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Activated Protein C-catalyzed Proteolysis of Factor VIIIa Alters Its Interactions within Factor Xase*

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Factor VIIIa, the cofactor for the factor IXa-dependent conversion of factor X to factor Xa, is proteolytically inactivated by activated protein C (APC). APC cleaves at two sites in factor VIIIa, Arg^{336}, near the C terminus of the A1 subunit; and Arg^{562}, bisecting the A2 subunit (Fay, P., Smudzin, T., and Walker, F. (1991) *J. Biol. Chem.* 266, 20139–20145). Factor VIIIa increased the fluorescence anisotropy of fluorescein-Phe-Phe-Arg factor IXa (Fl-FFR-FIXa; K_d = 42.4 nM), whereas cleavage of factor VIIIa by APC eliminated this property. Isolation of the APC-cleaved A1/A3-C1-C2 dimer ($A1^{336}$/A3-C1-C2), and the fragments derived from cleaved A2 subunit ($A2_N$/$A2_C$), permitted dissection of the roles of individual cleavages in cofactor inactivation. Intact A1/A3-C1-C2 dimer increased Fl-FFR-FIXa anisotropy and bound factor X in a solid phase assay, while these activities were absent in the $A1^{336}$/A3-C1-C2. However, the residues removed by this cleavage, Met^{337}–Arg^{372}, did not directly participate in these functions since neither a synthetic peptide to this sequence nor an anti-peptide polyclonal antibody blocked these activities using intact dimer. CD spectral analysis of the intact and truncated dimers indicated reduced α and/or β content in the latter. The A1/A3-C1-C2 dimer plus A2 subunit reconstitutes cofactor activity and produced a factor VIIIa-like effect on the anisotropy of Fl-FFR-FIXa. However, when A2 was replaced by the $A2_N$/$A2_C$ fragments, the resulting fluorescence signal was equivalent to that observed with the dimer alone. These results indicate that APC inactivates the cofactor at two levels within the intrinsic factor Xase complex. Cleavage of either subunit modulates the factor IXa active site, suggesting an essential synergy of interactive sites in factor VIIIa. Furthermore, cleavage of the A1 site alters the conformation of a factor X binding site within that subunit, thereby reducing the affinity of cofactor for substrate.

Factor VIII, the plasma protein deficient or defective in individuals with hemophilia A, is synthesized as a 300-kDa precursor protein (1, 2) with domain structure A1-A2-B-A3-C1-C2 (3). Factor VIII is processed to a series of divalent metal ion-linked heterodimers (4–6) by cleavage at the A3-B junction, generating a heavy chain minimally represented by the A1-A2 domains which may possess all or part of the B domain and a light chain consisting of the A3-C1-C2 domains.

Factor VIII functions as the cofactor for factor IXa in the intrinsic factor Xase[1] complex, where it increases the k_{cat} for conversion of factor X to factor Xa by several orders of magnitude (7). Factor VIII must first be activated, through limited proteolysis by thrombin, to the active cofactor form, factor VIIIa. Thrombin cleaves the factor VIII at two sites within the heavy chain, Arg^{740} and Arg^{372} (8). The former liberates the B domain or its fragments, while the latter bisects the A1 and A2 domains. Thrombin also cleaves near the N terminus of the light chain, at Arg^{1689} (8). Thus factor VIIIa is a heterotrimer of A1, A2, and A3-C1-C2 subunits.[2] The A1 subunit and the A3 domain retain the metal ion linkage and this stable dimer (A1/A3-C1-C2) (9, 10) is associated with the A2 subunit mainly through electrostatic forces (11). The factor VIIIa subunits can be isolated separately and are inactive. However, factor VIIIa activity can be reconstituted upon combining the isolated A1/A3-C1-C2 dimer and the A2 subunit (11–13).

Activated protein C, a potent anticoagulant, proteolytically inactivates the cofactors, factors VIIIa and Va, in a surface-dependent reaction (see Ref. 14 for review). The protease binds the light chain (-derived) subunit of the cofactors (15, 16) at a region localized to the C-terminal end of the A3 domain (A3-C1 junction) (17). Inactivation results from cleavage of the heavy chain (-derived) subunits. Activated protein C cleaves factor VIIIa rapidly at Arg^{562} (9), which bisects the A2 subunit, and slowly within the A1 subunit at Arg^{336} (8, 9), which releases a highly acidic region from the C terminus. The cleavage of the A2 subunit most closely correlates with factor VIIIa inactivation (9).

The functions of the activated protein C-catalyzed cleavages are not well understood. In this report, we examine the mechanisms for loss of cofactor activity following proteolytic inactivation of factor VIIIa. Analyses using activated protein C-

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[1] The abbreviations used are: factor Xase, the phospholipid-bound complex of factor VIIIa and factor IXa; BSA, bovine serum albumin; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; RVV-X, factor X activator from Russell's viper venom; Fl-FFR-FIXa, factor IXa modified in its active site with fluorescein-Phe-Phe-Arg chloromethyl ketone; $A2_N$/$A2_C$, the A2 subunit N- and C-terminal fragments, respectively, after activated protein C cleavage at Arg^{562}; $A1^{336}$/A3-C1-C2, the A1/A3-C1-C2 dimer cleaved by activated protein C at Arg^{336}; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

[2] Factor VIIIa subunits are designated relative to the domain sequence A1-A2-B-A3-C1-C2 (3) and are as follows: A1, residues 1–372; A2, residues 373–740; A3-C1-C2, residues 1690–2332. Noncovalent subunit associations are denoted by "/" and covalent associations are denoted "-."

cleaved factor VIIIa fragments allow dissection of the effect of each cleavage event relative to factor VIIIa function. Results of this study show that cleavage of both the A1 and A2 subunits affects the orientation of cofactor with the active site of factor IXa, whereas cleavage at A1 results in a reduced affinity of cofactor for substrate factor X.

