist. This posed the following questions 1). are these two forms of factor Va functionally identical, and 2). do they originate from two forms of circulating factor V in plasma or are they generated during activation by thrombin. In Chapter 6, we addressed these questions and we describe the separation and kinetic analysis of these two

forms of factor Va. We separated purified factor Va on a MonoS column, factor Va bound to the column and eluted in two separate peaks in a linear NH_4Cl gradient. Factor Va with a 74kD light chain eluted at 450 mM NH_4Cl (factor Va_1), whereas factor Va with the 71kD light chain eluted at 750 mM NH_4Cl (factor Va_2) indicating that the two forms differ considerably in charge. Approximately $1/3$ of the factor Va eluted as factor Va_1 and $2/3$ as factor Va_2 . When tested under reaction conditions suboptimal with respect to factor Xa and phospholipid concentrations the two factor Va forms express different cofactor activity in the prothrombinase complex. Using rates of prothrombin activation as a measure for the amount of factor Xa-Va complexes formed, an apparent K_d ($K_{1/2\text{Va}}$) for Xa-Va complex formation of 5.9 nM for factor Va_1 and 0.45 nM for factor Va_2 was obtained. These results were obtained with phospholipids containing a low percentage anionic phospholipid (DOPS/DOPC; 5/95; M/M; 2 μM). When the mole percentage PS is increased (to 20 mole% PS) the difference in factor Xa-Va complex formation disappear (K_d app 0.047 nM factor Va_1 versus 0.044 nM factor Va_2). However, even with these phospholipids differences between factor Va_1 and factor Va_2 can be observed provided the concentration of phospholipid is sufficient low. Thus at a 20-fold lower phospholipid concentration (0.1 μM) the K_d app for factor Xa-Va complex formation at the phospholipid membrane surface is 0.4 versus 0.044 nM for factor Va_1 and factor Va_2 respectively. This indicates that the different efficiencies by which factors Va_1 and Va_2 incorporate in the prothrombinase complex are at higher phospholipid concentrations compensated by an increased number of binding sites on the phospholipid membrane. At saturating factor Va concentrations prothrombin activation rates were essentially the same in these experiments, indicating that the two forms express the same catalytic activity in prothrombin activation but differ in their ability to form a Xa-Va complex at the phospholipid surface. Direct binding experiments to a planar phospholipid bilayer in an ellipsometer indicated that factor Va_2 bound with high affinity ($K_d = 0.53$ nM) whereas binding affinity of factor Va_1 was relatively low ($K_d = 24$ nM). These results show that factor Va_1 and Va_2 are not identical, but differ in their ability to assemble into a Xa-Va complex on a phospholipid membrane and this difference is caused by different binding properties. Purified plasma factor V also binds to MonoS and elutes in two (overlapping) peaks. Upon activation of the factor V containing fractions with thrombin, factor V eluting at a low salt concentration in the gradient yielded the 74kD light chain whereas factor V eluting at a higher salt concentration yielded the 71kD light chain. It is concluded that the basis for the two factor Va forms is already present in the factor

V molecule. When platelets are activated with thrombin and passed over MonoS, factor Va activity elutes at 450 and 750 mM NH_4Cl , respectively, indicating that platelet factor V is also heterogeneous. The conclusion from the experiments described in Chapter 6 therefore is that factor V is present in plasma and blood platelets in two forms which differ in the carboxyterminal domain of factor V (the domain from which the light chain originates). Upon activation these two factor Va forms neither differ in their interaction with prothrombin nor in their capacity to promote the catalytic activity of factor Xa, but exhibit considerable differences in their ability to assemble into a membrane-bound factor Xa-Va complex caused by a different affinity for the procoagulant membrane. This heterogeneity in factor V molecules might be introduced during post-ribosomal processing of factor V. Recently, Ortel and coworkers (1992) showed that the structural basis for the light chain doublet is found in the C2 domain. Furthermore, this group has shown that within the C2 domain of factor Va a phosphatidylserine-specific binding site is located (Ortel *et al.*, 1992). It is conceivable that this PS binding site is more exposed in the factor Va₂ form accompanied with a relatively increased positive charge of the light chain. Further experiments are needed to resolve the question what the structural difference is between the two factor Va forms. Differences at the carboxyterminus of factor V might be shown using C-terminal amino acid sequencing techniques. Alternatively differences in the carbohydrate moieties on the light chain may also be the cause of factor V heterogeneity. Questions arise whether there is a difference in activation of the two factor V forms. Furthermore, it is interesting to see whether activated platelets support Xa-Va complex formation with both factor Va forms to the same extend.

The light chain of factor Va is reported to be involved in binding of activated protein C (APC). In factor Va and VIIIa light chain a binding site for protein C has been identified. This APC binding site is located in the A3 domain of factor Va and factor VIIIa (Krishnaswamy *et al.*, 1986; Fay and Walker, 1989; Walker *et al.*, 1990). It is possible that the two factor Va light chains have different binding affinities for APC. This consequently results in differences in inactivation rates for the two factor Va forms. Another phenomenon is the effect of factor Va light chain on the activation of protein C. Salem and coworkers (1984) showed that factor Va light chain accelerate protein C activation by thrombin. Again it is not clear whether both factor Va forms accelerate this reaction to the same extend. The effects of both factor Va forms in the protein C pathway as described above remains to be examined. It might be expected that at the early stages of coagulation small amounts

of membrane surfaces for factor Va binding are formed. In this case, factor Va₂ (high affinity fraction) will bind first to the membranes forming a receptor for factor Xa. When more procoagulant membranes are generated (activated platelets) also factor Va₁ will bind. It is possible that factor Va₁ preferentially plays a role in the protein C pathway. In this light, it is conceivable that in patients with mild bleeding or thrombotic tendencies the ratio of the two factor Va forms is altered. The function of the two different forms of factor V (and factor Va) remains to be established.

