Altered Interactions between the A1 and A2 Subunits of Factor VIIIa following Cleavage of A1 Subunit by Factor Xa*

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Factor VIIIa consists of subunits designated A1, A2, and A3-C1-C2. The limited cofactor activity observed with the isolated A2 subunit is markedly enhanced by the A1 subunit. A truncated A1 (A1336) was previously shown to possess similar affinity for A2 and retain ~60% of its A2 stimulatory activity. We now identify a second site in A1 at Lys36 that is cleaved by factor Xa. A1 truncated at both cleavage sites (A137–336) showed little if any affinity for A2 (Kd > 2 μM), whereas factor VIIIa reconstituted with A2 plus A137–336/A3-C1-C2 dimer demonstrated significant cofactor activity (~30% that of factor VIIIa reconstituted with native A1) in a factor Xa generation assay. These affinity values were consistent with values obtained by fluorescence energy transfer using acrylodan-labeled A2 and fluorescein-labeled A1. In contrast, factor VIIIa reconstituted with A137–336 showed little activity in a one-stage clotting assay. This resulted in part from a 5-fold increase in Kd for factor X when A1 was cleaved at Arg336. These findings suggest that both A1 termini are necessary for functional interaction of A1 with A2. Furthermore, the C terminus of A1 contributes to the Kd for factor X binding to factor Xase, and this parameter is critical for activity assessed in plasma-based assays.

Factor VIII, a plasma protein that participates in the blood coagulation cascade, is deficient or defective in individuals with hemophilia A. Factor VIII functions as a cofactor for the serine protease, factor IXa, in the anionic phospholipid surface-dependent conversion of factor X to Xa. Factor VIII is synthesized as a multi-domain, single chain molecule (A1-A2-B-A3-C1-C2) (1) with a molecular mass of ~300 kDa (2, 3). Factor VIII is processed to a series of divergent metal ion-linked heterodimers by cleavage at the B-A3 junction, generating a heavy chain consisting of the A1-A2-B domains and a light chain consisting of the A3-C1-C2 domains. This procofactor is activated by cleavage at Arg72 twenty, Arg740, and Arg1689 by thrombin and factor Xa, converting the dimer into the factor VIIIa trimer composed of the A1, A2, and A3-C1-C2 subunits (4, 5). The resulting factor VIIIa heterotramer retains the metal ion-dependent linkage between the A1 and A3-C1-C2 subunits, whereas A2 is associated with a weak affinity by electrostatic interactions (5, 6). Factor VIIIa is unstable, and loss of activity is due to the dissociation of the A2 subunit from the A1/A3-C1-C2 dimer (5–7). Under physiological conditions, the Km for this interaction is ~260 nM (8, 9); however, at slightly acidic pH and low ionic strength, this interaction is facilitated by an ~10-fold increase in the affinity (Kd = 30 nM) (8).

The role of factor VIIIa in the intrinsic factor Xase is to bind factor IXa, which increases the kcat for factor Xa formation by several orders of magnitude compared with factor IXa alone (10). Interactive sites for factor IXa are localized to A2 and A3 domains (11–13). Recent studies have shown that modulation of factor IXa by the isolated A2 subunit enhances the kcat for factor Xa activation by ~100-fold (14) and that the isolated A1 subunit synergizes this effect (>15-fold) to alter the interaction of A2 subunit with the protease (15, 16).

Early evidence suggested that the C-terminal acidic region of A1 subunit (residues 337–372) represented an A2-interactive site and participated in A2 activation following thrombin activation (17). This region is also implicated in the binding of factor X (18), although the significance of this cofactor-substrate interaction is not well understood. The A1 subunit is cleaved at Arg336 by activated protein C (19), factor IXa (20, 21), and factor Xa (19). Proteolysis at this site correlates with inactivation of factor VIIIa. Thus, this cleavage is thought to represent a mechanism for the dampening of factor Xase activity. A recent study demonstrated that a truncated subunit lacking the C terminus of A1 (A1336) possessed similar affinity for A2 subunit and retained ~60% of the A2 stimulatory activity compared with native A1 (16). However, that study also suggested that the C-terminal region of A1 appeared critical for proper orientation of A2 subunit in factor VIIIa relative to factor IXa (16).