MATERIALS AND METHODS

Reagents—Recombinant factor VIII was generously provided by Debra Pittman of the Genetics Institute and by Dr. Jim Brown of Bayer Corp. The reagents α-thrombin and factors X and Xa (Enzyme Research Laboratories). Phe-Pro-Arg chloromethyl ketone (Calbiochem), activated protein C and RVV-X (Haematologic Technologies), Fl-FFR-FIXa (Molecular Innovations), the antibody ESH-8 (American Diagnostica), PSPCPE (Sigma), and the chromogenic substrate for factor Xa, S-2765 (N^{α}-benzoyloxycarbonyl-D-arginyl-L-glycyl-L-arginyl-*p*-nitroanilide dihydrochloride; Kabi/Chromogenix) were purchased from the indicated vendors. PSPCPE vesicles (20, 40, and 40%, respectively) were prepared as described previously (18). The monoclonal antibody R8B12 (11) and rabbit antibody prepared to factor VIII residues 337–372 (19) were described previously. Preparation of a rabbit antibody to a synthetic peptide corresponding to factor VIII residues 403–427 will be described elsewhere.

Proteins—Factor VIIIa (20) and factor VIIIa subunits (11) were prepared from recombinant factor VIII as described previously. Activated protein C-cleaved factor VIIIa was prepared as follows. Factor VIII (1.2 μM) in Buffer A (20 mM Hepes, pH 7.2, 100 mM NaCl, 2 mM $CaCl_2$, 0.01% Tween 20) was activated with thrombin (24 nM) and at peak activity, hirudin (6 units) was added to stop the reaction. PSPCPE vesicles (100 μg/ml) and activated protein C (120 nM) were then added, and the reaction was run at 37 °C for 30 min to inactivate the factor VIIIa. Activated protein C was inactivated with Phe-Pro-Arg chloromethyl ketone when factor VIIIa activity was less than 5% of initial. Purification of the $A1^{336}$/A3-C1-C2 dimer and $A2_N/A2_C$ fragments was accomplished using Mono S chromatography. The cleaved factor VIIIa was dialyzed against Buffer B (10 mM histidine, pH 6.5, 100 mM NaCl, 5 mM $CaCl_2$, 0.01% Tween) for 2 h at room temperature and applied to a Mono S column equilibrated in Buffer B. Protein was eluted with a 30-ml gradient of 0.1–1 M NaCl in Buffer B. Fractions containing $A2_N/A2_C$ (eluting at approximately 200 mM NaCl) and $A1^{336}$/A3-C1-C2 (eluting between 0.4 and 0.6 M NaCl) were pooled separately and dialyzed against Buffer B for 2 h. Pools were individually reapplied to the Mono S column equilibrated in Buffer B. The column was eluted with a 60-ml gradient of 0.1–1 M NaCl in Buffer B. Resultant $A2_N/A2_C$ and $A1^{336}$/A3-C1-C2 were concentrated using Ultrafree-MC microconcentrators (Millipore) with molecular weight cut-off values of 5000 and 10,000, respectively. The concentrators were pretreated with 5% Tween 20 for 2 h at room temperature prior to use. Yields of $A1^{336}$/A3-C1-C2 and $A2_N/A2_C$ were approximately 35 and 15%, respectively, of the predicted recovery.

Electrophoresis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using the method of Laemmli (21) with a Bio-Rad minigel system. Electrophoresis was carried out at 150 V for 1 h.

Assays—Factor VIIIa activity was determined using a one-stage clotting assay with substrate plasma that had been chemically depleted of factor VIII activity (22). Factor VIIIa reconstitution assays were performed using purified A1/A3-C1-C2 dimer and the A2 subunit (20 nM each). Reactions were run in Buffer A containing BSA (0.5 mg/ml) and inosithin (100 μg/ml) for 50 min at room temperature. Other additions are indicated in the appropriate figure legends. Protein concentrations were determined by the Coomassie Blue dye binding method of Bradford (23) using BSA as the standard.

Fluorescence Anisotropy—Fluorescence anisotropy measurements were made using a SPEX Fluorolog 212 spectrophotometer operated in L format. Excitation and emission wavelengths were 495 and 520 nm, respectively. Anisotropy measurements were made by manually rotating the polarizers and monitoring the fluorescence for 10 s at each position. Three determinations were made at each position and the average obtained. The microcell (200 μl) initially contained PSPCPE vesicles (50 μg/ml) in Buffer A as a blank and was subtracted from all subsequent measurements. After the blank was measured, Fl-FFR-FIXa (20 nM) was then added to the microcell and the initial anisotropy determined. Titrations were performed by consecutively adding the titrant and determining the anisotropy.