SAMENVATTING en SLOTOPMERKINGEN

Deze sectie is voornamelijk bedoeld voor diegenen die het Engels niet goed beheersen, en onbekend zijn met de biochemie van de bloedstolling. Daarom geef ik in deze Nederlandse samenvatting eerst een korte inleiding over wat bloedstolling is, daarna volgt een samenvatting van de hoofdstukken, afgesloten door suggesties voor nieuw onderzoek.

Als een bloedvat beschadigd wordt treed er bloedverlies op. Bloedstolling is de omzetting door het enzym trombine van het oplosbare fibrinogeen naar het onoplosbare fibrine. Het basale beschermingsmechanisme ter voorkoming van bloedverlies omvat verscheidene cellulaire en (bloed)plasmacomponenten. Daarnaast zijn er fysiologische effecten op de betrokken bloedvaten. Het stoppen van bloedingen in kleine bloedvaten kan spontaan gebeuren door het samentrekken van het bloedvat. In grote bloedvaten is een bloedprop gevormd uit bloedplaatjes en fibrine het belangrijkste mechanisme van bloedstelping. Deze hemostatische bloedprop wordt in eerste instantie gevormd door aggregatie van bloedplaatjes waarna fibrinedraden worden gevormd. Het volledig gevormde solsel bevat bloedplaatjes, rode en witte bloedlichaampjes in een fibrine netwerk. Het biochemische proces dat leidt tot bloedstolling is feitelijk een serie opeenvolgende activeringen waarbij inactieve pro-enzymen geactiveerd worden tot proteolytische (eiwitsplitsende) enzymen. Een typisch aspect van deze cascade is de grote versterking als gevolg van opeenvolgende reacties. De totale versterking van dit proces kan in de orde van 10^6 (miljoen) maal zijn. De enzymen die betrokken zijn bij de bloedstolling circuleren in de bloedstroom als pro-enzymen in een inactieve vorm. Ze worden geactiveerd tot actieve eiwitsplitsende enzymen door splitsing van een of twee peptide bindingen. Deze pro-enzymen worden aangeduid met Romeinse cijfers. De geactiveerde stollingsfactor wordt aangeduid door toevoeging van een kleine 'a'.

Het enzym trombine (factor IIa) wordt gevormd uit het pro-enzym protrombine (factor II), deze activering kan verlopen via twee routes, de intrinsieke

Figuur 1. Een vereenvoudigd schema van de extrinsieke bloedstolling. Als bloed in contact komt met weeßselfactor (WF) bindt factor VII uit bloed hieraan, het pro-enzym factor VII wordt omgezet naar de actieve vorm, factor VIIa. Factor VIIa activeert de pro-enzymen factor IX en X. Factor IXa activeert ook factor X en is daarmee een belangrijke verbinding tussen de extrinsieke en intrinsieke stolling. Factor X activeert protrombine (factor II) naar trombine (factor IIa) die fibrinogeen in het onoplosbare fibrine omzet. Trombine activeert de cofactoren V en VIII en het pro-enzym factor XI. (figuur naar Hemker and Lindhout, 1984)

en extrinsieke stolling.

De extrinsieke stolling wordt gezien als belangrijk bij het starten van het stolproces (Davie *et al.*, 1991). Het extrinsieke stollingsproces wordt gestart wanneer bloed in contact komt met *weeßselfactor* (WF, Figuur 1). Weeßsel direct achter de wand van een bloedvat hebben *weeßselfactor* gebonden op het celoppervlak. Bloed komt dus in contact met *weeßselfactor* als de binnenwand van

een bloedvat beschadigd raakt. *Weefselfactor* bindt dan het pro-enzym factor VII uit bloed, dan wordt factor VII actief (factor VIIa-WF complex) en kan vervolgens de proenzymen factor X en factor IX activeren (Figuur 1). Factor X wordt niet alleen door het VIIa-WF complex geactiveerd, maar ook door factor IXa. Deze reactie vormt een versterkingslus in de activering van factor X, waar extrinsieke en intrinsieke stolling bij elkaar komen. Factor Xa activeert dan protrombine (factor II) naar trombine (factor IIa). Trombine is het enzym dat uiteindelijk verantwoordelijk is voor de omzetting van het in oplossing zijnde fibrinogeen naar het onoplosbare fibrine, het uiteindelijke stolsel.

De intrinsieke stolling is vermoedelijk verantwoordelijk voor het gaande houden van de trombine vorming (Davie *et al.*, 1991). Trombine activeert het pro-enzym factor XI dat weer factor IX kan activeren. De intrinsieke stolling kan ook op gang gebracht worden door contact met lichaamsvreemde oppervlakken, bijvoorbeeld glas. Bij de stolling zijn ook zogenaamde cofactoren betrokken. De factoren V en VIII (zie figuur 1) zijn beide cofactoren en circuleren in het bloed in een inactieve vorm. Beide cofactoren worden geactiveerd door trombine. Deze

Tabel 1. Kinetische eigenschappen van de protrombinase (Xa-Va) en tienase ((IXa-VIIIa) complex bestanddelen. In beide gevallen is het duidelijk dat aanwezigheid van fosfolipide membranen de K_m verlaagt en dat de cofactoren de k_{cat} verhogen. (Gegevens afkomstig van Rosing *et al.*, 1980; van Dieijen *et al.*, 1981)

