Activated Protein C Cleavage of Factor Va Leads to Dissociation of the A2 Domain*

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Kenneth G. Mann‡, Matthew F. Hockin, Kelly J. Begin, and Michael Kalafatis

From the Department of Biochemistry, University of Vermont, College of Medicine, Burlington, Vermont 05405-0068

The products of cleavage of bovine factor Va by activated protein C (APC) in the presence and absence of phospholipid (25% phosphatidylserine, 75% phosphatidylcholine, PCPS) were evaluated using sedimentation velocity/equilibrium methods in the analytical ultracentrifuge and by immunoprecipitation using an antibody directed against the light chain of the factor Va molecule. The molecular weight and sedimentation coefficient of the associated heavy and light chains of factor Va, 173,000 (7.9 S) is reduced to 132,000 (7.1 S) by APC cleavage at Arg505 and Arg662. Complete cleavage of the factor Va heavy chain (with APC-PCPS) at Arg505, Arg662 and Arg306 results in a drastic change in the molecular weight observed for the product. Two products are resolved with sedimentation coefficients of 3.3 and 6.3 S with estimated molecular weights of 48,000 and 114,000, respectively. Immunoprecipitation studies showed that the products of factor Va cleavage at Arg505 and Arg662 (A1A2N, A2C) are mostly noncovalently associated and consequently immunoprecipitated with an antibody directed against the light chain of the factor Va molecule. In contrast, for factor Va cleaved at Arg505, Arg662 and Arg306 the precipitated complex consisted of the A1 domain (residues 1–306) and the light chain (residues 1537–2183) of factor Va (A1-LC). The fragments corresponding to residues 307–505 (A2N) and 506–662 (A2C) are found in the supernatant. The combined mass of these two products (48,000) is similar to the estimated mass of the 3.3 S fragment estimated from sedimentation velocity/equilibrium studies; while the combined mass of the 1–306 + 1537–2183 products corresponds to 114,000, the estimated mass of the 6.3 S fragment. These data lead to the conclusion that cleavages at Arg662, Arg505, and Arg306 of the factor Va molecule resulted in the dissociation of the entire A2 domain as two noncovalently associated fragments (A2N, A2C). Enzyme kinetic and light scattering data suggest that the complete inactivation of the factor Va molecule involves not only cleavage at Arg662 but also the dissociation of the A2 domain. These data also suggest that the complete APC inactivation of the factor Va molecule is analogous to the spontaneous inactivation of factor VIIIa, which occurs via the dissociation of the A2 domain.

Bovine factor V circulates in blood as a single chain 330,000 molecular weight protein (1), which is cleaved by thrombin to give rise to the active species, factor Va, which is composed of a heavy chain (A1A2, residues 1–713) and a light chain (A2C12, residues 1537–2183) (2, 3). The two subunits of the factor Va molecule are associated noncovalently (4) and contribute both the membrane receptor site for factor Xa, the serine protease component of the prothrombinase complex (5, 6) and an effector function which influences the kcat of the enzyme complex. As a consequence, this complex is 300,000-fold more efficient in the activation of plasma thrombin than factor Xa at potential physiologic concentration. In addition to the proteolytic activation steps which result in a 400-fold (at a minimum) increase in activity (7) over factor V, the factor Va molecule is subject to proteolytic regulation by activated protein C (APC)6 generated as a consequence of thrombin-thrombomodulin cleavage of plasma protein C (8). APC cleaves the bovine factor Va heavy chain at Arg505, Arg662 and Arg306 to produce an inactive species (9, 10). The inactivation cleavages occur in sequential fashion with cleavage at Arg505 preceding cleavages at Arg662 and Arg306. Once cleaved the resulting product, factor Va, is no longer capable of binding factor Xa or prothrombin (11). While the cleavages at Arg505 and Arg662 are both influenced by phospholipid (1-palmitoyl-2-oleoyl phosphatidylserine and 1-palmitoyl-2-oleoyl phosphatidylcholine, PCPS), the cleavage at Arg306 is dependent upon the presence of anionic phospholipids (10). The factor Va molecule cleaved only at Arg505 and Arg662 is still capable of binding factor Xa and influencing thrombin activation, but the affinity for factor Xa and the efficiency of prothrombin activation are reduced (10, 12). The present study was undertaken to examine the alterations in factor Va molecular composition associated with cleavage by APC.