Solid Phase Factor X Binding Assay—Microtiter wells were coated with ESH-8 (1 μg IgG/well), which recognizes residues 2248–2285 within the C2 region of the factor VIII light chain (24), in 15 mM Na_2CO_3/35 mM $NaHCO_3$, pH 9.6, at 4 °C overnight. Wells were washed three times (200 μl/well) with Buffer C (20 mM Hepes, pH 7.2, 5 mM $CaCl_2$, 100 mM NaCl, 0.1% Tween 20) and blocked with Buffer C containing 2% BSA (200 μl/well) for 1 h at room temperature. Either A1/A3-C1-C2 or $A1^{336}$/A3-C1-C2 (50 nM in Buffer C containing 1% BSA; 100 μl/well) was incubated in the wells for 2 h at room temperature. The plate was washed as above and factor X (0–2 μM) was then added (100 μl/well in Buffer C containing 1% BSA) and incubated at room temperature for 2 h. The plate was washed five times as above and RVV-X activator was added (0.5 μg/ml in 20 mM Tris, pH 7.2, 200 mM NaCl, 5 mM $CaCl_2$; 100 μl/well) and incubated for 15 min at room temperature. The chromogenic substrate S-2765 was then added (200 nM), and the plate was read at 405 nm for 20 min using a V_{max} microtiter plate reader (Molecular Devices). Rates of chromogen conversion were compared with a standard of factor Xa alone and the amount of factor X bound to the A1/A3-C1-C2 or $A1^{336}$/A3-C1-C2 was determined.

Circular Dichroism—Native and truncated forms of the A1/A3-C1-C2 dimers were dialyzed against 0.15 M NaCl and 5 mM $CaCl_2$ for 24 h (several changes) at 4 °C. The samples were filtered using Gelman Acrodisc filters (0.2 μm) that had been treated overnight with 5% Tween 20 and thoroughly rinsed with the dialysis solution before use. CD spectra were recorded from 200–250 nm at room temperature on a Jasco-710 CD spectrometer. Protein samples (45–50 μg/ml; ~400 nM) were scanned in a cylindrical quartz cuvette (1-mm path length) for an average time of 16 s at every 0.1 nm. For all CD spectra, a buffer base line was subtracted. The observed ellipticity (Θ_{obs}) was converted to a mean residue ellipticity (Θ), degree·cm^2 dmol^{-1}, using the relationship $\Theta = (\Theta_{obs} \cdot MRW)/(10 \cdot d \cdot c)$, where d is the path length in centimeters, c is the concentration in grams/milliliter and the mean residue weight (MRW) is 115.

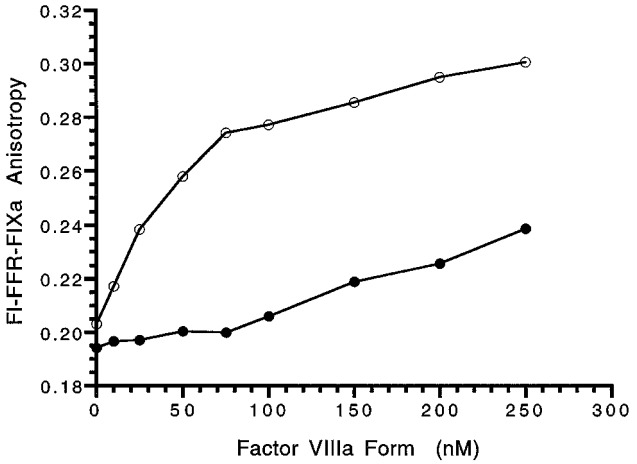

FIG. 1. **Fluorescence anisotropy of Fl-FFR-FIXa in the presence of cleaved or native factor VIIIa.** Anisotropy of Fl-FFR-FIXa was measured as described under "Materials and Methods." Reactions containing Fl-FFR-FIXa (20 nM) and PSPCPE (50 μg/ml) in buffer A were titrated with either native (*open circles*) or cleaved (*closed circles*) factor VIIIa at the indicated concentrations. Data for the intact factor VIIIa were fitted to a quadratic equation (43). Fitted constants are: K_d = 42.4 ± 18.7; number of sites (n) = 0.91 ± 1.2.

RESULTS

Effect of Activated Protein C Cleavage of Factor VIIIa—Since the fluorescence anisotropy of Fl-FFR-FIXa is differentially modulated by factor VIII and factor VIIIa (25, 26), this property was used as a spectroscopic assay to evaluate the effect of activated protein C on factor VIIIa interaction with Fl-FFR-FIXa. Cleaved or intact factor VIIIa was titrated into a sample containing Fl-FFR-FIXa (20 nM) and phospholipid, and the anisotropy was measured (Fig. 1). Intact factor VIIIa produced a saturable increase (K_d = 42.4 ± 18.6 nM) in the fluorescence anisotropy of Fl-FFR-FIXa. An incremental change of 0.097 was observed at near-saturating (250 nM) factor VIIIa. However, the activated protein C-cleaved factor VIIIa resulted in a markedly reduced effect on the Fl-FFR-FIXa anisotropy. Based upon competition experiments described below, the reduced anisotropy did not result from a reduced affinity of cleaved

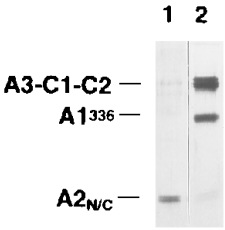

FIG. 2. **SDS-polyacrylamide gel electrophoresis of purified, activated protein C cleaved-factor VIIIa subunits.** $A2_N/A2_C$ and $A1^{336}$/A3-C1-C2 were isolated following Mono S chromatography and are shown in gel *lanes 1* and *2*, respectively. The gel was stained with silver nitrate.