Bestanddeel Protrombinase	K_m (μM)	V_{max} mol IIa·s ⁻¹ ·mol Xa ⁻¹	Relatieve katalytische Efficiëntie
Xa	84	0.011	1
Xa, Va	34	6.22	1,400
Xa, membraan	0.06	0.038	4,800
Xa, Va, membraan	0.21	32.0	1,130,00

Bestanddeel Tienase	K_m (μM)	V_{max} mol Xa·s ⁻¹ ·mol IXa ⁻¹	Relatieve Katalytische Efficiëntie
IXa	181	$1.8 \cdot 10^{-4}$	1
IXa, membraan	0.058	$4.1 \cdot 10^{-5}$	730
IXa, VIIIa, membraan	0.063	8.3	140,000,000

geactiveerde cofactoren, factor VIIIa en Va, hebben zelf geen enzymatische activiteit maar versterken de enzymwerking van de enzymatische stollingsfactor, respectievelijk factor IXa en factor Xa, waarmee ze een complex vormen (Tabel 2). De geactiveerde cofactoren binden dan met één deel van het eiwit aan een geschikt membraanoppervlak en een ander deel van het eiwit bindt dan aan een geactiveerd pro-enzym.

Het effect van de cofactor op de reactie kan beschouwd worden als het resultaat van drie effecten:

- 1) Een verhoging van de omzettingssnelheid (k_{cat}) van de reactie.
- 2) Het lokaliseren van het enzym-cofactor complex aan membraan oppervlakken.
- 3) Bevorderen van de substraatbinding aan het enzym-cofactor complex.

Door de laatste twee genoemde eigenschappen kan een cofactor beschouwd worden als een membraan receptor voor zowel enzym als substraat.

Een belangrijke eigenschap van de bloedstollingsreacties is, dat ze beperkt zijn tot membraanoppervlakken. Deze membranen moeten aan bepaalde eisen voldoen, willen ze geactiveerde bloedstollingsfactoren kunnen binden. Dit soort voor de bloedstolling geschikte oppervlakken worden geleverd door geactiveerde bloedplaatjes en door beschadigde oppervlakken van cellen in de wand van het bloedvat. De aanwezigheid van membranen veranderen de kinetische eigenschappen van de activering en inactivering van stollingsfactoren (zie tabel 1 en Hoofdstuk 3). De affiniteit voor het substraat wordt aanzienlijk verhoogd door de aanwezigheid van fosfolipide membranen (K_m gaat omhoog). De cofactoren factor Va en factor VIIIa hebben een relatief hoge bindingsaffiniteit voor fosfolipide membranen. Deze cofactoren kunnen worden beschouwd als 'receptor' voor de respectievelijke stollingsfactoren Xa en IXa op het membraanoppervlak. Een bijkomend voordeel van de lokalisatie op membraanoppervlakken is daardoor dat de reactiecomponenten in het stollingsproces elkaar gemakkelijker vinden.

Ongecontroleerde bloedstolling zou dodelijk zijn, daarom zijn er mechanismen aanwezig die de bloedstolling controleren en beperken tot de plaats van vaatwand beschadiging. Een van de controlesystemen is de aanwezigheid van enzymremmende stoffen in het bloed. Deze remmers zijn specifiek voor een bepaald enzym en leggen de activiteit van het enzym stil. Ze werken traag, waardoor er tijd zit tussen de start van de bloedstolling en de remming daarvan met als effect dat de stolling eerst goed op gang kan komen en daarna langzaam weer uitdooft.

Het andere systeem is de protein C route. De protein C route remt de bloedstolling sterk door selectieve inactivering van de cofactoren Va en VIIIa (Esmon, 1984). De protein C route omvat het pro-enzym protein C en de cofactor protein S. Het pro-enzym protein C moet net als de andere pro-enzymen eerst geactiveerd worden alvorens zijn functie uit te kunnen oefenen. Protein C wordt geactiveerd door trombine, zodat deze route pas geactiveerd wordt, als de bloedstolling goed op gang is gekomen en er voldoende trombine gevormd is. De cofactor protein S hoeft niet geactiveerd te worden.

Het doel van het onderzoek beschreven in dit proefschrift is om meer inzicht te krijgen in enkele van de mechanismen via welke de trombine vorming wordt gecontroleerd tijdens de hemostase en in het bijzonder door de plasma eiwitten factor V (Va), geactiveerd proteïne C en protein S.

Het eerste hoofdstuk geeft een overzicht van het mechanisme van de bloedstolling en een literatuur overzicht van de huidige inzichten met betrekking tot het werkingsmechanisme van deze eiwitten in de regulatie van protrombine-activering.

Zuivering van factor V uit menselijk (humaan) plasma is beschreven in hoofdstuk 2. Factor V circuleert in plasma in een concentratie van ongeveer 8 µg/ml (~ 25 nM). Vanwege de labiliteit van factor V is complete remming van proteases gedurende de isolatie procedure essentieel. De gehanteerde isolatie procedure voor factor V is ten dele gebaseerd op de procedure ontwikkeld door Dahlbäck (1980) en Suzuki *et al.* (1982). Wijzigingen die, wij hebben aangebracht in de procedure resulteerden in een verminderde isolatietijd, hogere opbrengst, hogere concentratie factor V en minder verontreiniging in het eind preparaat. Gel filtratie (methode om eiwitten op grootte te scheiden) wordt nu uitgevoerd met een Superdex-200 kolom gekoppeld aan een Pharmacia FPLC systeem. Een laatste ion-uitwisselings chromatografie (eiwitten worden op lading gescheiden) stap met een MonoS kolom op het FPLC systeem werd toegevoegd aan de procedure om de laatste onzuiverheden te verwijderen. Dit verbeterde onze factor V zuivering aanzienlijk in termen van snelheid, hoeveelheid en kwaliteit vergeleken met preparaten verkregen op lagedruk chromatografie materialen. Met behulp van deze procedure werd factor V meer dan 4000 maal gezuiverd uit plasma met een opbrengst van 20% in 55 uur.