In this study, we examine the intersubunit interactions of factor VIIIa employing a purified A1 subunit form possessing a newly identified cleavage site and designated as A137–336, following cleavage at both termini (Lys36 and Arg336) by factor Xa. These results demonstrate a role for the N terminus of the A1 subunit in preserving a conformation of the subunit necessary for functional interaction with the A2 subunit. Furthermore, these results define a role for the A1 C-terminal region in contributing to the Kd for substrate factor X binding to factor Xase. Thus, specific mechanisms for the loss of factor VIIIa function and down-regulation of factor Xase can now be attributed to individual cleavages of the A1 subunit by factor Xa.

MATERIALS AND METHODS

Reagents—Purified recombinant factor VIII preparations were generous gifts from Bayer Corp. (Berkeley, CA) and the Genetics Institute (Cambridge, MA). The monoclonal antibody 58.12 recognizing the N-terminal end of A1 (22) was a gift from Dr. James Brown, and monoclonal antibody C5 recognizing the C-terminal of A1 (23) was a gift from Drs. Carol Fulcher and Zaverio Ruggeri. Phospholipid vesicles contain-

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ing 20% PS, 40% PC, and 40% PE (Sigma) were prepared using N-acetylglucosamine (24). TAP was a gift from Dr. S. Krishnaswamy and reagents human α-thrombin, factor IXa, factor X, and factor Xa (Enzyme Research Laboratories, South Bend, IN) and the chromogenic Xa substrate S-2765 (Nα-benzoyl-Larginyl-L-arginyl-7-amino-4-methylcoumarin) were purchased from the indicated vendors. Acrylodan and fluorescein-5-

Isolation of Factor VIII Subunits—Each factor VIII subunit was isolated from recombinant factor VIII. Factor VIII (1.5 μM) was treated overnight at 4 °C in buffer containing 10 mM MES, pH 6.0, 0.25 M NaCl, 50 mM EDTA, and 0.01% Tween 20, and the light chain and heavy chain were isolated following chromatography on SP-Sepharose and Q-Sepharose (Amersham Biosciences). The purified heavy chain was cleaved by thrombin, and the A2 and A1 subunits were purified by fast protein liquid chromatography using a Hi-Trap Heparin column and a Mono-Q column as reported previously (16). The A3-C1-C2 subunit was prepared as previously described (18). The A137 N terminus of Factor VIII was isolated from recombinant factor VIII. Factor VIII (1.5 μM) in buffer containing 20 mM HEPES, pH 7.2, 100 mM NaCl, 1 mM CaCl2, and 0.01% Tween 20 was added to the purified A1 (11 μM) in a 1:10 (mol/mol) ratio in a buffer containing 20 mM HEPES, pH 7.2, 0.1 M NaCl, 5 mM CaCl2, and 0.01% Tween 20 and reacted overnight at 22 °C. The reaction was quenched using TAP at an 8–fold molar excess relative to factor Xa. The cleaved A1 subunit was fractionated by fast protein liquid chromatography using Fast-Track Hi-Trap chelating column (1.0 l), to which 100 μl of 0.1 M CuCl2 was added beforehand (27). The truncated A1 subunit was eluted at 0.2–0.3 M glycerol using a linear glycerol gradient (0–1.0 M) in 20 mM HEPES, pH 7.2, 0.3 M NaCl, and 0.01% Tween 20 and reacted overnight at 22 °C. The purity of truncated A1 subunit (A137–336) was >95% as determined by SDS-PAGE. A chromogenic assay revealed >95% residual factor Xa activity on TAP-purified A137–336. A truncated A137–336 subunit was prepared as described earlier (16).

