Peptide Bond Cleavages and Loss of Functional Activity during Inactivation of Factor Va and Factor VaR506Q by Activated Protein C*

Gerry A. F. Nicolaëst, Guido Tans†, M. Christella L. G. D. Thomassen‡, H. Coenraad Hemker†, Ingrid Pabinger§, Katalin Varadi%, Hans P. Schwarz%, and Jan Rosing‡

From the †Cardiovascular Research Institute Maastricht, University of Limburg, Maastricht, The Netherlands, the ‡Department of Hematology and Blood Coagulation, University Hospital, Vienna, Austria, and the §Immuno AG, Vienna, Austria

Factor V was purified from the plasma of an activated protein C (APC)-resistant patient who is homozygous for the mutation Arg506 → Gln (factor VaR506Q). Factor V R506Q was converted by thrombin into factor Va which was further purified yielding a factor Va preparation that had the same cofactor activity in prothrombin activation as normal factor Va. Inactivation of low concentrations of normal factor Va (1–5 nM) by 0.15 nM APC in the presence of phospholipid vesicles proceeded via a biphasic reaction that consisted of a rapid phase (k = 4.3 × 10^5 M^-1 s^-1), yielding a reaction intermediate with reduced cofactor activity that was fully inactivated during the subsequent slow phase (k = 2.3 × 10^4 M^-1 s^-1). Inactivation of factor VaR506Q proceeded via a monophasic reaction (k = 1.7 × 10^5 M^-1 s^-1). Immunoblot analysis showed that APC-catalyzed inactivation of factor Va occurred via peptide bond cleavages in the heavy chain. The rapid phase of inactivation of normal factor Va was associated with cleavage at Arg506 and full inactivation of factor Va required subsequent cleavage at Arg306. The slow monophasic inactivation of factor VaR506Q correlated with cleavage at Arg506. Cleavage at Arg506 in normal factor Va resulted in accumulation of a reaction intermediate that exhibited 40% cofactor activity in prothrombin activation mixtures that contained a high factor Xa concentration (5 nM). Compared with native factor Va, the reaction intermediate retained virtually no cofactor activity at low factor Xa concentrations (0.3 nM). This demonstrates that factor Va that is cleaved at Arg506 is impaired in its ability to interact with factor Xa. Michaelis-Menten kinetic analysis showed that cleavage at Arg506 in membrane-bound factor Va was characterized by a low k_m, for factor Va (20 nM) and k_cat = 0.96 s^-1. For cleavage at Arg506 in factor VaR506Q, the kinetic parameters were k_m = 196 nM and k_cat = 0.37 s^-1. This means that differences between APC-catalyzed inactivation of factors Va and VaR506Q become much less pronounced at high factor Va concentrations. When factor VaR506Q was inactivated by APC in the absence of phospholipids, cleavage at Arg579 of the heavy chain also contributed to factor Va inactivation. Comparison of rate constants for APC-catalyzed cleavage at Arg506, Arg506, and Arg679 in the absence of phospholipids indicated that phospholipids accelerated these cleavages to a different extent. This indicates that the binding of factor Va to phospholipids changes the accessibility of the cleavage sites and/or the sequence of peptide bond cleavage by APC.

Human blood coagulation factor Va is a heterodimeric glycoprotein (1) that consists of a heavy chain (M_r = 105,000) associated via a single Ca^2+ ion with a light chain (M_r = 74,000 or 72,000, cf. Ref 2). Factor Va is formed during hemostasis from the inactive procofactor V after limited proteolysis. Factor Va is an essential nonenzymatic cofactor of the prothrombin-activating complex, which further comprises the serine protease factor Xa, calcium ions, and a procoagulant membrane surface. Depending on the reaction conditions factor Va accelerates prothrombin activation 10^9–10^10-fold (3–5).

Proteolytic inactivation of factor Va by activated protein C (APC), 3 is one of the key reactions in the regulation of thrombin formation. APC-catalyzed cleavage of factor Va is stimulated by the presence of negatively charged membrane surfaces (6–8) and by the protein cofactor, protein S (8–10). The loss of cofactor activity of factor Va is associated with peptide bond cleavages in the heavy chain of factor Va at Arg306, Arg506, and Arg679 (11, 12). The physiologic importance of the down-regulation of factor Va activity by APC is underscored by the observation of recurrent thromboembolic events in individuals that are deficient in either protein C or protein S (13–15).

Since a first publication by Dahlbäck et al. (16), several laboratories have identified the occurrence of familial thrombophilia in a large group of patients with a poor anticoagulant response to APC (APC resistance). APC resistance is at least 10 times more common than all other known genetic thrombosis risk factors together and has an allelic frequency of ~2% in the Dutch population (17). The molecular defect in APC-resistant patients was recently demonstrated (18–21) to be linked to a single-point mutation in the factor V gene that causes an amino acid substitution (Arg506 → Gln) at a site that is cleaved during APC-catalyzed inactivation of factor Va (11).

In this paper we present a kinetic analysis of APC-catalyzed inactivation of human factor Va that was either obtained from

1 The abbreviations used are: APC, activated protein C; BCA, bicinchoninic acid; BSA, bovine serum albumin; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-sn-glycero-3-phosphoserine; factor VaR506Q, abnormal factor Va in which Arg506 is replaced by Gln; p-NPG, p-nitrophenyl-p'-guanidino-benzoate hydrochloride; RVV-X, purified factor X activator from Russel’s viper venom; PAGE, polyacrylamide gel electrophoresis; S2238, D-Phe-(pipecolyl)-Arg-pNA; S2366, L-pyroGlu-Pro-Arg-pNA.

* This work was supported by Program Grant 900-526-192 from the Dutch Organisation for Scientific Research (NWO). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Dept. of Biochemistry, University of Limburg, P. O. Box 616, 6200 MD Maastricht, The Netherlands. Tel.: 31-43-801678; Fax: 31-43-670988.
normal plasma or from the plasma of a patient who is homozygous for the mutation (Arg\textsuperscript{206} → Gln). Parallel experiments were performed in which changes of functional activity of factor Va during inactivation by APC were correlated with the cleavage of peptide bonds in the factor Va molecule. This study was undertaken in order to elucidate the consequences of the molecular defect in factor V for the regulation of in vivo prothrombin activation.