Protein C is in geactiveerde vorm een belangrijke regulator van factor Va

(zie Hoofdstuk 4) en VIIIa activiteit. Activering van proteïne C door niet fysiologische activatoren is van belang omdat deze activatoren gebruikt kunnen worden als gereedschap bij het bestuderen van structuur-functierelaties van proteïne C, bij het maken van geactiveerd proteïne C (APC) uit proteïne C, en bij het ontwikkelen van functioneel proteïne C analyse bepalingen. Huidige kwantitatieve proteïne C bepalingen methoden gebruiken een proteïne C activator geïsoleerd uit het gif van de slang *Agkistrodon contortrix contortrix*. Deze reactie wordt sterk geremd door calcium ionen en (althans in het geval van menselijk proteïne C) door natriumchloride (NaCl ;keukenzout). Dit verschijnsel is in het bijzonder van belang voor het gebruik van deze activator in kwantitatieve proteïne C test systemen waardoor, door deze gevoeligheid, goed gedefinieerde reactie condities nodig zijn voor wat betreft ionsterkte en vrije calcium concentraties.

Hoofdstuk 3 beschrijft de zuivering en karakterisering van een proteïne C activator uit het slangegif van *Agkistrodon halys halys*. De proteïne C activator uit het *Agkistrodon halys halys* gif werd 533 voudig gezuiverd doormiddel van ionuitwisselingschromatografie op QAE-Sephadex A-50, affiniteitschromatografie op aprotinin-Sepharose (eiwitten worden gescheiden op grond van de specifieke bindingsaffiniteit voor het kolommateriaal, in dit geval aprotinine) en ionuitwisselingschromatografie op een MonoQ kolom in een FPLC systeem. Het gezuiverde enzym is een eiwit bestaande uit een enkele keten met een moleculair gewicht van 36.000 Dalton. Activering van proteïne C is het gevolg van een specifieke splitsing in de zware keten van het molecuul. In het geval van proteïne C activering door zijn fysiologische activator thrombine, is deze splitsing geïdentificeerd als de verwijdering van een klein fragment van 1.400 Dalton van de aminoterminalen zijde van de zware keten (Kisiel, 1979). De proteïne C activator gezuiverd uit *Agkistrodon halys halys* gif activeert proteïne C hoogst waarschijnlijk via hetzelfde mechanisme. De proteïne C activator vertoont een hoge amidolytische activiteit voor de chromogene substraten S2302 en S2238. De activiteit van de activator wordt niet beïnvloed door thiolprotease of metalloprotease remmers. Aanzienlijke remming is daarentegen gemeten wanneer de proteïne C activator werd geïncubeerd met de (serine) protease remmer benzamidine en soybean trypsin inhibitor (een trypsine remmer geïsoleerd uit de soja boon), en vrijwel complete remming met de actieve centrum titrant pNPGb en de remmer PPACK. Dit is een indicatie dat de proteïne C activator van *Agkistrodon halys halys* een serine en een histidine residu in het actieve centrum bevat hetgeen het enzym als een serine protease klassificeert. De gezuiverde protease kan zowel menselijk als runder

protein C activeren. Activering van menselijk protein C met een redelijke snelheid gebeurt alleen in een calcium vrij reactie medium bij een lage ionische sterkte (weinig 'zout' aanwezig). Calcium ionen remmen de activering van menselijk protein C met een schijnbare K_i van 0.8 mM. Natriumchloride (keukenzout) remt ook sterk de activering van menselijk protein C (met een K_i schijnbaar van 20 mM NaCl). Hiermee in tegenstelling wordt de activatie van runder protein C nauwelijks beïnvloed door variaties in calcium of zout (natriumchloride) concentraties in het reactie medium. De schijnbare K_i 's voor calcium ion and zout (natriumchloride) remming van runder protein C activering zijn respectievelijk >10 mM and 220 mM. Kinetische analyse van menselijk protein C activering door de slangegif activator (in een calcium vrij medium en bij lage zout concentratie (ionsterkte)) onthulde een schijnbare K_m voor protein C van $0.52 \mu\text{M}$ en een omzettingssnelheid (k_{cat}) van ruim 10 moleculen protein C per minuut (0.17 s^{-1}). De gevonden catalytische efficiëntie (k_{cat}/K_m) is $3.3 \cdot 10^5 \text{ M}^{-1}\text{s}^{-1}$. Bij hogere ionsterkte wordt de activerings snelheid van menselijk protein C lineair met de protein C concentratie hetgeen duidt op een sterke stijging van de K_m met stijgende ionsterkte. Runder protein C wordt geactiveerd met een K_m van $0.056 \mu\text{M}$ en een omzettingssnelheid van 14 moleculen protein C per minuut (k_{cat} is 0.24 s^{-1}). De catalytische efficiëntie van runder protein C activering ($k_{\text{cat}}/K_m = 4.3 \cdot 10^6 \text{ M}^{-1}\text{s}^{-1}$) is 13 maal beter dan van menselijk protein C activering. De reden daarvoor is een lagere K_m van de gif activator voor runder protein C. Deze cijfers zijn een indicatie voor een grote conformatie en/of een structuurverschil tussen menselijk en runder protein C bij fysiologische zout sterkte. Het lijkt er bovendien op dat in tegenstelling tot runder protein C, de conformatie van menselijk protein C aanzienlijk verandert met veranderende ionsterkte. Protein C activering door trombine wordt ook geremd door calcium ionen, maar als thrombomoduline aanwezig is, heeft deze reactie juist calcium nodig.