Labeling of A1 and A2 Subunits—To obtain fluorescein-labeled A1 (Fl-A1) or A137–336, purified A1 (20 μM) or A137–336 (6.5 μM) subunits in 20 mM HEPES, pH 7.2, 100 mM NaCl, 1 mM CaCl2, and 0.01% Tween 20 were reacted with a 50-fold molar excess of fluorescein 5-maleimide in the dark at 22 °C for 4 h. The unbound fluorescein 5-maleimide was removed by dialyzing the reaction mixture at 4 °C overnight in the above buffer. Acrylodan-labeled A2 (Ac-A2) was obtained from reacting factor VIII (1.5 μM) in the above buffer with a 10-fold molar excess of acrylodan. Labeling conditions and removal of unbound acrylodan were as described above. Isolated Ac-A2 subunit was purified from labeled, EDTA-treated factor VIII following thrombin cleavage using the combination of SP-Sepharose, Q-Sepharose, and Hi-Trap heparin chromatography steps as described above. Approximately 0.9–1.1 mol of fluorescein 5-maleimide was incorporated/mol of A1 or A137–336 subunit. The incorporation of acrylodan into A2 subunit was 1.0–1.2 mol/subunit.

Reconstitution of Factor VIII Activity—The A137–336-C1-C2 dimer was reconstituted with 500 mM A1-A3-C1-C2 and variable levels of A1 form overnight at 4 °C in 20 mM HEPES, pH 7.2, 0.3 mM NaCl, 25 mM CaCl2, and 0.01% Tween 20. The dimer solution was diluted 10-fold, and factor VIIIa was formed by the addition of 20 μM A2 subunit in 20 mM MES, pH 7.2, 100 mM NaCl, 50 mM CaCl2, and 0.01% Tween 20. The reconstitution reactions were run at 22 °C, and the resulting factor VIIIa activity was determined after 15 min by a one-stage clotting assay using factor VIII-deficient plasma (28).

Factor Xa Generation Assays—The rate of conversion of factor X to factor Xa was monitored in a purified system (29). For assays examining the effect of A1 cleavage on factor VIIIa reconstitution from A2 subunit plus A137–336-C1-C2 dimers comprised of different forms of A1, the dimers and 20 mM A2 subunit were reconstituted and incubated with 20 nM factor IXa and 100 μM PS-PC-PE vesicle for 30 s in the above buffer. The reactions for either assay were initiated with the addition of 500 nM factor X. The aliquots were removed at appropriate times to assess residual levels of product formation and added to tubes containing EDTA (final concentration, 50 mM) to stop the reaction. The rates of factor Xa generation were determined by the addition of the chromogenic substrate, S-2765 (final concentration, 0.46 mM). The reactions were read at 405 nm using a V_max microtiter plate reader (Molecular Devices, Sunnyvale, CA). All of the reactions were run at 22 °C.

Electrophoresis and Western Blotting—SDS-PAGE was performed on a 10% gel using the procedure of Laemmli (30). Electrophoresis was carried out using a Bio-Rad minigel apparatus at 150 V for 1 h. The bands were visualized following staining with GelCode Blue Stain Reagent (Pierce). Alternatively, the protein was transferred to a polyvinylidenefluoride membrane using a Bio-Rad mini-trans blot apparatus at 50 V for 2 h in buffer containing 20 mM CAPS, pH 11, and 10% (v/v) methanol. The protein was probed using the 58.12 and C5 monoclonal antibodies followed by goat anti-mouse alkaline phosphatase-linked secondary antibody. The signal was detected using the ECF system (Amersham Biosciences), and the blots were scanned at 570 nm using Storm 860 (Molecular Devices).

RESULTS

Isolation and Purification of Factor Xa-cleaved A1 Subunit—Factor Xa cleaves the A1 subunit (residues 1–372) of human factor VIIIa at Arg336 yielding a truncated subunit lacking the C-terminal domain rich in acidic amino acids (19). Because this cleavage inactivates cofactor activity, an examination of the A1-associated activities following interaction of the isolated subunit with factor Xa was undertaken. We observed that an extended interaction of the subunit with protease (10:1 molar ratio) converted A1 into a subunit of ~40 kDa, which was significantly smaller than the A1 subunit truncated at Arg336 (~45 kDa). Pretreatment of factor Xa with a molar excess of TAP resulted in no generation of either fragment, indicating that both cleavages were catalyzed by factor Xa (results not shown).