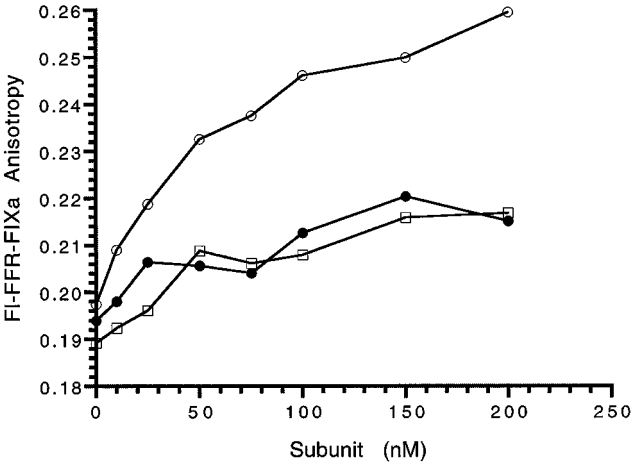

FIG. 3. **Fluorescence anisotropy of Fl-FFR-FIXa in the presence of A1/A3-C1-C2, $A1^{336}$/A3-C1-C2, or factor VIII light chain.** Reactions containing Fl-FFR-FIXa (20 nM) and PSPCPE (50 μg/ml) in buffer A were titrated with A1/A3-C1-C2 (*open circles*), $A1^{336}$/A3-C1-C2 (*closed circles*), or light chain (*open squares*) at the indicated concentrations.

factor VIIIa for factor IXa. That the loss of the factor VIIIa-dependent change in Fl-FFR-FIXa anisotropy correlated with activated protein C-catalyzed cleavage of factor VIIIa indicated that activated protein C abolished the ability of the factor VIIIa to properly modulate the active site of factor IXa.

Role of Specific Cleavages in Factor VIIIa—To dissect the mechanism of cofactor inactivation by activated protein C, we prepared and separately isolated the proteolytically-derived products following digestion of factor VIIIa. Two products (shown in Fig. 2) were obtained following Mono S chromatography. Cleavage at Arg^{336} (9) liberates the C-terminal acidic region of the A1 subunit to generate the metal ion-linked dimer designated $A1^{336}$/A3-C1-C2. This truncated dimer eluted in a broad peak from the Mono S column. Activated protein C bisects the A2 subunit at Arg^{562} (9) to yield two fragments, designated $A2_N$ and $A2_C$. These latter fragments co-eluted early during Mono S chromatography. The presence of both A2 fragments in this preparation was verified by Western blotting (not shown), using monoclonal antibody R8B12 (11), that recognizes an epitope in $A2_C$ (9) and a rabbit antipeptide antibody prepared to factor VIII residues 403–427 that reacts with $A2_N$.

To examine effects of A1 cleavage, the A1/A3-C1-C2 or $A1^{336}$/A3-C1-C2 dimer was titrated into a sample containing Fl-FFR-FIXa (20 nM) and PSPCPE vesicles (50 μg/ml). Results (Fig. 3) show that the A1/A3-C1-C2 dimer produced a saturable increase in the anisotropy of Fl-FFR-FIXa with an incremental increase of 0.063 at 200 nM dimer. When $A1^{336}$/A3-C1-C2 was used in place of the native dimer, there was minimal change (~0.02) in the anisotropy of the Fl-FFR-FIXa. This affect was similar to that observed using isolated factor VIII light chain, which contains the high affinity binding site for factor IXa (27). Thus, it appears the activated protein C cleavage at the A1 site eliminated the contribution of the A1 subunit to modulate the active site of factor IXa. However, the acidic region of the A1 subunit (residues 337–372), which is removed following activated protein C cleavage at Arg^{336} (9), did not appear to be directly involved in this interaction, since neither high concentrations (50 μM) of synthetic peptide $FVIII^{336-372}$ corresponding to this region, nor a rabbit antibody made against this peptide (19), affected the ability of the intact A1/A3-C1-C2 to modulate the anisotropy of the FL-FFR-FIXa (data not shown). Therefore, cleavage of the acidic terminal region of A1 may result in some conformational change that renders this subunit unable to modulate the Fl-FFR-FIXa active site.

An alternate possibility is that the cleavage at the A1 site resulted in a marked reduction in the affinity of $A1^{336}$/A3-C1-C2 for Fl-FFR-FIXa. This potential was remote based upon a similar fluorescence effect observed with the isolated factor VIII light chain. However, factor VIIIa reconstitution assays were performed to assess the possibility of disparate binding following cleavage at this site. Factor VIIIa can be reconstituted from isolated subunits (11–13), and active site-modified factor IXa stably enhances this reconstitution at physiologic pH (28). Since $A1^{336}$/A3-C1-C2 plus A2 subunit cannot reconstitute factor VIIIa activity (9), the capacity of this cleaved dimer to inhibit the factor IXa-dependent enhancement of factor VIIIa reconstitution from native subunits was determined. Factor VIIIa subunits, A2 and A1/A3-C1-C2 (20 nM each), were reacted with various concentrations of $A1^{336}$/A3-C1-C2 in the absence or presence of Fl-FFR-FIXa (20 nM) (Fig. 4). The $A1^{336}$/A3-C1-C2 dimer did not affect the reconstitution of subunits in the absence of Fl-FFR-FIXa, consistent with the requirement for the acidic region of A1 for association with A2 subunit (9). However, the cleaved dimer inhibited the Fl-FFR-FIXa-dependent enhancement of factor VIIIa reconstitution with 50% inhibition observed at ~70 nM $A1^{336}$/A3-C1-C2. This result suggested a somewhat weaker affinity (~3-fold) of factor IXa for $A1^{336}$/A3-C1-C2 compared with native A1/A3-C1-C2. Similar results to those obtained with the cleaved dimer were observed using isolated factor VIII light chain (data not shown). Therefore, cleavage at the A1 site does not appear to significantly affect the binding of $A1^{336}$/A3-C1-C2 to Fl-FFR-FIXa, but does eliminate its ability to induce an A1 subunit-dependent change in the Fl-FFR-FIXa active site.