Factor Va cofactor activiteit *in vivo* (in levende organismen) wordt afgebroken door geactiveerd protein C (APC) in samenwerking met protein S. In hoofdstuk 4 hebben we het effect van calcium, fosfolipiden (kunstmatige nabootsing van cel oppervlakken) en protein S op de kinetische parameters van APC-gecatalyseerde factor Va geanalyseerd. Hierbij gebruikmakend van gezuiverde menselijke eiwitten. In overeenstemming met eerdere publicaties vonden wij dat factor Va inactivering door APC niet afhankelijk is van de aanwezigheid van fosfolipiden en protein S. De reactie, in afwezigheid van fosfolipiden, vereist de aanwezigheid van calcium. Bij de optimale calcium

concentratie (2 mM) verloopt de reactie met een tweede orde reactie constante van $6.1 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$. De inactiveringsreactie in een fosfolipide vrij reactie medium heeft een hoge K_m voor factor Va ($>78 \text{ nM}$). APC-gekatalyseerde factor Va inactivering wordt aanzienlijk versneld door negatief geladen membraan oppervlakken. De mate waarin de fosfolipiden de reactie stimuleren is afhankelijk van de fosfolipide concentratie en samenstelling. Optimale snelheden van factor Va inactivering worden verkregen in aanwezigheid van $10 \text{ }\mu\text{M}$ fosfolipide vesicles met een hoog percentage anionische (negatief geladen) fosfolipiden. Onder deze reactie condities werd de APC gekatalyseerde factor Va inactivering gekarakteriseerd door een schijnbare tweede orde constante van $1.4 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$. Dit is 230 maal hoger dan de reactiesnelheid in afwezigheid van fosfolipiden in het reactie medium. Het stimulerend effect van fosfolipiden op APC gekatalyseerde factor Va inactivering vertoont een duidelijk optimum voor fosfolipid concentratie en fosfolipid (DOPS/DOPC) compositie. Membranen die enkel zijn opgebouwd uit het neutrale fosfolipide fosfatidylcholine zijn niet in staat de APC gekatalyseerde factor Va inactivering te versnellen. Dus om factor Va inactivering te stimuleren moeten membranen negatief geladen (anionische) fosfolipiden bevatten. Het vermogen om factor Va inactivering te stimuleren is niet beperkt tot fosfatidylserine (een negatief geladen fosfolipide)bevattende membranen aangezien membranen met andere negatief geladen fosfolipiden ook de factor Va inactivering aanzienlijk stimuleren. Het vermogen van verschillende fosfolipiden om de factor Va inactivering te stimuleren neemt toe met de volgorde fosfatidylethanolamine < olie zuur < fosfatidyl zuur < fosfatidylglycerol < fosfatidylmethanol < fosfatidylserine. Versnelling van de APC gekatalyseerde factor Va inactivering door negatief geladen membranen is waarschijnlijk een gevolg van het feit dat APC en factor Va allebei binden aan dergelijke membranen. Dit maakt de inactivering van membraan gebonden factor Va door membraan gebonden APC mogelijk. De schijnbare K_m voor factor Va is aanzienlijk verlaagd in aanwezigheid van fosfolipide membranen in het reactiemedium. Als de concentratie fosfolipiden wordt verhoogd is er een parralelle verhoging van de K_m . Ook de maximale reactie snelheid ($V_{\max}$) van APC gekatalyseerde inactivering van factor Va blijkt een functie te zijn van de fosfolipide concentratie. De $V_{\max}$ neemt toe met de concentratie fosfolipide aanwezig in het reactie medium en bereikt een plateau bij $10 \text{ }\mu\text{M}$ fosfolipide.

Dit suggereert duidelijk, dat in aanwezigheid van fosfolipide (membraan)oppervlak gebonden factor Va geïnactiveerd wordt door oppervlak gebonden APC. Bovendien geven de experimenten beschreven in Hoofdstuk 4

duidelijk aan dat bevordering van proteïne S voor het binden van APC aan membranen van geringe betekenis is in het gezuiverde menselijke systeem.

In een model waarin proteïne S de binding van APC aan fosfatidylserine bevattende membranen bevordert zou men verwachten dat proteïne S het stimulerend effect bij voorkeur zou laten zien bij lage fosfolipide concentraties en op membranen met weinig fosfatidylserine, met andere woorden onder die condities waarbij de snelheid van factor Va inactivering wordt beperkt door de binding van APC aan het membraan. De afwezigheid van stimulatie onder beperkende condities mag beschouwd worden als een ondersteuning van de alternatieve hypothese dat proteïne S het vermogen van factor Xa om factor Va te beschermen tegen inactivering door APC opheft, of dat andere eiwitten (bijvoorbeeld een plasma proteïne S bindend eiwit) nodig zijn voor het tot volle expressie brengen van de antistollings activiteit van proteïne S (Walker, 1986). Bovendien is het mogelijk dat proteïne S andere antistollings eigenschappen bezit, zoals de recentelijk beschreven APC onafhankelijke interactie van proteïne S met factor Va (Heeb *et al.*, 1993). Het is duidelijk dat het werkings mechanisme van proteïne S als antistollings eiwit nog niet vastgesteld is.

Proteolytische enzymen uit slangegif blijken uitstekend gebruikt te kunnen worden als gereedschap bij het bestuderen van structuur en functie relaties in stollingsfactoren. Een proteolytisch enzym instaat tot het inactiveren van factor Va is eerder geïsoleerd uit het gif van de slang *Naja naja oxiana* (NO) (Gerads *et al.*, 1992). Het enzym uit *Naja naja oxiana* gif verandert factor Va in een molecuul met sterk verminderde cofactor activiteit (factor Va_(NO)). Incuberen van factor Va met het gif protease resulteert in een tijds afhankelijk verlies van factor Va activiteit met een stabiele overblijvende activiteit van 10-15%.