MATERIALS AND METHODS

HEPES, Sepharose CL-4B, cyanogen bromide, bovine serum albumin, PS, and PC were purchased from Sigma. Bovine factor Va, bovine factor Xa, and bovine APC were purified as described previously (13, 14). Phospholipid vesicles composed of 75% PC and 25% PS were prepared as described previously (15). The concentration of the phospholipid vesicles in each preparation was determined by phosphorous assay. Dansylarginine 4-ethyl piperidine amide (DAPA) was a gift from Haemalogic Technologies, Essex Junction, VT. Factor Va protein concentrations were estimated spectrophotometrically by absorbance at 280 nm, using ε280nm = 17,416. The compositions of the products of APC cleavage were assessed via SDS-polyacrylamide gel electrophoresis using 5–15% gradient gels under reducing conditions and stained with Coomassie Brilliant Blue (17).

Bovine factor Va was extensively dialyzed against 20 mM HEPES, 0.15 M NaCl, 5 mM CaCl2, pH 7.4 (HBS-Ca2+). Factor Va (2 μM) was incubated with bovine APC (40 nM) in the presence or absence of 20 μM PCPS vesicles. Following a 150-min incubation the reaction was terminated by addition of diisopropyl fluorophosphate to 1 mM.

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† To whom correspondence should be addressed: Dept. of Biochemistry, C401, Given Bldg., University of Vermont, College of Medicine, Burlington, VT 05405-0068. Tel.: 802-656-2220; Fax: 802-862-8229.

‡ The abbreviations used are: APC, activated protein C; PS, 1-palmityl-2-oleoyl phosphatidylserine; PC, 1-palmityl-2-oleoyl phosphatidylcholine; PCPS, phosphatidylcholine, phosphatidylserine vesicles; DAPA, dansylarginine, 4-ethyl piperidine amide; LC, light chain; HC, heavy chain.
versus g decrement coefficients (s20, w samples at 20 °C using a scan interval of 6 min. Weight average sedimentation ultracentrifuge runs. For all calculations a
thesized on 5 to 15% SDS-polyacrylamide gels, prior to and after the analysis. Contributions for multiple species resolved by sedimentation (APC-supplied by Beckman with the X-LI. Estimations of the relative absorbance area ratio for
w i t h1 m l o f 2 0 m M HEPES, 0.15 m NaCl, 5 m M CaCl₂. Elution of the chain antibody, αHFV#1, was coupled to Sepharose CL-4B beads (23). Approximately 30 μg of the factor Va treated with APC (≥PCPS vesicles) was incubated with 300 μl of αHFV-1 beads in 150 μl of buffer. Incubation was allowed to proceed for 30 min with occasional stirrings. Following 30 min of incubation the samples were centrifuged at 10,000 × g for 5 min. The recovered supernatant was saved for analyses of the unbound material. The antibody beads were washed three times with 1 ml of 20 mM HEPES, 0.15 m NaCl, 5 mM CaCl₂. Elution of the beads was performed with 2% SDS. The proteins were visualized on a 5 to 15% polyacrylamide gel electrophoresis (17).

The APC enzyme concentration dependence of the rate of factor Va inactivation process was evaluated by mixing factor Va (1 μM) PCPS (20 μM) and HBS-Ca⁺⁺ at 37 °C with APC over a concentration range from 0 to 5 μM. Following a 3-min incubation period an aliquot of the reaction was removed for assessment of remaining activity in the prothrombin activation assay utilizing 10 nM factor Xa. The remaining sample was quenched into SDS buffer and analyzed by SDS-polyacrylamide gel electrophoresis (17).