Role of A2 Cleavage—While the A1/A3-C1-C2 produces a clear increase in the anisotropy of the active site-modified enzyme, it possesses no coagulant activity, since the A2 subunit is required for functional cofactor (10, 11). In Fig. 5, the A2 subunit was titrated into a reaction containing Fl-FFR-FIXa (20 nM) and A1/A3-C1-C2 (50 nM), and the change in Fl-FFR-FIXa anisotropy was measured. The change in the anisotropy in the reconstitution reaction ($\Delta r = 0.047$) was similar to the magnitude change seen when factor VIIIa (50 nM) was used in place of the free subunits ($\Delta r = 0.055$, Fig. 1). Therefore, the A2 subunit markedly contributes to the factor VIIIa-dependent increase in Fl-FFR-FIXa anisotropy. When the $A2_N/A2_C$ fragments were used in place of the intact subunit, no further anisotropy change was observed upon addition to the reaction containing Fl-FFR-FIXa and A1/A3-C1-C2 (Fig. 5). Furthermore, the $A2_N/A2_C$ fraction failed to reconstitute factor VIIIa coagulant activity in the presence of A1/A3-C1-C2 dimer (data not shown). Since the two fragments represent no loss in mass compared with the intact A2 subunit (9), these results suggest that the activated protein C cleavage of this subunit abolishes the A2-dependent affect on the factor IXa active site.

Effect of Cofactor Cleavage on the Interaction with Factor X—In addition to activated protein C-catalyzed proteolysis of

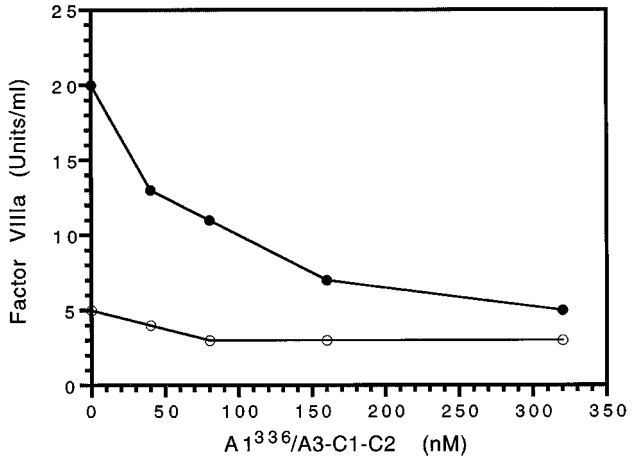

FIG. 4. **Effect of A1[336]/A3-C1-C2 on factor VIIIa reconstitution.** A1/A3-C1-C2 (20 nM) and A2 subunit (20 nM) were incubated with the indicated concentrations of A1[336]/A3-C1-C2 in buffer A containing 0.5 mg/ml BSA and 100 μg/ml inosithin in the presence (*closed circles*) and absence (*open circles*) of Fl-FFR-FIXa (20 nM) for 50 min at room temperature. Factor VIIIa activity was determined using a one-stage clotting assay.

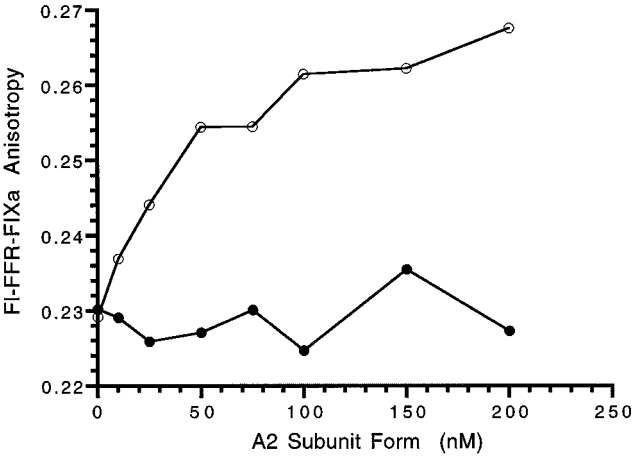

FIG. 5. **Effect of A2 cleavage on the factor VIIIa-dependent fluorescence anisotropy of Fl-FFR-FIXa.** Reactions containing Fl-FFR-FIXa (20 nM), A1/A3-C1-C2 (50 nM), and PSPCPE (50 μg/ml) in buffer A were titrated with either the intact A2 subunit (*open circles*) or $A2_N/A2_C$ (*closed circles*) at the indicated concentrations.