In Hoofdstuk 5 beschrijven we de structurele veranderingen in factor Va als gevolg van incubatie met het *Naja naja oxiana* enzym. De ontstane verandering in cofactor activiteit van factor Va_(NO) werd daarna geanalyseerd. SDS-PAGE heeft aan het licht gebracht dat de moleculaire massa van de zware keten veranderde van 105 kD naar ongeveer 100 kD na incubatie met het gif protease. De lichte keten verandert niet na incubatie van factor Va met de NO protease. Binding studies aan vlakke fosfolipide dubbellen in een ellipsometer lieten zien dat factor Va and factor Va_(NO) met gelijkwaardige affiniteit binden aan het fosfolipide oppervlak (K_d 's van respectievelijk 4.2 en 2.8 nM). Uit dit experiment concluderen we dat de verlaagde cofactor activiteit van factor Va_(NO) in de protrombine activering niet veroorzaakt wordt door verlaagde bindingsaffiniteit van factor Va_(NO) aan

fosfolipide membranen. N-terminale aminozuur sequentieanalyse onthulde dat de factor $Va_{(NO)}$ preparaten naast de bekende termini van de zware en lichte keten van factor Va een nieuwe terminus was gevormd met de aminozuur volgorde Asp-Arg-Leu-Glu-Pro-Glu. Vergelijking van de nieuw gevormde N-terminus in factor $Va_{(NO)}$ met de uit de literatuur bekende complete aminozuur volgorde van factor V laten zien dat het slangegif enzym de zware keten splitst tussen His⁶⁸²-Asp⁶⁸³. Hierdoor wordt een stuk van 27 aminozuren afgesplitst van de carboxyterminale zijde van de zware keten. De cofactor activiteiten van factor Va en factor $Va_{(NO)}$ zijn met elkaar vergeleken door het meten van het vermogen om factor Xa gekatalyseerde protrombine activering te ondersteunen in aanwezigheid van membranen (fosfolipiden) en calcium. Factor Va and $Va_{(NO)}$ allebei stimuleren de binding van factor Xa aan negatief geladen membraan oppervlakken. De hoeveelheden van factor Va en $Va_{(NO)}$ die nodig zijn voor half maximale vorming van het protrombinase complex verschilt aanzienlijk (0.7 nM factor Va *versus* 9.42 nM factor $Va_{(NO)}$). Verdere kinetische analyses tonen dat verzadigende hoeveelheden van beide vormen van factor Va in een zelfde k_{cat} voor protrombin activering resulteerde. De K_m voor protrombine bepaald bij verzadigende concentraties van factor $Va_{(NO)}$ is ongeveer 3 maal hoger dan in het geval dat een verzadigende concentratie factor Va werd gebruikt (0.87 μ M *versus* 0.24 μ M). Deze gegevens geven aan dat factor $Va_{(NO)}$ sterk verzwakt is in zijn mogelijkheid om met factor Xa een interactie aan te gaan en dit geldt in mindere mate voor de interactie met protrombine. In combinatie met de kinetische gegevens geeft dit aan dat het kleine carboxyterminale fragment van de zware keten van factor Va nodig is voor een optimale interactie van factor Va met protrombine en factor Xa.

Het is al lange tijd bekend dat gezuiverd factor Va op PA-gels (een methode om eiwitten op grootte te scheiden en door kleuring zichtbaar te maken; zie b.v. hoofdstuk 2, figuur 1) een zware keten van 105kD en twee enigszins verschillende lichte ketens van respectievelijk 74 en 71kD laat zien. Omdat factor Va bestaat uit een complex met een zware en een lichte keten, moeten er twee verschillende vormen van factor Va bestaan. Dit stelde ons voor de volgende vragen 1) Zijn deze twee vormen van factor Va functioneel identiek en 2) Zijn ze afkomstig van twee vormen in bloed circulerend factor V of worden ze gegenereerd tijdens activatie door trombine.

In Hoofdstuk 6 richten we ons op deze vragen en beschrijven we de scheiding en kinetische analyse van de twee factor Va vormen. We scheiden gezuiverd factor Va op een MonoS kolom, factor Va bond aan de kolom en liet los

in gescheiden pieken in een lineair zout (ammoniumchloride; NH_4Cl) gradient. Factor Va met een 74kD lichte keten liet los van de kolom bij 450 mM NH_4Cl (factor Va_1) factor Va met de 71kD lichte keten spoelde daarentegen van de kolom bij 750 mM NH_4Cl (factor Va_2). Dit geeft aan dat de twee factor Va vormen aanzienlijk in lading verschillen. Ongeveer $1/3$ van de factor Va elueerde als factor Va_1 en $2/3$ als factor Va_2 . De twee factor Va vormen vertonen verschillende cofactor activiteiten in het protrombinase complex als ze getest worden onder suboptimale omstandigheden voor wat betreft de factor Xa en fosfolipide concentraties. Gebruikmakend van de snelheid van protrombine activering als een maat voor de hoeveelheid factor Xa-Va complexen gevormd, werd een schijnbare K_d ($K_{1/2\text{Va}}$) voor Xa-Va complex vorming van 5.9 nM voor factor Va_1 en 0.45 nM voor factor Va_2 gevonden. Deze resultaten werden verkregen met membranen waarin een laag percentage negatief geladen fosfolipide aanwezig was (DOPS/DOPC; 5/95; M/M; 2 μM). Wanneer het percentage van de negatief geladen fosfolipide werd verhoogd (tot 20 mole% PS) verdween het verschil in de factor Xa-Va complex vorming (K_d schijnbaar 0.047 nM factor Va_1 versus 0.044 nM factor Va_2). Maar zelfs bij deze membranen kan een verschil tussen factor Va_1 en factor Va_2 gevonden worden als de concentratie fosfolipiden maar laag genoeg wordt. Bij een 20 maal lagere fosfolipide concentratie (0.1 μM) is de K_d schijnbaar voor factor Xa-Va complex vorming is respectievelijk 0.4 versus 0.044 nM for factor Va_1 and factor Va_2 .