Light scattering measurements were made in a photon counting Fluoromax II SPEX fluorimeter (Edison, NJ). Scattering intensity was measured at 320 nm (excitation and emission) with a band pass of 5 nm. Experiments were performed in degassed and filtered (0.2 μM) HBS-Ca⁺⁺ buffer in a fluorimeter cuvette with gentle stirring. Factor Va (100 nM) was incubated with 20 μM PCPS and the scattering intensity monitored. The decrease in scattering intensity upon addition of APC (20 nM) was continuously monitored over 30 min (24). A first order fit of the data was achieved using a single exponential fit. In control experiments, the addition of 10 nM APC to PCPS vesicles in the absence of factor Va resulted in no detectable change in light scattering intensity under the experimental conditions used.

RESULTS AND DISCUSSION

Bovine factor Va was chosen for these studies because of its relative abundance and stability which permits reasonably dependable physical analyses of the protein. The species studied included factor Va, factor Va cleaved by APC, 5 mM Ca⁺⁺, in the

Functionally defined apparent dissociation constants (Kd,app) for factor Xa binding to factor Va-PCPS were inferred from an assay that employs purified reagents and measures thrombin formation (18). In a typical experiment a mixture of prothrombin (1.4 μM) PCPS vesicles (20 μM) and DAPA (3 μM) were incubated in the dark to allow equilibration. At selected time intervals, an aliquot of the mixture was added to a cuvette containing factor Va product (0.5 nM) and the reaction initiated by various concentrations of factor Xa. Thrombin formation was measured using a Perkin Elmer MFP-44A spectrophotometer using excitation and emission wavelengths of 280 and 550 nm (DAPA-thrombin fluorescence) (18). The initial rate of thrombin formation for each factor Xa concentration was calculated and plotted as a function of the factor Xa concentration using the software PRIZM (GraphPad™, San Diego, CA), assuming that factor Xa has one binding site for factor Va.

Sedimentation studies were conducted using a Beckman Optima XL-I analytical ultracentrifuge equipped with UV-visible absorbance scanning and Rayleigh Interference Optics. Sedimentation velocity experiments were performed at 50,000 rpm using an An50-Ti, 8-hole rotor in 12-mm double-sector cells that housed sapphire windows. Data were collected using absorbance optics (λ = 280 nm). The dialysate (see above) was used in the cell reference sector. All velocity analyses were performed simultaneously. The measurements were made on 400-μl samples at 20 °C using a scan interval of 6 min. Weight average sedimentation coefficients (s20, w) were determined by second moment analysis (19). g*(s) analyses were conducted as described previously (20). In both instances the absorbance versus time scans were analyzed on an IBM PS350 using the software package Microlab Origin version 3.78 supplied by Beckman with the X-LI. Estimations of the relative absorbance contributions for multiple species resolved by sedimentation (APC-PCPS cleavage) were made by graphical integration of the g*(s) plots.

Sedimentation equilibrium studies were performed using the high speed meniscus depletion method as described by Yphantis (21). Experiments were performed at 15,500 rpm for 14 h at 19.9 °C. The attainment of equilibrium was assessed empirically by the observation of identical gradients over a 3-h period. Molecular weight average estimates were evaluated using the Optima XL software package Microlab Origin Version 3.78 from Beckman. The reaction products were visualized on 5 to 15% SDS-polyacrylamide gels, prior to and after the analytical ultracentrifuge runs. For all calculations a t of 0.72 was used (22).

Estimates of the molecular weights of species present in heterogeneous mixtures were accomplished by two approaches. One approximation made use of the relationship between sedimentation coefficient and molecular weight for nearly globular proteins.

\[
\frac{S_1}{S_0} = \left( \frac{M_1}{M_0} \right)^{2/3}
\]

(Eq. 1)

In this instance the sedimentation coefficients of the two species observed are taken from the g*(s) analyses of the sedimenting boundaries.

In the second approximation the sedimentation equilibrium profile
absence of phospholipid (designated factor VaAPC, cleavage at Arg505, Arg506, and Arg1752/1753), and factor Va cleaved by APC, 5 mM Ca2+ and PCPS. Unlike human factor Va the bovine factor Va.