EXPERIMENTAL PROCEDURES

Materials—Hepes, Tris, bovine serum albumin, and Russell’s viper venom were purchased from Sigma. DOPC and DOPS were obtained from Avanti Polar Lipids. The chromogenic substrates S2238 and S2366 were supplied by Chromogenix, Mölndal, Sweden. D-Pro-Phe-Arg-CH\textsubscript{2}Cl was obtained from Calbiochem and p-NPGB was from Nutritional Biochemicals. Micro BCA protein assay kits were from Pierce. Fast protein liquid chromatography equipment and column materials used for protein purification were purchased from Pharmacia, Uppsala, Sweden.

Proteins—Human coagulation factors used in this study were isolated from fresh frozen plasma. Human prothrombin and factor X were purified according to DiScipio et al. (22). Human thrombin was prepared from prothrombin activation described by Pletcher and Nelsenstuen (23). Human factor Xa was obtained from purified factor X after activation with RVV-X and isolation of the activation mixture by affinity chromatography on soybean trypsin inhibitor-Sepharose (24). RVV-X was purified from Russell’s viper venom according to Schiffman et al. (25). Protein C was purified and activated as described by Schellekens et al. (26). The first steps of the purifications of human factor V and factor V\textsuperscript{R506Q} were essentially as described by Dahlbäck (27) with minor modifications (28). The factor V (in 25 mM Hepes, pH 7.5, 50 mM NaCl, 5 mM CaCl\textsubscript{2}) thus obtained was applied to a Resource S column and eluted with 20 ml of a linear gradient (50–1000 mM NaH\textsubscript{2}CO\textsubscript{3}) in 25 mM Hepes (pH 6.5), 5 mM CaCl\textsubscript{2}. Aliquots from column fractions were incubated for 15 min with 5 nM thrombin and factor Va. Factor Va-containing fractions were pooled. Factor Va was prepared by incubating factor V (33 μg/ml) for 20 min with 5 mM thrombin in 25 mM Hepes (pH 7.5), 175 mM NaCl, and 5 mM CaCl\textsubscript{2}. After activation 15 μg D-Pro-Phe-Arg-CH\textsubscript{2}Cl was added to inhibit thrombin. Factor Va was further subjected to fast protein liquid chromatography on a Mono S column (HR 5/5) at room temperature (2). The factor Va bound to the Mono S column was eluted with 15 ml of a linear gradient (50–1000 mM NaH\textsubscript{2}CO\textsubscript{3}) in 25 mM Hepes (pH 7.5), 5 mM CaCl\textsubscript{2}. Factor Va activity eluted from the column in two well-separated protein peaks containing factor Va with light chains of M\textsubscript{r} = 74,000 and 71,000, respectively (2). The two factor Va containing peaks were pooled and brought into 25 mM Hepes (pH 7.5), 175 mM NaCl, 5 mM CaCl\textsubscript{2} by a buffer exchange on a PD-10 column. Factor V and factor Va from an APC-resistant patient were purified by the same methods. The patient was a female with a history of deep vein thrombosis and pulmonary embolism. Plasma from the patient was obtained by plasmapheresis. The APC-resistance ratio was 1.31 (Coatest APC-resistance kit, Chromogenix, Mölndal, Sweden). The patient was identified as homozygous for the substitution G\textsuperscript{506} → A in the factor V gene (arginine acid substitution Arg\textsuperscript{206} → Gln in factor V) by genetic analysis in genomic DNA samples isolated from peripheral blood (29).

Protein Concentrations—Protein concentrations were determined with the micro BCA protein assay kit (30). Molar thrombin and factor Xa concentrations were determined by active-site titration with p-NPGB (31, 32). Prothrombin concentrations were determined after complete activation of prothrombin with Echis carinatus viper venom and quantitation of the loss of thrombin with p-NPGB. APC concentrations were determined with S2366 using kinetic parameters reported by Sala et al. (33). Factor V and factor Va were quantitated as described below.

Phospholipid Vesicle Preparations—Small unilamellar phospholipid vesicles were prepared as described earlier (2). Phospholipid concentrations were determined by phosphate analysis (34).

Gel Electrophoresis and Immunoblot Analysis—Purity of protein preparations was analyzed by SDS-PAGE on 8% acrylamide slab gels (35) and staining with Coomassie Brilliant Blue R-250. The inactivation patterns of factor Va were analyzed after SDS-PAGE, electrophoretic transfer of proteins from the gel to Immobilon-P membranes, and visualization with a polyclonal rabbit-antibody directed against the heavy chain of factor Va and goat-anti-rabbit IgG conjugated with alkaline phosphatase as described by Blake et al. (36).

Factor Va Assay—Factor Va cofactor activity was quantitated by determining the rate of factor Xa-catalyzed prothrombin activation in reaction mixtures that contained a limiting amount of factor Va, a saturating concentration factor Xa, and a fixed (limiting) concentration of factor Va and varying amounts of factor Xa. Factor Xa, factor Va, and phospholipid vesicles were preincubated for 5 min at 37°C in 25 mM Hepes (pH 7.5), 175 mM NaCl, 2 mM CaCl\textsubscript{2}, and 5 mg/ml BSA. Prothrombin activation was started by the addition of prothrombin (preincubated at 37°C in the same buffer) and rates of prothrombin activation were determined with the chromogenic substrate S2238 (4). The apparent K\textsubscript{d} for dissociation of the membrane-bound factor Xa-Va complex (K\textsubscript{DVA}) was obtained from plots of the rate of prothrombin activation as a function of the factor Xa concentration that were fitted to the equation for a single-site binding isotherm (hyperbola) via non-linear least squares regression analysis.