Dit betekent dat de verschillende efficiënties waarmee de factoren Va_1 and Va_2 in het protrombinase complex incorporeren bij hogere fosfolipideconcentraties, gecompenseerd worden door een verhoogd aantal bindingsplaatsen. Bij verzadigende factor Va concentraties zijn de snelheden van protrombine activering voor al deze experimenten gelijk. Dit wijst er op dat de twee factor Va vormen dezelfde catalytische activiteit hebben in protrombine activering maar verschillen in de mogelijkheid om Xa-Va complexen te vormen op een fosfolipide oppervlak. Directe bindingsexperimenten aan vlakke fosfolipide dubbellen in een ellipsometer lieten zien dat factor Va_2 bond met hoge affiniteit ($K_d = 0.53$ nM) terwijl de bindingsaffiniteit van factor Va_1 vrij laag was ($K_d = 24$ nM). Deze resultaten geven duidelijk aan dat factor Va_1 and Va_2 niet identiek zijn maar verschillen in de mogelijkheid om te assembleren in een Xa-Va complex op een fosfolipide membraan en dat dit verschil veroorzaakt wordt door een verschil in membraan bindings eigenschappen. Gezuiverd factor V bind ook aan MonopS en spoelt in een zout gradient van de kolom in twee (overlappende) pieken. Na

activering van de factor V bevattende fracties met trombine bleek de factor V die het eerste van de kolom spoelde factor Va met een 74 kD lichte keten opleverde terwijl de bij meer zout eluerende factor V resulteerde in een factor Va met een 71 kD licht chain. Hieruit werd geconcludeerd dat de basis voor de twee factor Va vormen reeds aanwezig is in het factor V molecuul. Als trombine geactiveerde bloedplaatjes op een MonoS kolom gebracht wordt blijkt dat factor Va activiteit van de kolom elueert bij respectievelijk 450 and 750 mM zout (NH_4Cl). Dit geeft aan dat bloedplaatjes factor V ook heterogeen is.

De conclusie uit de experimenten beschreven in Hoofdstuk 6 is dat factor V aanwezig in plasma en bloedplaatjes in twee vormen voorkomt die verschillen in het carboxyterminale domein van factor V (Dat is het domein waaruit de lichte keten gevormd wordt). Na activering verschillen de twee factor Va vormen niet in de interactie met protrombine of in de capaciteit om de catalytische activiteit van factor Xa te bevorderen, maar ze verschillen wel aanzienlijk in de mogelijkheid om te assembleren in een membraan gebonden factor Xa-Va complex veroorzaakt door verschil in bindingsaffiniteit voor membranen. Deze heterogeniteit in het factor V molecuul zou geïntroduceerd kunnen zijn tijdens post-ribosomale behandeling van factor V.

Recentelijk heeft Ortel en zijn medewerkers laten zien dat de structurele basis van de twee lichte ketenvormen op het C2 domein ligt. Hij liet verder zien dat op dit C2 domein van factor Va een fosfatidylserine specifieke bindingsplaats is gelocaliseerd (Ortel *et al.*, 1992). Het is aannemelijk dat deze fosfatidylserine bindingsplaats beter beschikbaar is in factor Va₂, gepaard gaande met een relatief verhoogde positieve lading van de lichte keten.

Nadere experimenten zijn nodig om de vraag op te lossen wat het structurele verschil is tussen de twee factor Va vormen. Verschillen aan de carboxyterminale zijde van factor V zouden zichtbaar gemaakt kunnen worden met de recent ontwikkelde C-terminale aminozuur volgorde bepalingstechnieken. Verschillen in de koolhydraat ketens aan de lichte keten kunnen ook de oorzaak zijn van de factor V heterogeniteit. Een interessante vraag is of er een verschil is in activering van de twee factor V vormen. Verder is het interessant om te zien of op geactiveerde bloedplaatjes factor Xa-Va complex vorming voor beide factor Va vormen verschillend is.

In de literatuur is gerapporteerd dat de lichte keten van factor Va betrokken is bij de binding van geactiveerd proteïne C (APC) aan factor Va. Zowel in factor Va en factor VIIIa lichte keten is een bindingsplaats geïdentificeerd. Deze APC

bindingsplaats ligt op het A3 domein van factor Va en VIIIa (Krishnaswamy *et al.*, 1986; Fay and Walker, 1989; Walker *et al.*, 1990). Het is mogelijk dat de twee factor Va vormen verschillende bindingsaffiniteit hebben voor APC. Dit heeft tot gevolg dat er verschillende inactiverings snelheden zijn voor de twee vormen van factor Va. Een ander fenomeen is het effect van de lichte keten van factor Va op de activering van proteïne C. Salem en medewerkers (1984) lieten zien dat de factor Va lichte keten de activering van proteïne C door trombine versnelt. Ook hier is het (nog) niet duidelijk of beide factor Va vormen in gelijke mate deze reactie versnellen. De effecten van beide factor Va vormen in de proteïne C route, zoals hierboven beschreven, moeten nog onderzocht worden. Verondersteld mag worden dat in de vroege fase van de bloedstolling kleine hoeveelheden geschikte membraan oppervlakken voor factor Va binding worden gevormd. In dat geval zal factor Va₂ (de hoge affiniteit fractie) als eerste zal binden aan het membraan waar het een receptor vormt voor factor Xa. Als er meer geschikte membraan oppervlakken worden gegenereerd (geactiveerde bloedplaatjes) zal ook factor Va₁ binden. Het is mogelijk dat factor Va₁ een belangrijke rol speelt in de proteïne C route. In dit licht is het aannemelijk dat in patiënten met lichte bloedings of trombose neigingen de verhouding van de twee factor Va vormen mogelijk is veranderd. De functie van de twee verschillende factor V (en factor Va vormen) moet nog opgehelderd worden.