The relative absorbance contributions of a and b to the concentration profiles in sedimentation equilibrium experiments were inferred from integration of the areas from the $g^a(s)$ plot. The values of 48,000 and 114,000 provided the best fit to the apparent molecular weights.

TABLE I
Products of APC cleavage of bovine factor Va

| Factor Va species | Composition | Designation | $M_r$ (SDS-PAGE) | $K_{d(app)}$ for factor Xa | Velocity at saturating factor Xa |
|------------------|-------------|-------------|------------------|---------------------------|-------------------------------|
| Va               | 1–713       | Heavy chain (HC) | 94,000           | 0.6 ± 0.13                | 710 ± 40                     |
| VaAPC            | 1–505       | A1–A2A2c   | 70,000           |                           |                               |
|                  | 506–662     | A2c        | 20,000           |                           |                               |
|                  | 663–713     | COOH terminus | –6,000          | 1.3 ± 0.25               | 145 ± 7                      |
| VaAPC/PCPS       | 1–306       | A1         | 40,000           |                           |                               |
|                  | 307–505     | A2A2N     | 28,000           |                           |                               |
|                  | 506–662     | A2c        | 20,000           |                           |                               |
|                  | 663–713     | COOH-terminus | –6,000          | No binding               |                               |
|                  | 1537–1752/3 | LCN       | 30,000           |                           |                               |
|                  | 1753/4–2183 | LCy       | 48/46,000        |                           |                               |

a The velocity is given in nm Ha/min/0.5 nm Hase assuming that at saturating concentrations of factor Xa all factor Va (0.5 nM) will be saturated with factor Xa and the maximum enzyme concentration of active enzyme is 0.5 nM prothrombinase.

b Estimated from

\[
\frac{S_1}{S_0} = \frac{M_a}{M_b}^{\alpha/\beta}
\]

using the mass and $s_{20,w}$ data of Laue (footnote c) for component a. The $s_{20,w}$ and mass used for component b were the Va heavy chain values.

c Reported by Laue et al. (28).

d Fragment mass, assuming a mixture of two noninteracting “ideal” components.

$g^a(s)$ analyses indicated two components, a with $s_{20,w}$ 6.3 and b with $s_{20,w}$ 3.3. The molecular weight estimated for intact factor Va (173,000) is in reasonable agreement to the sum of the heavy and light chain masses estimated from SDS gel electrophoresis (168,000) (Table I) and consistent with the molecular weight reported by Laue and co-workers for bovine factor Va (28). The product of APC cleavage of factor Va (minus PCPS) displayed a heterogeneous molecular weight distribution in sedimentation equilibrium with weight average molecular weight of 132,000. The light chain fragments are known to remain intact following cleavage at Arg1753/1753 (9), and the cleavage at Arg1753 gives rise to fragments of 153,000 and 20,000.
sedimentation data assuming an “ideal” noninteracting two component model. These collected data suggest that following cleavage at Arg 306 the entire A2 domain dissociates from the remainder of the molecule (A1zLC) and exists primarily as the noncovalently associated product A2 NzA2C.

The identical materials used in the sedimentation experiments (Fig. 1) were subjected to immunoprecipitation analyses using an anti-factor Va light chain antibody coupled to Sepharose. The results of this experiment are shown in Fig. 3. The factor Va molecule cleaved at Arg 505, Arg 662, and Arg 1752/1753 (lane 2) is quantitatively immunoprecipitated by the Sepharose coupled anti-light chain antibody (lane 4). No products are found in the supernatant (lane 3). These results are consistent with the conclusion of reversible association of the products of APC cleavage at Arg 505, Arg 662, and Arg 1752/1753. A similar immunoprecipitation experiment conducted with the material cleaved using APC PCPS (lane 5) reveals that while the light chain fragments (LCC and LCN) and the A1 domain are bound to the immunoprecipitate (lane 7) the A2N and A2C fragments are found in the supernatant (lane 6).