Purification of this fragment was problematic because it did not bind to either Mono S or Mono Q columns under low salt conditions. However, it was adsorbed by a Cu²⁺-bound Hi-Trap chelating column utilizing the copper binding of His residues (27) and was eluted quantitatively and with high purity (>95%) with 0.2–0.3 M glycerol. Fig. 1A shows gel electrophoresis of A1, A137–336, and the ~40-kDa subunit. A chromogenic assay revealed <0.1% residual factor Xa activity or TAP (used to inactivate the factor Xa) present in the purified fraction. This fragment was further characterized by Western blot analysis, which employed anti-A1 domain monoclonal antibodies specific for the N- and C-terminal sequences. Although native
Interestingly, the residue specified at cycle number 5 (Asn 41) A1 (N-C5 clonal antibody specific for the N-terminal region) (1636), A1336 form with A2 (these data suggest a weak intersubunit interaction for this A1 subunit in stimulating A2 cofactor activity (58.12, left panel) and for the C-terminal region (C5, right panel).

A1 and A1336 subunits were reactive with monoclonal antibody 58.12 (an antibody directed to residues 1–337) —equivalent affinity for the A2 subunit (Kd 58.12 (an antibody directed to residues 1–337) —equivalent affinity for the A2 subunit (Kd 58.12), the 40-kDa A1 was not identified (Fig. 1B, left panel). Use of the C5 antibody (specific for residues contained with 337–372) showed reactivity with the native A1 subunit only and did not react with either the 40-kDa A1 form or A1336 (Fig. 1B, right panel). These findings indicated that the 40-kDa A1 subunit was truncated at both termini.

Identification of the N-terminal Cleavage Site—To identify the site of cleavage that results in the N-terminal truncation, the ~40-kDa A1 form was subjected to 10 cycles of automated N-terminal sequence analysis (Table I). Results from this analysis indicated that this A1 form was derived from cleavage at Lys36-Ser37. Thus, cleavage at this site was consistent with no failure to identify this residue provides direct support for utilization of Asn41 for high affinity interaction with A2 subunit and subsequent stimulation of A2 activity.

Reconstitution of Factor VIIIa Forms—Although the A137–336 possessed little ability to directly stimulate the A2 subunit, an additional series of experiments was performed to assess the capacity of this A1 form to reconstitute factor VIIIa following association of A3-C1-C2 and A2 subunits. Factor VIIIa was reconstituted in a two-step procedure. In the first step the A1/A3-C1-C2 dimer form was prepared by reacting varying amounts of the A1 forms and 500 nM A3-C1-C2 subunit in the presence of Ca2+ overnight at 4 °C. The resultant dimer solutions were diluted 10-fold and reacted with the A2 subunit (20 nM) to generate the factor VIIIa heterotrimers. Reconstituted factor VIIIa activity was determined in a factor Xa generation assay initiated by the addition of factor IXa (20 nM) and factor X (500 nM) as described under “Materials and Methods.” These data are illustrated in Fig. 3 and Table II. The Vmax values obtained at saturating levels of factor VIIIa reconstituted from native A1 and A1336 with A3-C1-C2 plus A2 subunits were 26.9 ± 0.9 and 13.0 ± 0.7 min−1, respectively. These relative activity values were similar to results obtained above for the direct stimulation of the A2 subunit. The Kd value for the intersubunit interaction with A2 using native A1/A3-C1-C2 dimer was 202 ± 21 nM, a value similar to the earlier reported values obtained under physiological conditions (~260 nM) (8, 9). Furthermore, the Kd value (299 ± 51 nM) for A2 subunit interaction with the A137/336/A3-C1-C2 dimer was similar to that of native A1.