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ABBREVIATIONS and GLOSSARY

APC	Activated Protein C.
Apparent value	Quantity that is constant only while specified concentrations are held constant.
BSA	bovine serum albumin
Chromozym-TH	N-Tosyl-L-glycyl-L-prolyl-L-arginine-p-nitroanilide
DEAE	diethylaminoethyl: Chemical group used in anion-exchange chromatography
(DO)PC	(1,2-dioleoyl)-sn-glycero-3-phosphocholine
(DO)PS	(1,2-Dioleoyl)-sn-glycero-3-phosphoserine
EDTA	ethylenediaminetetraacetic acid
FPLC	fast liquid protein chromatography
HEPES	hydroxyethylpiperazineethanesulfonic acid: Good buffer, $pK_a=7.5$.
I-2581	N $^{\alpha}$ -dansyl-(p-guanidino)-phenylalanine-piperidine hydrochloride: Reversible inhibitor of thrombin.
k_{cat}	Catalytic constant: limiting rate divided by total enzyme concentration: also turnover number.
k_{cat}/K_m	Catalytic efficiency: The k_{cat}/K_m determines the specificity for competing substrates, and sets a lower limit on the rate constant for the association of enzyme and substrate.
K_d	Dissociation constant: the free concentration of a substance which result in half maximal binding.
K_i	Inhibitor constant: inhibitor concentration at which the rate of an enzyme catalyzed reaction is half of the rate without inhibitor.
K_m	Michaelis constant: substrate concentration at which the rate of an enzyme catalyzed reaction is half of the limiting rate.
M_r	Relative molecular weight
PMSF	Phenylmethanesulfonyl fluoride
p-NPGB	p-nitrophenyl-p'-guanidino-benzoate hydrochloride: Suicide substrate used for active site titration of trypsin-like enzymes.
PPACK	D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone: Irreversible inhibitor of serine proteases with high affinity for thrombin ($K_i^{thrombin}=2.5$ nM). Chloromethylketones alkylate the active site histidine of the catalytic triad Ser-His-Asp.
QAE	Quaternary aminoethyl: Chemical group used in anion-exchange chromatography
RVV-V	purified factor V activator from Russell's viper venom (Schiffman <i>et al.</i> , 1969; Tokunaga <i>et al.</i> , 1988)
RVV-X	purified factor X activator from Russell's viper venom (Schiffman <i>et al.</i> , 1969)
S2222	H-D-isoleucyl-L-glutamyl-L-glycyl-L-arginine-p-nitroanilide dihydrochloride

S2238	H-D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride: Chromogenic substrate used for thrombin.
S2251	H-D-valinyl-L-leucyl-L-lysine-p-nitroanilide dihydrochloride
S2288	H-D-isoleucyl-L-prolyl-L-arginine-p-nitroanilide dihydrochloride
S2302	H-D-Prolyl-L-phenylalanyl-L-arginine-p-nitroanilide dihydrochloride: Chromogenic substrate used for kallikrein and factor XIIa.
S2337	N-benzoyl-L-isoleucyl-L-glutamyl-piperidyl-glycyl-L-arginine-p-nitroanilide hydrochloride: Chromogenic substrate used for factor Xa.
S2366	L-pyroglutamyl-L-prolyl-L-arginine-p-nitroanilide hydrochloride: Chromogenic substrate used for activated protein C (APC).
S2765	N ^α -Benzylcarbonyl-D-arginyl-L-glycyl-L-arginine-p-nitroanilide hydrochloride
SDS	sodium dodecyl sulfate
SDS-PAGE	polyacrylamide gel electrophoresis in the presence of SDS
Tris	Tris(hydroxymethyl)amino-methane: Buffer $pK_a=8.3$.
V_{max}	Limiting rate: rate approached by an enzyme-catalyzed reaction when the substrate concentrations become very large.

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Harry

Curriculum vitae

Harry Bakker werd geboren op 26 februari 1958 te Krimpen aan den IJssel. Hij behaalde het MAVO diploma in 1975 aan de 'Willem van Oranje' MAVO te Hengelo. In hetzelfde jaar begon hij de analisten opleiding aan de School voor Laboratorium Personeel in Hengelo, die in 1979 werd afgesloten met het HBO-A diploma Klinische Chemie. In dat jaar startte hij zijn studie Biologie aan de Rijksuniversiteit Groningen. In 1987 werd het doctoraal diploma met als specialisatie Moleculaire Genetica behaald. Zijn twee doctoraalvakken waren Moleculaire Genetica en Fysiologische Chemie. Tot december 1988 was hij werkzaam bij de vakgroep Fysiologische Chemie van de Rijksuniversiteit Groningen. Daarna trad hij in dienst als Assistent in Opleiding bij de vakgroep Biochemie van de Rijksuniversiteit Limburg, alwaar hij het onderzoek verrichtte dat heeft geleid tot dit proefschrift.