The issues then arise as to whether ultimate inactivation is associated with cleavage at position Arg 306 or whether inactivation is associated with cleavage or the dissociation of the components A2NzA2C which together comprise the entire A2 domain of the factor Va heavy chain or both the cleavage and dissociation process. Fig. 4 presents an analysis of the inactivation of factor Va as a function of APC concentration. It can be seen in this plot that the reaction is decidedly nonlinear with respect to enzyme concentration, ultimately becoming independent of enzyme at the highest concentrations evaluated (1.5–5 μM). These inactivation analyses are fundamentally coupled anti-light chain antibody (lane 4). No products are found in the supernatant (lane 3). These results are consistent with the conclusion of reversible association of the products of APC cleavage at Arg 505, Arg 662, and Arg 1752/1753. A similar immunoprecipitation experiment conducted with the material cleaved using APC PCPS (lane 5) reveals that while the light chain fragments (LCC and LCN) and the A1 domain are bound to the immunoprecipitate (lane 7) the A2N and A2C fragments are found in the supernatant (lane 6).

The issues then arise as to whether ultimate inactivation is associated with cleavage at position Arg 306 or whether inactivation is associated with cleavage or the dissociation of the components A2N + A2C, which together comprise the entire A2 domain of the factor Va heavy chain or both the cleavage and dissociation process. Fig. 4 presents an analysis of the inactivation of factor Va as a function of APC concentration. It can be seen in this plot that the reaction is decidedly nonlinear with respect to enzyme concentration, ultimately becoming independent of enzyme at the highest concentrations evaluated (1.5–5 μM). These inactivation analyses are fundamentally
more complicated than the analysis of the spontaneous inactivation factor VIIIa since the inactivating reaction is lipid dependent and because the identification of the factor Va inactivation associated with cleavage at Arg306 must be conducted by quantitative analysis of thrombin generation (at high factor Xa concentrations) rather than simply by a clotting assay. The inset in Fig. 4 illustrates plots of the activity loss, post heavy chain cleavage for two APC concentrations in the experiment described in Fig. 4. These points correspond to the activity losses in the intervals following complete cleavage of the heavy chain, at 2.5 and 1 min following the addition of 1.7 μM (triangle) and 5 μM (squares) APC, respectively. In both cases activity remained after the factor Va heavy chain was entirely cleaved as assessed by gel analyses. However, the activity still remaining (~20%) decays at a rate (k = 0.4 min⁻¹) independent of the enzyme concentration. These data suggest an enzyme independent process, consistent with dissociation of the A2 domain fragments, is associated with complete activity loss. However since the rate of the cleavage-independent activity loss overlaps the Arg306 cleavage rate it is not possible to assign a totally independent value to the residual activity present in the factor Va cleaved at Arg305 and Arg306 product prior to dissociation.

The rate of mass reduction following APC inactivation of factor Va was studied using right angle light scattering. The inset in Fig. 4 illustrates plots of the activity loss, post heavy chain cleavage for two APC concentrations in the experiment described in Fig. 4. These points correspond to the activity losses in the intervals following complete cleavage of the heavy chain, at 2.5 and 1 min following the addition of 1.7 μM (triangle) and 5 μM (squares) APC, respectively. In both cases activity remained after the factor Va heavy chain was entirely cleaved as assessed by gel analyses. However, the activity still remaining (~20%) decays at a rate (k = 0.4 min⁻¹) independent of the enzyme concentration. These data suggest an enzyme independent process, consistent with dissociation of the A2 domain fragments, is associated with complete activity loss. However since the rate of the cleavage-independent activity loss overlaps the Arg306 cleavage rate it is not possible to assign a totally independent value to the residual activity present in the factor Va cleaved at Arg305 and Arg306 product prior to dissociation.