Interestingly, although isolated A137–336 had little ability to directly stimulate the A2 subunit, likely in part a consequence of a weak affinity interaction for A2, reconstitution of A137–336/ A3-C1-C2 dimer plus A2 subunits yielded a form of factor VIIIa that demonstrated significant cofactor activity. This activity (8.0 ± 0.3 min−1) was ~30% the value compared with factor VIIIa formed with native A1 and ~60% of the activity formed with A1336. Furthermore, the A137–336/A3-C1-C2 dimer showed equivalent affinity for the A2 subunit (Kd = 245 ± 35 nM) compared with the values obtained for the native dimer and A137/336/A3-C1-C2 dimer. This association of the A137–336 subunit with A3-C1-C2 resulted in a >10-fold affinity enhancement compared with that observed for isolated A137–336 subunit. These results indicate that both a physical and functional interaction of the A137–336 subunit with A2 requires association of this A1 form with the A3-C1-C2 and suggests that this interaction is mediated by an A3-C1-C2-dependent change in the conformation of the A137–336 subunit.

Reconstituted Factor VIIIa Activity Measured in a One-stage Clotting Assay—Activity of the reconstituted factor VIIIa composed of either native A1 or A137–336 forms was measured in a one-stage clotting assay as described under “Materials and Methods.” Compared with factor VIIIa reconstituted with native A1 form, little if any activity was observed for that with the A137–336 form (Fig. 4). This result was in contrast to the results obtained in a factor Xa generation assay with factor VIIIa comprised of the A137–336 form, which yielded ~30% of the activity of factor VIIIa containing native A1. Similarly, we previously reported no activity of factor VIIIa composed of A1336 in a one-stage clotting assay (16), whereas this form showed ~60% of native activity in a factor Xa generation assay in the current study. Thus, the failure to observe factor VIIIa activity with trimer reconstituted from the A137–336 form using a one-stage clotting assay was consistent with the earlier result. Overall these results suggest that the lack of the C-terminal region of A1 leads to disparate results in the two assay systems.

**Fig. 1.** SDS-PAGE and Western blots of native and cleaved A1 forms. A, the purified subunits were run on an 8% gel followed by staining with GelCode Blue. Lane 1, molecular mass markers; lane 2, native A1 subunit; lane 3, A1336; lane 4, A137–336. B, Western blot of the A1 (lane 1), A1336 (lane 2), and A137–336 (lane 3) using anti-A1 monoclonal antibody specific for the N-terminal region (58.12, left panel) and for the C-terminal region (C5, right panel).
Effects of A1 Cleavage on Factor VIIIa Subunit Interactions

Activity of Factor VIIIa Forms at Variable Substrate Concentration—Because the above results suggest altered activities of the cofactor forms possessing C-terminal truncations in A1 subunit and because this region of factor VIII contains a factor X-interactive site (18), a study was undertaken to evaluate the role of substrate factor X concentration relative to factor VIII function. We hypothesized that an increase in $K_m$ for factor X as a result of a C-terminal truncation in factor VIIIa leading to a disrupted interaction with factor X would have a more pronounced effect on the one-stage assay in which the factor X concentration is ~25% the plasma level or ~40 nM compared with the factor Xa generation assay, which uses a concentration of substrate yielding $V_{max}$ conditions (29) (500 nM as described under “Materials and Methods”).

For this analysis, 500 nM native A1 or A137–336 subunit and

TABLE I
Amino-terminal sequence analysis of the ~40-kDa A1 subunit

| Cycle no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|-----------|---|---|---|---|---|---|---|---|---|----|
| A1 residues 37–46 (K) | S | F | P | F | N | T | S | V | V | Y |
| 40-kDa fragment | (7.6) | (6.1) | (4.8) | (4.8) | (ND)$^b$ | (3.2) | (1.9) | (2.8) | (3.3) | (2.0) |

$^a$—, not identified.
$^b$ ND, not detected.

Activity of Factor VIIIa reconstituted with A1 forms. The A1 form/A3-C1-C2 dimer was reconstituted with 500 nM A3-C1-C2 and the indicated levels of A1 (open circles), A1336 (closed circles), or A137–336 (squares) subunit. After incubation with 5 nM factor IXa and 10 $\mu$M PS-PC-PE, the reaction was initiated with the addition of 500 nM factor X as described under “Materials and Methods.” The initial rates of factor Xa generation were plotted as functions of A1 concentration and fitted to the quadratic equation by nonlinear least squares regression (45).