factor VIIIa affecting its ability to modulate the factor IXa active site, the intrinsic factor Xase complex formed with cleaved cofactor may possess altered interaction with its substrate, factor X. To examine this possibility, we utilized the observation by Lollar *et al.* (29) that inclusion of factor X further increased the anisotropy of Fl-FFR-FIXa in complex with factor VIIIa. Consistent with the earlier report (29), factor VIIIa supported a factor X-dependent increase in Fl-FFR-FIXa anisotropy (total increase = 0.103). However, the factor VIIIa cleaved by activated protein C failed to support this effect (results not shown). The loss of a factor X-dependent effect appeared to result from cleavage at the A1 site based upon comparison of the A1/A3-C1-C2 with A1[336]/A3-C1-C2 (Fig. 6). When factor X was titrated into the reaction containing Fl-FFR-FIXa (20 nM) and either A1/A3-C1-C2 or A1[336]/A3-C1-C2 (100 nM), only the intact dimer could support the factor X-dependent increase in anisotropy. This result suggested a role for the acidic C-terminal of A1 in modulating the factor X-dependent contribution to the anisotropy of the factor IXa active site and/or contributing to the binding site on the cofactor for factor X.

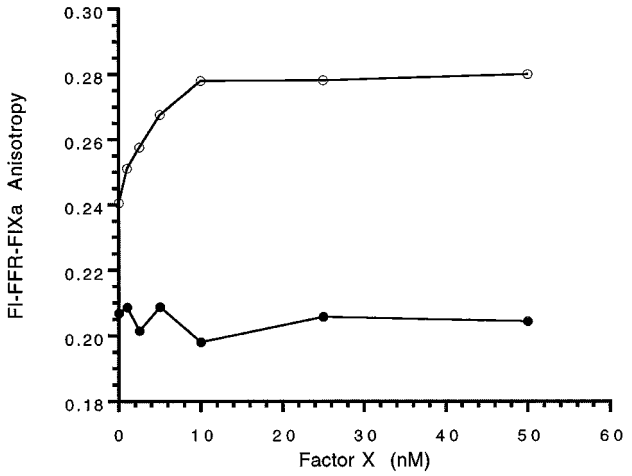

FIG. 6. **Effect of A1/A3-C1-C2 cleavage on the factor X-dependent change in Fl-FFR-FIXa anisotropy.** Reactions containing Fl-FFR-FIXa (20 nM), PSPCPE (50 μg/ml), and either A1/A3-C1-C2 (100 nM; *open circles*) or A1[336]/A3-C1-C2 (100 nM; *closed circles*) in buffer A were titrated with factor X at the indicated concentrations.

To determine whether the cleaved dimer retained the capacity to bind factor X, a microtiter plate binding assay was developed. Briefly, factor VIII-derived subunits were incubated in wells that had been coated with a capture antibody, ESH-8. Various concentrations of factor X were then added to the wells. The amount of factor X bound to the immobilized factor VIII-derived protein was determined following reaction with RVV-X (to convert bound zymogen to the active protease) and subsequent addition of the factor Xa chromogenic substrate, S-2765. Results (Fig. 7) showed a linear response only with the intact dimer. No significant binding was observed with the cleaved dimer or either light chain form. These results suggested that the intact A1 subunit possesses an interactive site for factor X. In a similar experiment, reaction of intact dimer with saturating levels of the anti-FVIII[337–372] polyclonal IgG (as judged by enzyme-linked immunosorbent assay) prior to addition of factor X did not effect the binding of substrate to A1/A3-C1-C2 (data not shown). This result suggested that the binding site for factor X is not contained within the acidic C-terminal region of A1, since this region would be blocked by antibody. Rather, the presence of these residues may be required to maintain the conformation of the factor X binding site.

Conformational Analysis—The above results suggested that the acidic C-terminal region of the A1 subunit is important for the conformation of the factor X binding site as well as the conformation of the region(s) responsible for the characteristic anisotropic effect of A1/A3-C1-C2 on Fl-FFR-FIXa. These inferences were supported by CD analysis of A1[336]/A3-C1-C2 and A1/A3-C1-C2 (Fig. 8). The removal of residues Met[337]–Arg[372] from A1/A3-C1-C2 altered the secondary structure of the protein. The observed decrease in ellipticity in the region of 200–222 nm for the truncated dimer compared with the native form suggested a reduction in its α-helix and β-sheet content.

DISCUSSION

In this report, we examined the mechanism by which activated protein C-catalyzed proteolysis of factor VIIIa abolishes its cofactor activity in the intrinsic factor Xase. Cleavage of factor VIIIa does not appear to significantly perturb the affinity of the cofactor for enzyme. However, based on fluorescence anisotropy measurements using Fl-FFR-FIXa, these cleavages alter the interaction of factor VIIIa relative to the factor IXa active site. Furthermore, while A1/A3-C1-C2 dimer binds factor X in a solid phase binding assay, the A1[336]/A3-C1-C2 dimer