The rate of mass reduction following APC inactivation of factor Va was studied using right angle light scattering. The data presented in the Fig. 5 illustrate the light scattering change associated with addition of APC (20 nM) to a factor Va (100 nM), PCPS (20 μM) reaction mixture. A dramatic decrease in light scattering intensity occurs upon addition of APC to the factor Va-phospholipid vesicle complex. At these concentrations the cleavage reactions would have been complete within approximately three minutes an interval accounting for about 70% of the total scattering intensity reduction. Thus cleavage and dissociation are occurring on roughly the same time scale. A 1st order exponential approximation to the data would suggest a dissociation rate k = 0.5 min⁻¹ similar to the rate of the nonenzymatic factor Va decay rate (Fig. 4, inset).

The collected data lead to the conclusion depicted in Fig. 6. The complete inactivation of the factor Va molecule which occurs with cleavage of the heavy chain at Arg306, Arg505, and Arg662 results in the dissociation of the A2 domain fragments from the remainder of the factor Va molecule. The larger product at 6.3 S is a noncovalently associated trimeric complex composed of A1-LC (Mr 114,000). This product is incapable of binding factor Xa or prothrombin or serving in the prothrombinase complex (Fig. 2). The dissociated product (s) corresponds to the entire A2 domain, has a s20,W of 3.3 and is composed of two peptides A2b and A2c, with a combined molecular weight of 48,000. It is likely, based upon the molecular weight estimated from the s20,W of the component b and the best fit of sedimentation equilibrium data (Table II) that the two fragments, are noncovalently associated, most likely in a reversible interaction.

The observation that the inactivating cleavage at Arg306 results in the dissociation of the A2 domain suggest that the inactivated product, factor Vai, is equivalent in gross structure to the inactive factor VIIIa product which results following the thrombin activation cleavage of factor VIII at Arg272 (29). This required activation cleavage in factor VIII (at Arg372) results in the subsequent dissociation of the A2 domain and the spontaneous inactivation of factor VIIIa (30, 31). The factor Va APC cleavage at Arg306 produces a result similar to the Arg272 cleavage in factor VIII resulting in dissociation of the A2 domain and loss of factor Va activity. The process for factor Va occurs at a faster rate (>0.5 min⁻¹) than the process for factor VIIIa A2 dissociation (0.32 min⁻¹) (32).

The mechanism of complete inactivation of factor Va by APC-PCPS has been controversial. The present data are consistent with the mechanism illustrated in Fig. 6 in which activity is totally lost following cleavage at Arg306 and the largely irreversible dissociation of the A2 domain fragments. Under the experimental conditions described in the present study (≥6 μM Va) it is clear that any affinities of the ultimate cleavage products for each other (if present at all) are relatively weak. However, since the dissociation of the A2 fragment is most likely the direct cause of the total factor Va inactivation, it is entirely possible that the rate-limiting step of factor Va inactivation can be shifted from peptide bond (Arg306) cleavage to A2 domain fragment dissociation by other constituents of the reaction mixture. In addition the products of the cleavage at Arg306 also appear to engage in a reversible binding phenomena which may also be influenced by third component binding. For example, it has been reported and confirmed by numerous studies that factor Xa stabilizes the factor Va molecule from inactivation by APC (33–36). Similarly it has been shown that factor IXa stabilizes the thrombin activated factor VIIIa molecule presumably by preventing dissociation of the A2 subunit (37, 38). The stabilization of factor Va by factor Xa may be, in part, a consequence of an altered dissociation of the products following APC cleavage. If this is indeed the case the presence of factor Xa or prothrombin during APC inactivation may be major confounding elements in kinetic evaluations of factor Va inactivation but elements that are biologically relevant.

The ultimate deconvolution of the relative activity losses associated with factor Va cleavage at position 306 and the activity loss associated with the dissociation of the fragments of the A2 domain is a matter that will require further extensive experimental analysis. However the present results clearly indicate that the kinetics of APC inactivation of factor Va are associated with complex processes which influence both the binding of the enzyme factor Xa and the effector function of factor Va in the prothrombinase complex and include both the proteolysis and the dissociation of fragments of the A2 domain ultimately leading to a totally inactivated factor Va molecule.

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