Fig. 2. Stimulation of A2 activity by A1 forms. Cofactor activity of the isolated A2 subunit was measured in a factor Xa generation assay. A2 subunit (400 nM) was reacted with indicated levels of A1 (open circles), A1336 (closed circles), or A137–336 (squares) subunit. After incubation with 5 nM factor IXa and 10 $\mu$M PS-PC-PE, the reaction was initiated with the addition of 500 nM factor X as described under “Materials and Methods.” The initial rates of factor Xa generation were plotted as functions of A1 concentration and fitted to the quadratic equation by nonlinear least squares regression (45).

Fig. 3. Factor VIIIa reconstituted with A1 forms. The A1 form/A3-C1-C2 dimer was reconstituted with 500 nM A3-C1-C2 and the indicated levels of A1 (open circles), A1336 (closed circles), or A137–336 (squares) overnight at 4 °C. The reaction was diluted 10-fold, and factor VIIIa was formed by addition of 20 nM A2 subunit. After incubation with 20 nM factor IXa and 10 $\mu$M PS-PC-PE, the reaction was initiated with the addition of 500 nM factor X as described under “Materials and Methods.” The initial rates of factor Xa generation were plotted as a function of A1 concentration and fitted to the quadratic equation by nonlinear least squares regression.

A3-C1-C2 subunit were reconstituted as described above. The preformed dimers were diluted 10-fold and reacted with 20 nM A2 subunit, followed by the addition of 20 nM factor IXa. The reactions were initiated using varying amounts of factor X as described under “Materials and Methods.” The results of this experiment are shown in Fig. 5. The $V_{max}$ values obtained from the fitted curve for factor VIIIa forms composed of native A1 and A137–336 subunits were 21.8 ± 4.1 and 7.2 ± 0.3 min$^{-1}$, respectively, consistent with the data described above. The $K_m$ values for factor X obtained with factor Xase comprised of the A1-containing factor VIIIa (41.3 ± 3.3 nM) was 5-fold lower compared with that for A137–336-containing factor VIIIa (206 ± 19 nM). Overall, these parameters result in a marked reduction in catalytic efficiency ($V_{max}/K_m$) for factor Xase of the truncated A1 (0.035 min$^{-1}$ nM$^{-1}$) compared with native factor Xase (0.53 min$^{-1}$ nM$^{-1}$). These results also indicate that for reactions containing the latter factor VIIIa form, the factor X concentration is ~20% the $K_m$ value in the one-stage assay. Taken together, these results support a role for the C-terminal region of A1 in factor Xase substrate binding and are consistent with the hypothesis that the disparity observed in the two assays when using C-terminal truncated factor VIIIa forms reflects, in part, a rate-limiting substrate concentration in the plasma-based assay.

Energy Transfer between Ac-A2 and Fl-A1 Subunit Forms—In an earlier report, we demonstrated that reconstitution of factor VIIIa using fluorophore-labeled subunits can be
acrylodan-labeled intact factor VIII as described under reconstitution assay (32), the Ac-A2 subunit was isolated from inactive subunit that failed to yield functional factor VIIIa in a cation of the isolated A2 subunit with acrylodan results in an analysis.

The A1/A3-C1-C2 (33). Because modification of the isolated A2 subunit with acrylodan results in an inactive subunit that failed to yield functional factor VIIIa in a reconstitution assay (32), the Ac-A2 subunit was isolated from acrylodan-labeled intact factor VIII as described under “Materials and Methods.”