The kinetic parameters (K\textsubscript{m} and V\textsubscript{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 of factor Va 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 regression analysis.

Curve Fitting of Pseudo-first Order Time Courses of Factor Va Inactivation—Time courses of factor Va or factor Va\textsuperscript{R506Q} inactivation by APC were determined by following the loss of cofactor activity of factor Va as a function of time. Data reported in the literature (11, 12) and in this paper demonstrate that the loss of factor Va cofactor activity can proceed via two pathways:

\[
\text{Factor Va} \rightarrow \text{Factor Va}_{\text{int}} \rightarrow \text{Factor Va}_{\text{R506Q}}
\]

(Eq. 1)

\[
\text{Factor Va} \rightarrow \text{Factor Va}_{\text{int}} \rightarrow \text{Factor Va}_{\text{R506Q}}
\]

(Eq. 2)

in which factor Va\text sub int is a reaction intermediate with partial cofactor activity, factor Va\text sub R506Q is a reaction product that has no cofactor activity, k\textsubscript{1} is the first-order rate constant for cleavage of peptide bond 1, k\textsubscript{2} is the second-order rate constant for cleavage of peptide bond 2 in factor Va\text sub int, and k\textsubscript{3} is the second-order rate constant for cleavage of peptide bond 2 in native factor Va.

Under first order conditions, i.e. conditions at which the inactivation rate is directly proportional to the factor Va and APC concentrations, the loss of cofactor activity is described by Equation 3 (36):

\[
\frac{dV_{V}}{dt} = V_{V_{0}} \cdot e^{-k_{1}t} + B + V_{V_{a}} \cdot \left( k_{2} \cdot e^{-k_{2}t} \right) - \left( 1 - e^{-k_{3}t} \right)
\]

(Eq. 3)

in which \(V_{Va}\) is the cofactor activity determined at time \(t\), \(V_{Va_{int}}\) is the cofactor activity determined before APC is added, \(B\) is the cofactor activity of factor Va\textsubscript{int} (expressed as fraction of the cofactor activity of native factor Va), and \(k_{1}, k_{2},\) and \(k_{3}\) are the rate constants defined above. The rate constants and the cofactor activity of factor Va\textsubscript{int} were obtained by fitting the data to Equation 3 using non-linear least squares regression analysis.

In the case of normal factor Va inactivation, \(k_{3}\) appeared to be correlated with cleavage of the peptide bond at Arg\textsuperscript{206}. Therefore, time courses of normal factor Va inactivation were fitted with a fixed \(k_{3}\) obtained from kinetic analysis of factor Va\textsuperscript{R506Q} inactivation.

In some experiments (factor Va inactivation in free solution) there was a slow loss of factor Va cofactor activity in the absence of APC. In these cases the spontaneous loss of cofactor activity (0.4%/min) was added to \(k_{3}\).

RESULTS

Peptide Bonds in Factor Va Susceptible to Proteolysis by APC—Recently it was reported (11) that APC can cleave 3

21159

The Journal of Biological Chemistry

Downloaded from jrnc.org by UCB - Universiteit Maastricht on July 27, 2007. Copyright © 2007 by The Journal of Biological Chemistry.
peptide bonds in the heavy chain of human factor Va that are located at Arg<sup>306</sup>, Arg<sup>506</sup>, and Arg<sup>679</sup>. Fig. 1 represents a schematic diagram of the heavy chain of factor Va and the molecular weights of possible cleavage products. In the present paper we will refer to the rate constants associated with the indicated peptide bond cleavages as $k_{306}$, $k_{506}$, and $k_{706}$, respectively.

Structural and Functional Properties of Factor Va Purified from Normal and from APC-resistant Plasma—Factor V and thrombin-activated factor Va were purified from normal and homozgyous APC-resistant plasma as described under “Experimental Procedures.” The thrombin-activated factor Va and factor Va<sup>R506Q</sup> preparations had identical gel electrophoretic properties with the characteristic heavy chain with $M_r = 105,000$ and a light chain doublet at $M_r = 74,000$ and 71,000 (data not shown).

Factor Va and factor Va<sup>R506Q</sup> had similar cofactor activities in prothrombin activation. Kinetic analysis showed that prothrombin was activated in the presence of 25 μM phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) with the following kinetic parameters: $K_m$ for prothrombin = 0.13 μM, $k_{cat} = 8570 \text{ min}^{-1}$ and $K_{i/Va}$ of Xa-Va complex formation = 0.083 nM for prothrombinase complexes with normal factor Va and $K_m$ for prothrombin = 0.12 μM, $k_{cat} = 8450 \text{ min}^{-1}$ and $K_{i/Va}$ of Xa-Va complex formation = 0.105 nM for prothrombinase complexes with factor Va<sup>R506Q</sup> (data not shown).

This means that the factor Va assay that we used, which measures the cofactor activity of factor Va in prothrombin activation, allows quantitative comparison of factor Va and factor Va<sup>R506Q</sup>.

**Fig. 1.** Schematic representation of APC cleavage sites in the heavy chain of factor Va. The heavy chain of human factor Va contains three peptide bonds that can be cleaved by APC and that are located at Arg<sup>306</sup>, Arg<sup>506</sup>, and Arg<sup>679</sup>. The molecular weights of possible cleavage products correspond to those given in other publications (11, 12).

Inactivation of Factor Va and Factor Va<sup>R506Q</sup> by APC

Kinetic Analysis of APC-catalyzed Inactivation of Membrane-bound Factor Va and Factor Va<sup>R506Q</sup>—Time courses of APC-catalyzed inactivation of factor Va and factor Va<sup>R506Q</sup> in the presence of phospholipid vesicles are shown in Fig. 2. At a low APC concentration (0.15 nM) normal factor Va was inactivated via a biphasic reaction during which 50% of the factor Va activity was lost within 3 min. Factor Va<sup>R506Q</sup> is inactivated at a considerably lower rate requiring more than 45 min for 50% loss of cofactor activity (Fig. 2A). When a similar experiment was performed at a high APC concentration (1.5 nM) inactivation of factor Va<sup>R506Q</sup> appeared to occur via a monophasic reaction (the data points could be fitted with a single exponential), whereas the inactivation of normal factor Va still proceeded via a biphasic reaction (Fig. 2B).