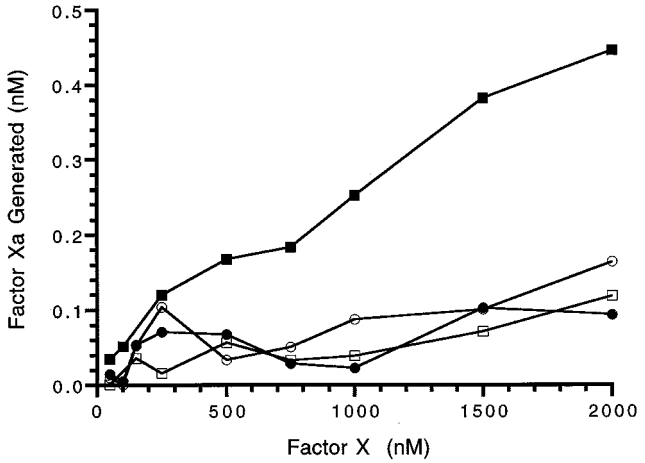

FIG. 7. **Binding of factor X to factor VIII-derived proteins.** Factor X was titrated into microtiter wells containing immobilized A1/A3-C-C2 (*closed squares*), $A1^{336}$/A3-C1-C2 (*closed circles*), factor VIII light chain (*open squares*), and A3-C1-C2 (*open circles*). Binding is presented as nM factor Xa formed following conversion of the bound zymogen to active serine protease with RVV-X and assay with S-2765.

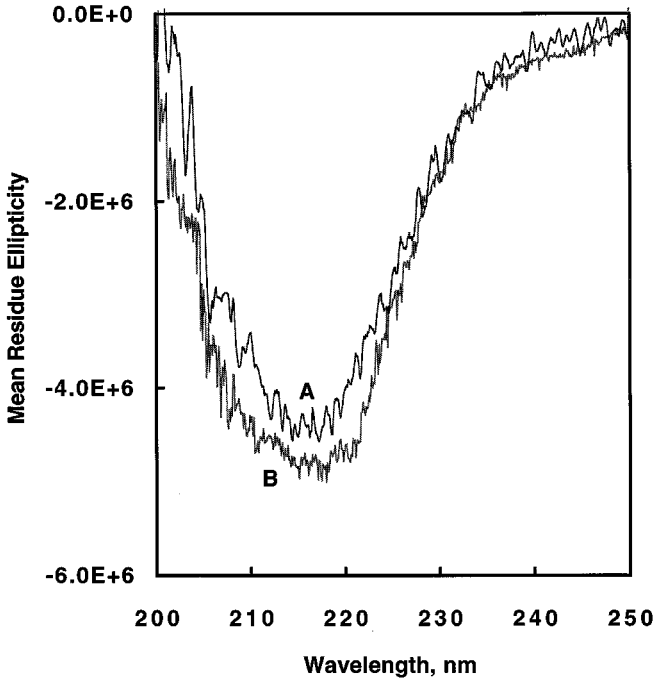

FIG. 8. **CD analysis of $A1^{336}$/A3-C1-C2 and A1/A3-C1-C2 dimers.** The samples were prepared as described under "Materials and Methods." Ellipticity is expressed in mean residue ellipticity, degree·cm^2 $dmol^{-1}$ for the truncated (*curve A*) and native (*curve B*) dimer forms.

exhibits a marked reduction in this activity. Thus, proteolysis by activated protein C results in inhibition of cofactor function at two levels, altered orientation with enzyme and reduced affinity for substrate.

Activated protein C catalyzes cleavage at two sites in factor VIIIa (9). From time course studies, Arg^{562} in the A2 subunit is cleaved initially to yield two fragments of similar size representing residues Ser^{373}–Arg^{562} ($A2_N$) and Gly^{563}–Arg^{740} ($A2_C$). This cleavage closely correlates with the loss of cofactor activity. While cleavage at Arg^{336} in the A1 subunit of the A1/A3-C1-C2 dimer lags behind cleavage at the A2 site, initial cleavage within A2 is not required for this event to occur. Thus the proteolytic events are not stringently ordered. Cleavage at the A1 site liberates a C-terminal acidic region and markedly weakens the affinity of the A2/dimer interaction (9). Analysis using a synthetic peptide corresponding to this region (Met^{337}–Arg^{372}) indicated that this segment contains an A2 interactive site (19). Since proteolysis of A2 requires its association with the A1/A3-C1-C2 dimer on the phospholipid surface, cleavage at Arg^{336} in the A1 subunit prior to cleavage of A2 in some factor VIIIa molecules results in the persistence of low levels of intact A2, as a result of it having dissociated from the dimer.

While no activated protein C-catalyzed cleavage of the factor VIII light chain-derived subunit, A3-C1-C2, is observed (9), this subunit is essential for efficient catalysis since it contains a binding site for the protease (16). This site was mapped to the C-terminal end of the A3 domain in factor VIII as well as in factor V (17). The A3-C1-C2 subunit also contains a high affinity site for factor IXa which probably lies N-terminal to the activated protein C site (27). Both proteases apparently compete for binding to factor VIIIa. Using active site-modified proteases, we showed that activated protein C blocked the factor IXa-dependent enhancement of factor VIIIa reconstitution from isolated subunits, whereas factor IXa blocked the factor VIIIa-dependent fluorescence enhancement of dansyl Glu-Gly-Arg-modified activated protein C (30). Mesters *et al.* (31) found that a peptide derived from activated protein C (residues 142–155) bound factor Va and inhibited both protease-catalyzed inactivation and factor Va-dependent clotting activity of factor Xa. This competition likely reflects the capacity of factors IXa and Xa to protect their respective cofactors from activated protein C (30, 32–34).