assessed and intersubunit affinity can be quantitated by following fluorescence resonance energy transfer (32). A similar approach was undertaken to examine interactions of isolated A2 with the native and truncated A1 subunit forms. The A2 subunit and the A1 subunit forms were labeled with the sulf-hydryl-specific probes, acrylodan and fluorescein-5-maleimide, respectively, utilizing the presence of a free cysteine residue in the A1 (Cys310) and A2 (Cys692) domains (33). Because modification of the isolated A2 subunit with acrylodan results in an inactive subunit that failed to yield functional factor VIIIa in a reconstitution assay (32), the Ac-A2 subunit was isolated from acrylodan-labeled intact factor VIII as described under “Materials and Methods.” The incorporation of acrylodan into A2 subunit was 1.0–1.2 mol/subunit. Direct labeling of isolated A1 subunit with fluorescein 5-maleimide yielded an active subunit with similar incorporation of fluorescein 5-maleimide into A1 or A1\(^{-336}\) subunit (0.9–1.1 mol/subunit). Reconstitution of the Ac-A2 subunit and Fl-A1 form/A3-C1-C2 dimer retained >80% specific activity compared with factor VIIIa prepared from unlabeled subunits (data not shown).

Fluorescence experiments were conducted at pH 6.0, which enhances the intersubunit affinity (8), using 200 nM Ac-A2 and varying amount levels of the Fl-A1 form as described under “Materials and Methods.” The fluorescence emission spectrum of the Ac-A2 subunit (fluorescence donor) overlaps with the excitation spectrum of the Fl-A1 form (fluorescence acceptor). Upon binding of Ac-A2 to the Fl-A1 form, the fluorescence intensity of the acrylodan is quenched, and the extent of this quenching is an indicator of the spatial separation between donor and acceptor fluorophores (34). The relative fluorescence intensities were recorded and integrated at \(\lambda = 460–480\) nm, and percentage of donor quenching was calculated based on the excitation spectrum of the Fl-A1 form (fluorescence acceptor). This value obtained using the physical assay was equivalent to intersubunit affinity determined using a functional assay \((432 \pm 66\) nM). This value obtained using the physical assay was equivalent to intersubunit affinity determined using a functional assay \((K_f = 428\) nM; see Table II). However, little donor quenching was observed for the Ac-A2-Fl-A1\(^{-336}\) subunit pairing (~7%), and this effect was not saturable over the range of acceptor concentrations employed, suggestive of little association of Ac-A2 with the truncated A1. Thus, the affinity values obtained with the physical assay support the functional affinities determined by the factor Xa generation assay and are consistent with a marked increase in \(K_f\) (>4-fold) following cleavage of Lys\(^{346}\).

**Influence of A3-C1-C2 in the Energy Transfer between Ac-A2 and Fl-A1 Subunits**—To further assess the role of the A3-
were formed by reacting 2 μM A3-C1-C2 and Fl-A1 37 has been shown to cleave the C-terminal region of A1 at a site altered conformation in the trimer formed with the truncated A1 between the fluorophores with an apparent closer separation. However, cleavage at Arg336, a site also attacked by activated low following reconstitution of factor VIII with native heavy chain (7). That cleavage at this site was benign to cofactor function following reconstitution of factor VIII with native heavy chain (7). The results obtained from the energy transfer studies compared with earlier studies demonstrating a lack of competition of factor VIIIa light chain (A3-C1-C2) with the A1/A3-C1-C2 dimers for A2 subunit as assessed by the energy transfer between Fl-A1 form/A3-C1-C2 dimer and Ac-A2. Fl-A1/A3-C1-C2 and Fl-A137–336/A3-C1-C2 dimers were formed by reacting 2 μM A3-C1-C2 with 2 μM Fl-A1 or Fl-A137–336, respectively. Ac-A2 (100 nM) was reconstituted with varying amounts of the dimer forms as described under “Materials and Methods,” and the results are shown in Fig. 7 and Table III. Donor quenching was observed for either dimer form, indicative of association of Ac-A2 subunit. The levels of the percentage of donor quenching at saturating Fl-labeled dimer consisting of A1 and A137–336 were 55.6 ± 2.5 and 29.9 ± 1.6%, respectively. These results suggest an altered spatial separation between the fluorophores with an apparent closer separation existing for the native subunits. This result is consistent with an altered conformation in the trimer formed with the truncated A1 form that may reflect reduced cofactor activity.