The time courses of factor Va and Va<sup>R506Q</sup> inactivation (% factor Va activity versus time) were not affected by varying the concentrations of factor Va or Va<sup>R506Q</sup> between 0 and 5 nM. Since the rates of factor Va inactivation were also directly proportional to the amount of APC present (0.05–5 nM) we concluded that factor Va and Va<sup>R506Q</sup> inactivation were first-order in both factor Va(Va R506Q) and APC. This allows calculation of apparent second-order rate constants for factor Va inactivation from time courses such as shown in Fig. 2.

On the basis of literature data (11, 12) and experiments presented below it can be postulated that the biphasic time courses of inactivation of normal factor Va presented in Fig. 2 result from rapid cleavage at Arg<sup>506</sup> yielding a reaction intermediate that possesses partial cofactor activity in prothrombin activation. This reaction intermediate is fully inactivated by subsequent slow cleavage at Arg<sup>679</sup>. Inactivation of factor Va<sup>R506Q</sup> likely proceeds via a monophasic reaction that is the result of slow cleavage at Arg<sup>679</sup>.

The time courses of inactivation could indeed be fitted (Fig. 2, solid lines) using an equation that describes the time-dependent loss of cofactor activity when factor Va is inactivated via the random ordered two-step reaction model described under “Experimental Procedures.” The fits of the particular experiments presented in Fig. 2 yielded the following second-order rate constants: $k_{506} = 4.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, $k_{306} = 1.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, and 40% cofactor activity for the reaction intermediate (factor Va cleaved at Arg<sup>506</sup>) in the case of normal factor Va inactivation and $k_{306} = 1.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and a reaction product that has virtually lost its cofactor activity in the case of factor Va<sup>R506Q</sup> inactivation.

Immunoblot Analysis of APC-catalyzed Inactivation of Membrane-bound Factor Va and Factor Va<sup>R506Q</sup>—Generation of heavy chain fragments during APC-catalyzed factor Va and factor Va<sup>R506Q</sup>.

**Fig. 2.** Inactivation of factor Va and factor Va<sup>R506Q</sup> by APC in the presence of phospholipids. 0.3 nM purified human factor Va (A) or factor Va<sup>R506Q</sup> (B) was incubated with (A) 0.15 nM APC or (B) 1.5 nM APC and 25 μM phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl<sub>2</sub>, and 5 μg/ml BSA at 37 °C. At the indicated time points factor Va activity was determined as described under “Experimental Procedures.” The solid lines represent exponential curves obtained after fitting the data using Equation 3 given under “Experimental Procedures.” The rate constants and activity of the reaction intermediate were determined by nonlinear least-squares regression of the data.
factor Va<sup>R506Q</sup> inactivation was analyzed by SDS-PAGE, transfer to Immobilon-P membranes, and visualization of the reaction products with a polyclonal antibody directed against the heavy chain domain of human factor Va (Fig. 3). In the description of the gel patterns presented below, the formation of reaction products was associated with peptide bond cleavages at Arg<sup>679</sup>, Arg<sup>506</sup>, or Arg<sup>679</sup> on the basis of molecular weights of heavy chain fragments indicated in Fig. 1.

During the initial phase of inactivation of normal factor Va, reaction products with M<sub>r</sub> of 75,000 and 26,000/28,000 were formed, which is indicative of cleavage at Arg<sup>506</sup> (Fig. 3A). Prolonged incubation of factor Va with a high APC concentration resulted in the disappearance of the M<sub>r</sub> = 75,000 reaction intermediate (cleavage at Arg<sup>506</sup>) and formation of reaction products with M<sub>r</sub> of 45,000 and 30,000, respectively. The fragment with M<sub>r</sub> = 30,000 stains poorly with our polyclonal antibody and was hardly visible on the gel. During the final stage of the inactivation reaction the peptide bond at Arg<sup>679</sup> was also cleaved, as is indicated by the disappearance of the M<sub>r</sub> = 26,000/28,000 doublet and the formation of low molecular weight products migrating with the dye front in the gel.

The immunoblot of the time course of APC-catalyzed inactivation of membrane-bound factor Va<sup>R506Q</sup> (Fig. 3B) differs from that of normal factor Va. Inactivation of factor Va<sup>R506Q</sup> correlated with the appearance of only two reaction products with M<sub>r</sub> = 60,000/62,000 and M<sub>r</sub> = 45,000, which corresponds with cleavage at Arg<sup>506</sup>. The immunoblot further demonstrates that cleavage at Arg<sup>679</sup> did not significantly contribute to factor Va<sup>R506Q</sup> inactivation since no additional bands became visible in the heavy chain or M<sub>r</sub> = 54,000/56,000 region of the immunoblot. This would have been expected if cleavage at Arg<sup>679</sup> had occurred.

The immunoblot analysis supports the model for APC-catalyzed proteolysis of factor Va presented in the previous section. The rapid phase of the time course of inactivation of normal factor Va is associated with cleavage at Arg<sup>506</sup> and the subsequent slow phase is due to cleavage at Arg<sup>306</sup>. Inactivation of factor Va<sup>R506Q</sup> proceeds via a monophasic reaction which is explained by cleavage at Arg<sup>306</sup>

APC-catalyzed inactivation of Factor Va and Factor Va<sup>R506Q</sup> in the absence of phospholipids—In order to quantify the effect of the membrane on the peptide bond cleavages during factor Va and factor Va<sup>R506Q</sup> inactivation by APC we have also determined time courses of factor Va inactivation in the absence of phospholipids under pseudo-first order conditions (Fig. 4). It was not possible to fit these time courses of inactivation with a single exponential. For both factor Va and factor Va<sup>R506Q</sup> it was necessary to postulate that inactivation proceeded via a biphasic reaction with a partially active reaction intermediate.