Factor Va is cleaved by activated protein C at multiple sites homologous to those in factor VIIIa. Three sites are cleaved in the human factor Va heavy chain, Arg^{306}, Arg^{506}, and Arg^{679} (35). Recent studies using a mutant factor V where Arg^{506} is replaced by Gln (36) indicated that cleavage at the Arg^{506} site does not contribute to inactivation but rather is required for efficient cleavage at the remaining sites. Furthermore, activated protein C cleaves the light chain of bovine factor Va (at residue Arg^{1773}; see Ref. 37) yielding fragments of 30 and 48 kDa (38). Thus, subtle differences exist in both effect and location of proteolysis following interaction of protease with the two cofactors.

Earlier studies examining the mechanism of activated protein C-catalyzed inactivation of bovine factor Va indicated that proteolysis affected cofactor binding to both factor Xa and prothrombin. Guinto and Esmon (39) showed that isolated factor Va heavy chain bound the immobilized prothrombin, whereas both chains were required for binding the factor Xa. However, following reactions with activated protein C, both of these functions were lost. Lucklow *et al.* (40) observed that the affinity of factor Va heavy chain for prethrombin 1 was reduced by at least 100-fold following reaction with activated protein C. Loss of factor Va affinity for prothrombin following reaction with activated protein C likely results from a conformational change rather than cleavage at a binding site based upon the observation that prothrombin failed to protect factor Va from proteolytic inactivation (32, 33).

Isolation of factor VIIIa products following reaction with activated protein C and reconstitution studies have permitted examination of the contribution of each cleavage to cofactor inactivation. Activated protein C cleavage at Arg^{336} in the A1 subunit is functionally equivalent to cleavage by factor IXa, which also attacks that site (41). This event results in weakened affinity for the A2 subunit, presumably due to loss of the A2 interactive site contained within residues 337–372 (19). Furthermore, in this study we show that this truncation of the A1 subunit markedly reduced the A1/A3-C1-C2-dependent increase in anisotropy of Fl-FFR-FIXa. This latter effect is likely

the result of conformation changes in the A1 and/or A3-C1-C2 subunits yielding altered orientation of cofactor with enzyme.

Results in the present study show minimal affect on affinity for factor IXa following cleavage of factor VIIIa. The $A1^{336}$/A3-C1-C2 dimer effectively inhibited the factor IXa-dependent enhancement of factor VIIIa reconstitution. A somewhat higher concentration of cleaved dimer was required to yield 50% inhibition of factor IXa-dependent cofactor reconstitution from intact dimer plus A2 subunit. This result may reflect a slightly reduced affinity of dimer for factor IXa following cleavage at that site. Alternatively, inhibitory activity may be reduced due to partial denaturation during its purification (as suggested by its broad elution peak). One reason for the apparent disparity with the analogous factor Va-factor Xa interaction described above is that bovine factor Va light chain is cleaved by activated protein C, whereas factor VIIIa A3-C1-C2 subunit is not. Since this subunit contains a high affinity site for factor IXa (27), cleavage within the homologous chain of factor Va could perturb its binding factor Xa.

Factor VIIIa A1 subunit likely contains a binding site for factor X. Factor X bound to the immobilized A1/A3-C1-C2 dimer in a dose-dependent manner, whereas no appreciable factor X binding was observed using $A1^{336}$/A3-C1-C2. This result is consistent with the role of factor Va heavy chain (contiguous A1-A2 domains) in binding the substrates prothrombin (39) and prethrombin 1 (40), as well as loss of this function following reaction with activated protein C. At present we cannot exclude direct participation of A3-C1-C2 in the factor X interaction, since this activity may be perturbed by its association with the antibody. The acidic C-terminal region of A1 probably does not directly contribute to binding, since prior reaction of A1/A3-C1-C2 dimer with a polyclonal antibody to this segment had no effect on subsequent reaction with factor X. Thus, loss of substrate binding activity following A1 cleavage may result from a conformational change triggered by loss of the acidic region. This conclusion is supported by CD analysis showing reduced α-helix and/or β-sheet content in the cleaved dimer. One possible explanation for the altered conformation is that acidic residues in the C-terminal region of A1 form salt links with other residues in the A1/A3-C1-C2 dimer which stabilize certain structural elements. Therefore, this segment in A1 subunit appears critical for cofactor activity in that it contributes to the retention of A2 subunit (19) as well as modulates the active conformation of the cofactor.

Although the A1/A3-C1-C2 dimer causes incremental increases in the anisotropy of the FIXa active site and the FX-dependent increase in this parameter, both values are further increased when A2 subunit is present to reconstitute factor VIIIa. However, purified $A2_N$/$A2_C$ fragments, in the presence of A1/A3-C1-C2, neither reconstitute factor VIIIa activity as judged by clotting assay nor modulate the A2-dependent incremental increases in the anisotropy parameters. Abrogation of these A2-dependent activities following cleavage at Arg^{562} is consistent with the sequence in and around the scissile bond representing a factor IXa interactive site. Recent results from our laboratory have shown that factor IXa selectively blocks activated protein C-catalyzed cleavage at Arg^{562} (30). Furthermore, synthetic peptides spanning this region inhibit factor Xase activity and the capacity of factor IXa to stabilize factor VIIIa (42), as well as block the A2-dependent component of the increase in the anisotropy of Fl-FFR-FIXa by factor VIIIa (43). Since this cleavage temporally precedes cleavage at the A1 site, these results confirm the role of A2 cleavage as a primary cause for factor VIIIa inactivation.

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