To correlate the loss of cofactor activity with peptide bond cleavages in the heavy chain we analyzed the product generation pattern by immunoblot analysis of samples taken from the inactivation mixture at selected time intervals (Fig. 5). Compared with the peptide bond cleavage pattern of membrane-bound factor Va a striking difference was observed. In the absence of phospholipids cleavage at Arg<sup>679</sup> occurred at appreciable rates in factor Va<sup>R506Q</sup> (Fig. 5B). This is concluded from the time-dependent appearance of an additional band in the heavy chain region of the gel.

Thus, it appears that in the case of factor Va<sup>R506Q</sup> inactivation proteolysis at Arg<sup>679</sup> and Arg<sup>306</sup> contributed to factor Va inactivation and that cleavage at Arg<sup>679</sup> preceded cleavage at Arg<sup>306</sup>. Fitting the time course of APC-catalyzed factor Va<sup>R506Q</sup> inactivation (Fig. 4, solid lines) to the equation for a biphasic reaction with a partially active intermediate yields rate constants of 8.0 × 10<sup>3</sup> M<sup>-1</sup> s<sup>-1</sup> and 1.0 × 10<sup>3</sup> M<sup>-1</sup> s<sup>-1</sup> for k<sub>679</sub> and k<sub>306</sub>, respectively, and 70% cofactor activity for the reaction intermediate that has been cleaved at Arg<sup>679</sup>. Immunoblot analysis (Fig. 5A) indicates that during the time courses of normal factor Va inactivation (Fig. 4) only peptide bond cleavages at Arg<sup>506</sup> and Arg<sup>306</sup> significantly contributed to the loss of factor Va cofactor activity. Inspection of the immunoblot that represents inactivation of normal factor Va reveals that cleavage at Arg<sup>679</sup> had a minor contribution to the loss of factor Va cofactor activity. As a first approximation we have, therefore, also fitted this time course with a biphasic exponential. This fit (Fig. 4, solid line) yielded rate constants of 1.3 × 10<sup>3</sup> M<sup>-1</sup> s<sup>-1</sup> and 4.0 × 10<sup>2</sup> M<sup>-1</sup> s<sup>-1</sup> for cleavage at Arg<sup>506</sup> and Arg<sup>306</sup>, respectively, and a reaction intermediate with 40% cofactor activity. It should be emphasized that the values of the latter rate constants may be slightly overestimated due to the...
TABLE I

Rate constants of APC-catalyzed peptide bond cleavages in factor Va and factor VaR506Q

Rate constants of APC-catalyzed inactivation of factor Va and factor VaR506Q were obtained by fitting time courses of factor Va or factor VaR506Q inactivation such as presented in Figs. 2 and 4 to Equation 3 given under "Experimental Procedures." The rate constants for normal factor Va were calculated from the half-time of disappearance of the Michaelis-Menten kinetic parameters. The rate constants and activity of the reaction intermediate were determined by nonlinear least-squares regression analysis of the data.

<table>
<thead>
<tr>
<th>Peptide Bond</th>
<th>Normal factor Va</th>
<th>Factor VaR506Q</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-PL +PL</td>
<td>-PL +PL</td>
</tr>
<tr>
<td>$k_{506}$</td>
<td>$1.4 \times 10^6$</td>
<td>$4.3 \times 10^7$</td>
</tr>
<tr>
<td>$k_{306}$</td>
<td>$3.7 \times 10^6$</td>
<td>$2.3 \times 10^6$</td>
</tr>
<tr>
<td>$k_{679}$</td>
<td>$5.9 \times 10^3$</td>
<td>$7.6 \times 10^3$</td>
</tr>
</tbody>
</table>

a PL, 25 μM phospholipid vesicles composed of 10% DOPS and 90% DOPE.
b ND, not determined.

FIG. 4. Inactivation of factor Va and factor VaR506Q by APC in the absence of phospholipids. 19 nM purified human factor Va (A) or factor VaR506Q (B) was incubated with 390 nM APC in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl$_2$, and 5 mg/ml BSA at 37°C. At the indicated time points factor Va activity was determined as described under "Experimental Procedures." The solid lines represent exponential curves obtained after fitting the data using Equation 3 given under "Experimental Procedures." The rate constants and activity of the reaction intermediate were determined by nonlinear least-squares regression analysis of the data.

The rate constants for APC-catalyzed Cleavage of Peptide Bonds during Inactivation of Free and Membrane-bound Factor Va and Factor VaR506Q—The rate constants for APC-catalyzed cleavage at Arg$_{306}$, Arg$_{506}$, and Arg$_{679}$ in factor Va and factor VaR506Q are summarized in Table I. It was not possible to calculate rate constants for cleavage at Arg$_{679}$ in normal factor Va from the time courses of inactivation presented in Figs. 2 and 4. However, from the disappearance of the Michaelis-Menten kinetic parameters (time points between 10 and 60 min, data not shown) it could be estimated that cleavage at Arg$_{679}$ in free factor Va occurred with $k_{679} = 7.6 \times 10^3$ M$^{-1}$ s$^{-1}$ (t$_{1/2} = 16$ min at 156 nM APC) and that the same peptide bond in membrane-bound factor Va was cleaved with $k_{679} = 7.6 \times 10^3$ M$^{-1}$ s$^{-1}$ (t$_{1/2} = 30$ min at 50 nM APC).

The data presented in Table I show that phospholipids stimulated the APC-catalyzed peptide bond cleavages in factor Va to a different extent. Phospholipids had no effect on $k_{679}$. Furthermore, it is clear that cleavage at Arg$_{306}$ in free factor VaR506Q occurred at a lower rate than in normal factor Va. For membrane-bound factor VaR506Q and factor Va this difference was annulled. These observations are indicative for specific effects of phospholipids and/or prior cleavage at Arg$_{506}$ on the cleavages at Arg$_{306}$ and Arg$_{679}$ (see “Discussion”).

Kinetic Parameters for APC-catalyzed Cleavage at Arg$_{506}$ and Arg$_{679}$ in Membrane-bound Factor Va and Factor VaR506Q—The differences in rate constants for cleavage at Arg$_{506}$ and Arg$_{679}$ in membrane-bound factor Va must result from differences between the Michaelis-Menten kinetic parameters ($k_{cat}$ and/or $K_m$) for these peptide bond cleavages. In order to obtain information on the kinetic basis of the differences we have determined the kinetic parameters, $K_m$ and $k_{cat}$, by measuring the initial rates of factor Va inactivation (between 0 and 20% loss of cofactor activity) at varying factor Va or factor VaR506Q concentrations. The experiment with normal factor Va yields the $k_{cat}$ and $K_m$ for cleavage at Arg$_{506}$ since this peptide bond is preferentially cleaved during the initial phase of inac-
Inactivation of Factor Va and Factor Va<sup>R506Q</sup> by APC in the presence of phospholipids. Initial rates of factor Va inactivation were determined at varying concentrations of factor Va (1) or factor Va<sup>R506Q</sup> (2) that were incubated with 0.28 nM APC (1) or 1 nM APC (2) and 25 μM phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol) in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl<sub>2</sub>, and 5 mg/ml BSA at 37 °C. After different time intervals the factor Va activity was determined as described under “Experimental Procedures” and initial rates of factor Va inactivation were expressed as nm factor Va inactivated/min/nM APC (assuming that normal factor Va is converted into an intermediate with 40% cofactor activity). The solid lines represent a fit of the data according the Michaelis-Menten equation with $K_m = 20 \text{ nM}$ and $V_{\text{max}} = 57.6 \text{ nM}$ factor Va inactivated/min/nM APC ($k_{\text{cat}} = 0.37 \text{ s}^{-1}$) for factor Va<sup>R506Q</sup> (2).

**Fig. 6.** Lineweaver-Burk plots for inactivation of factor Va and factor Va<sup>R506Q</sup> by APC in the presence of phospholipids.

Cofactor Activity of Factor Va after Cleavage at Arg<sup>506</sup> by APC—Pseudo-first-order time courses of inactivation of normal factor Va (Figs. 2 and 4) could only be fitted in a reaction model that consisted of two steps with the formation of a reaction intermediate (factor Va cleaved at Arg<sup>506</sup>) that retained partial cofactor activity.

The actual cofactor activity of factor Va is dependent on its interaction with procoagulant membranes, factor Xa, prothrombin, and on its ability to increase the catalytic activity ($k_{\text{cat}}$) of factor Xa (25–40). To examine which of these factors was impaired in factor Va that is cleaved at Arg<sup>506</sup>, we have characterized the functional properties of the inactivation intermediate. To this end normal factor Va was inactivated by APC in the absence of phospholipids (cf. Fig. 4) and after completion of the first phase of inactivation (Arg<sup>506</sup> cleavage) the reaction mixture was diluted and chromatographed on a Mono S column. The reaction intermediate bound to Mono S and eluted in a NH<sub>4</sub>Cl gradient as an overlapping doublet between 700 and 800 mM NH<sub>4</sub>Cl. These column fractions contained almost pure inactivation intermediate (Fig. 7B).

**Fig. 7.** Cofactor activities of factor Va and factor Va cleaved at Arg<sup>506</sup>. Purified human factor Va (40 nM) was incubated with 15 nM APC in 25 mM Hepes (pH 7.5), 175 mM NaCl, 3 mM CaCl<sub>2</sub>, and 5 mg/ml BSA at 37 °C. After 45 min the inactivation mixture was diluted 10-fold and applied to a Mono S column. Bound protein was eluted with 20 ml of a linear NH<sub>4</sub>Cl gradient (50–1000 mM) in 25 mM Hepes (pH 7.5). Immuno blot analysis of factor Va (A) and the fraction eluting between 700 and 800 mM NH<sub>4</sub>Cl (B) is shown in the inset. Aliquots taken from the inactivation mixture after 0 min (native factor Va) and from the column fraction were used to determine rates of prothrombin activation at (A) 1.1 nM factor Va (1) or (B) 1.2 nM factor Va cleaved at Arg<sup>506</sup> (2) in a reaction mixture containing 25 μM phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol), 0.5 μM prothrombin, and varying concentrations of factor Xa in 25 mM Hepes (pH 7.5), 175 mM NaCl, 2 mM CaCl<sub>2</sub>, and 5 mg/ml BSA at 37 °C. Rates of prothrombin activation were determined as described under “Experimental Procedures” and were corrected for thrombin formed in the absence of factor Va. The solid lines represent hyperbolas obtained after fitting the data with $K_{\text{cat}}$ = 0.088 nM and $V_{\text{max}} = 50.2 \text{ nmol of IIa/min/μg}$ of factor Va ($k_{\text{cat}} = 151 \text{ s}^{-1}$) for factor Va (1) and $K_{\text{cat}}$ = 3.9 nM and $V_{\text{max}} = 28.7 \text{ nmol of IIa/min/μg}$ of protein ($k_{\text{cat}} = 72 \text{ s}^{-1}$) for the column fraction (factor Va cleaved at Arg<sup>506</sup>) (2). Inset, double reciprocal plots of the same data.

To compare the abilities of factor Va and the purified inactivation intermediate to assemble with factor Xa into a membrane-bound prothrombinase complex we determined initial steady-state rates of prothrombin activation at limiting amounts of factor Va or factor Va cleaved at Arg<sup>506</sup> as a function of the factor Xa concentration (Fig. 7, A and B). Low amounts of factor Xa were required for full expression of prothrombinase activity in the case of normal factor Va ($K_{1/2}\text{Xa} = 0.088 \text{ nM}$). With factor Va that was cleaved at Arg<sup>506</sup> much higher factor Xa concentrations ($K_{1/2}\text{Xa} = 3.9 \text{ nM}$) were needed to obtain maximal prothrombinase activity. The $V_{\text{max}}$ of prothrombin activation obtained with the purified inactivation intermediate was lower than that determined for native factor Va. The $V_{\text{max}}$ values could be converted into $k_{\text{cat}}$ values on the basis of the protein contents of the titrated fractions. This yielded $k_{\text{cat}}$ values of 153 and 96 s<sup>-1</sup> for native and cleaved factor Va, respectively.

The interaction of the membrane-bound factor Xa-Va complex with prothrombin was not affected by cleavage at Arg<sup>506</sup>.
Inactivation of Factor Va and Factor Va\(^{R506Q}\) by APC

Fig. 8. The effect of assay conditions and factor Va concentration on the loss of cofactor activity during factor Va inactivation by APC. Purified human factor Va (•) or factor Va\(^{R506Q}\) (□) was incubated with APC and phospholipid vesicles in 25 mM Heps (pH 7.5), 175 mM NaCl, 3 mM CaCl\(_2\), and 5 mg/ml BSA at 37°C. At the indicated time points factor Va activity was determined as described under “Experimental Procedures” in assay mixtures that contained 10 nM or 0.4 nM factor Xa. A, inactivation mixture: 0.8 nM factor Va (•) or 0.8 nM factor Va\(^{R506Q}\) (□), 0.15 mM APC, 25 \(\mu\)M phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol); cofactor assay mixture: 0.4 nM factor Xa. B, inactivation mixture: 210 nM factor Va (•) or 210 nM factor Va\(^{R506Q}\) (□), 2.7 mM APC, 25 \(\mu\)M phospholipid vesicles (DOPS/DOPC, 10/90, mol/mol); cofactor assay mixture: 10 nM factor Xa.

since the \(K_m\) values for prothrombin were virtually the same for prothrombinase complexes with normal and cleaved factor Va (data not shown).

From these data we conclude that APC-catalyzed cleavage at Arg\(^{506}\) of the heavy chain of factor Va results in the formation of a reaction product that has lost part of its cofactor activity mainly because of a reduction of its affinity for factor Xa.

The Effect of the Factor Va Concentration and the Assay Conditions on Observed Time Courses of Inactivation of Membrane-bound Factor Va and Factor Va\(^{R506Q}\) by APC—The data presented in this paper demonstrate that differences in rate constants of cleavage at Arg\(^{506}\) and Arg\(^{306}\) are primarily due to differences in the \(K_m\) for these peptide bond cleavages. This means that at high factor Va concentrations, where the rates of cleavage approach \(V_{\text{max}}\), differences in time courses of inactivation of factor Va and factor Va\(^{R506Q}\) should become less pronounced.

We have also shown (Fig. 7) that the relative cofactor activities of native factor Va and factor Va cleaved at Arg\(^{506}\) strongly depend on the factor Xa concentration presented in the factor Va assay. It was demonstrated that factor Va that is cleaved at Arg\(^{506}\) will exhibit much lower cofactor activity than native factor Va when assayed at factor Xa concentrations below \(K_{\text{VXa}}\) (3.9 nM).

These observations predict that the observed time courses of factor Va or factor Va\(^{R506Q}\) inactivation by APC will depend: 1) on the factor Va concentration in the inactivation mixture and 2) on the assay conditions at which the loss of cofactor activity of factor Va is followed. Compared with factor Va\(^{R506Q}\), normal factor Va will be more rapidly inactivated at low than at high factor Va concentrations. The apparent loss of cofactor activity of normal factor Va will be even more rapid and approach complete loss of activity much faster when samples from the inactivation mixture are assayed at a factor Xa concentration at which the inactivation intermediate has no activity, i.e. \([\text{factor Xa}] < 10^{-3}\) nM (\(K_{\text{VXa}}\)).

These predictions were confirmed in an experiment in which time courses of factor Va inactivation were determined at low (Fig. 8A) or high concentrations of factor Va or factor Va\(^{R506Q}\) (Fig. 8B) and in which samples from the same inactivation mixture were assayed for the loss of cofactor activity in assay mixtures that contained a low (Fig. 8A) or a high factor Xa concentration mixture (Fig. 8B). As predicted, the differences in APC-catalyzed loss of cofactor activity of normal factor Va and factor Va\(^{R506Q}\) were most pronounced when factor Va was inactivated at a low concentration and assayed in a prothrombin activation mixture that contained a low factor Xa concentration (Fig. 8A). Differences in time courses of inactivation of factor Va and factor Va\(^{R506Q}\) were indeed greatly reduced when high factor Va concentrations were inactivated by APC and the loss of cofactor activity was monitored in prothrombin activation mixtures that contained a high factor Xa concentration (Fig. 8B).

DISCUSSION

Proteolytic inactivation of membrane-bound factor Va by APC requires cleavage of two peptide bonds located at Arg\(^{506}\) and Arg\(^{306}\) in the heavy chain domain of factor Va (Fig. 1). At low factor Va concentrations cleavage at Arg\(^{506}\) occurs at a rate that is approximately 20-fold higher than the rate of cleavage at Arg\(^{306}\). This results in the accumulation of a reaction intermediate that is cleaved at Arg\(^{306}\) and that exhibits partial cofactor activity in prothrombin activation (Fig. 2). Cleavage at Arg\(^{306}\), which may occur both in the reaction intermediate and in native factor Va (see below), results in a complete loss of cofactor activity.

The cofactor activity of the factor Va derivative cleaved at Arg\(^{506}\) strongly depends on the factor Xa concentration in the prothrombin activation mixture in which factor Va is assayed. Cofactor activity of the reaction intermediate in prothrombin activation is only observed at high factor Xa concentrations. Compared with native factor Va, full expression of cofactor activity of factor Va that is cleaved at Arg\(^{506}\) requires a 45-fold higher factor Xa concentration. This indicates that cleavage at Arg\(^{506}\) in the heavy chain of factor Va affects its interaction with factor Xa and results in a considerable loss of affinity for factor Xa.

Factor Va\(^{R506Q}\) obtained from factor V purified from the plasma of a homozygous APC-resistant patient is slowly inactivated by APC (cf. Ref 12). This factor Va molecule lacks the cleavage site at Arg\(^{506}\), due to replacement of Arg by Gln (18–21). Time courses of inactivation of low concentrations of membrane-bound factor Va\(^{R506Q}\) could be fitted with an equation for a single exponential curve. Immunoblotting experiments (Fig. 3B) show preferential cleavage at Arg\(^{506}\) during the inactivation of membrane-bound factor Va\(^{R506Q}\). These data indicate that APC inactivates factor Va\(^{R506Q}\) by a single cleavage at position Arg\(^{506}\) which results in the formation of a reaction product that has virtually lost its cofactor activity in prothrombin activation.

Under the conditions of our experiments (low factor Va concentrations) cleavage at Arg\(^{679}\) has a negligible contribution to the loss of cofactor activity in the case of APC-catalyzed inactivation of membrane-bound factor Va or factor Va\(^{R506Q}\). This is concluded from the fact that cleavage at Arg\(^{679}\) (i.e. disappear-
Inactivation of Factor Va and Factor Va\textsuperscript{R506Q} by APC

... of the M\textsubscript{r} = 26,000/28,000 fragment or appearance of doublet bands in the heavy chain of the M\textsubscript{r} = 54,000/56,000 region of immunoblots) is only observed after time intervals at which more than 90% of the cofactor activity of factor Va is already lost.

These data suggest that inactivation of membrane-bound factor Va and factor Va\textsuperscript{R506Q} by APC proceeds via the pathways depicted below.

\[ \text{Factor Va} \rightarrow \text{Factor Va}\text{int} \rightarrow \text{Factor Va}_{\text{int}} \rightarrow \text{Factor Va} \]

\[ k_{506} = 4.3 \times 10^{7} M^{-1} s^{-1} \]

\[ k_{506} = 1.7 \times 10^{6} M^{-1} s^{-1} \]

\[ k_{306} = 2.3 \times 10^{6} M^{-1} s^{-1} \]

\[ k_{306} = 1.7 \times 10^{6} M^{-1} s^{-1} \]

In this scheme factor Va\text{int} exhibits partial cofactor activity and factor Va is a reaction product that has no detectable cofactor activity. We propose that APC-catalyzed cleavage at Arg\textsuperscript{506} and Arg\textsuperscript{306} in membrane-bound factor Va occurs in a random fashion and that cleavage at Arg\textsuperscript{506} is not affected by prior cleavage at Arg\textsuperscript{506}. This is inferred from the fact that the rate constants for cleavage at Arg\textsuperscript{506} calculated from time courses of inactivation of normal and APC-resistant factor Va are approximately the same (\( k_{506} = 2.3 \times 10^{7} M^{-1} s^{-1} \) and 1.7 \( \times 10^{6} M^{-1} s^{-1} \) for normal Va and factor Va\textsuperscript{R506Q}, respectively).

Michaelis-Menten analysis provided additional information regarding the mechanism that accounts for the observed difference between \( k_{506} \) and \( k_{306} \). The kinetic parameters determined for cleavage at Arg\textsuperscript{506} in normal factor Va were: \( K_{m} \) for factor Va = 20 nM and \( k_{cat} = 0.96 \text{ s}^{-1} \). The peptide bond at Arg\textsuperscript{506} in factor Va\textsuperscript{R506Q} was cleaved with \( K_{m} = 196 \text{ nM} \) and \( k_{cat} = 0.37 \text{ s}^{-1} \). The fact that the calculated catalytic efficiencies \( (k_{cat}/K_{m}) \) have the same values as the second-order rate constants calculated for cleavage at Arg\textsuperscript{506} in normal factor Va and for cleavage at Arg\textsuperscript{306} in factor Va\textsuperscript{R506Q} verifies that the Michaelis-Menten kinetic parameters are indeed associated with the indicated peptide bond cleavages. These data suggest that the differences in rate constants for cleavage at Arg\textsuperscript{506} and Arg\textsuperscript{306} mainly result from differences in the functional activity of factor Va and its degradation products is assessed in prothrombin activation mixtures that contain varying concentrations of coagulation factors, while our conclusions are based on experiments at low factor Va concentrations (<10 nM) and assessment of cofactor activity of factor Va in prothrombin activation mixtures that contained varying concentrations of coagulation factors.

To emphasize the importance of reaction and assay conditions, we demonstrated that the apparent loss of factor Va cofactor activity during inactivation by APC indeed strongly depends on the concentration at which factor Va is inactivated and on the prothrombinase conditions at which the loss of cofactor activity is monitored (Fig. 8). Time courses of factor Va and factor Va\textsuperscript{R506Q} inactivation at a high factor Va concentration, in which the loss of cofactor activity of factor Va is followed in prothrombin activation mixtures that contain a high factor Xa concentration, show relatively little difference (Fig. 8B and Ref. 12). However, when factor Va is inactivated at a low concentration and when its cofactor activity is assayed in a prothrombin activation mixture that contains a low amount of factor Xa, the differences between factor Va and factor Va\textsuperscript{R506Q} are profound (Fig. 8A). This observation may have significant physiological consequences. If thrombotic events result from ongoing coagulation at low concentrations of factor Va and factor Va (the plasma factor V concentration is ~25 nM), down-regulation of the cofactor activity of factor Va by APC will be more efficient in the case of normal factor Va and will be maximally impaired in case of factor Va\textsuperscript{R506Q}. This underscores the increased risk for venous thromboembolism in individuals whose plasmas are APC-resistant as a consequence of a factor V phenotype in which Arg\textsuperscript{506} is substituted by Gln.

Acknowledgments—We thank Lico Hoekema and Dr. J. OseW. P. Govers-Rijnslag for technical assistance.

REFERENCES

39. Deleted in proof