The $K_d$ values calculated for interaction of Ac-A2 with Fl-A1/A3-C1-C2 and Fl-A137–336/A3-C1-C2 dimer were similar (72.2 ± 7.8 and 85.2 ± 10.8 nM, respectively) and 5-fold less when A3-C1-C2 was included. Comparison of binding energy values calculated for the interaction of A2 with native A1 ($K_d = 432$ nM, 8.7 kcal mol$^{-1}$) and with A1/A3-C1-C2 ($K_d = 72$ nM, 9.7 kcal mol$^{-1}$) indicate that 90% of the binding energy for A2 association within the factor VIIIa heterotrimer is derived from interaction with the A1 subunit. Furthermore, the N- and C-terminal truncations in A1 contribute little to the direct binding of A2 in the factor VIIIa heterotrimer. Therefore, the lack of interaction of A2 with the isolated A137–336 compared with high affinity binding of A2 to the dimer containing these A1 truncations suggest that interactions within the dimer promote a change in conformation in A1, facilitating its interaction with A2.

DISCUSSION

Factor Xa, a potent activator of factor VIII (35), has been shown to cleave human factor VIII at sites identical to those attacked by thrombin during cofactor activation. Factor Xa also attacks Arg1721 in the A3 domain of the A3-C1-C2 subunit and has been shown to cleave the C-terminal region of A1 at a site tentatively identified as Arg336 (19). Earlier studies employing a factor VIII light chain cleaved by factor Xa at Arg1721 showed that cleavage at this site was benign to cofactor function following reconstitution of factor VIII with native heavy chain (7). However, cleavage at Arg336, a site also attacked by activated protein C (19, 36) and factor Xa (20, 21), correlates with inactivation of the cofactor and down-regulation of factor Xase activity. The mechanisms for activity loss following cleavage at Arg336 are not fully understood but include reduced interaction of factor VIIIa with substrate factor X (37) and altered interaction with A2 subunit, as evidenced by reduced stimulation of activity associated with the A2 subunit (16). Thus, subsequent proteolytic attack of the cofactor by generated factor Xa would yield a reaction product limiting further factor Xa generation by a self-dampening mechanism.

C1-C2 subunits in the inter-A1-A2 interaction, we performed energy transfer analysis between Ac-A2 and the A1/A3-C1-C2 dimer reconstituted from the Fl-A1 subunit forms and A3-C1-C2. Fl-A1/A3-C1-C2 and Fl-A137–336/A3-C1-C2 dimers were formed by reacting 2 μM A3-C1-C2 with 2 μM Fl-A1 or Fl-A137–336, respectively. Ac-A2 (100 nM) was reconstituted with varying amounts of each dimer form. Figure 7 shows the fluorescence energy transfer between Fl-A1 form/A3-C1-C2 dimer and Ac-A2. Fl-A1/A3-C1-C2 or Fl-A137–336/A3-C1-C2 dimer was reconstituted by reacting 2 μM A3-C1-C2 and 2 μM Fl-A1 (open circles) or Fl-A137–336 (closed circles). Ac-A2 (100 nM) was reacted with varying amounts of each dimer for 30 min as described under “Materials and Methods.” % Donor Quenching refers to the fluorescence intensity of Ac-A2 reconstituted with the dimer relative to that of Ac-A2 alone. The data were fitted as described above.

In this work we examined the intersubunit interactions of factor VIIIa employing an A1 subunit form following terminal digest with factor Xa. Electrophoretic analysis of a terminal A1 cleavage fragment revealed truncation at its C terminus consistent with the loss of residues 337–372, as well as cleavage at a second prominent factor Xa-catalyzed site at Lys36–Ser37 as determined by N-terminal sequence analysis. The resultant A137–336, truncated by 36 residues at both its termini, showed markedly reduced affinity for the A2 subunit and no apparent capacity to stimulate the limited cofactor activity associated with A2 subunit. This result is in contrast to observations in this report as well as an earlier study (16) using an A1 subunit truncated only at its C terminus, A1336, which demonstrated similar affinity of this A1 form compared with native A1 for A2 subunit and showed a high level (~60%) of A2 stimulatory activity. Thus, these results identify the N-terminal region of A1 as participating either directly or indirectly in the interaction with A2 subunit.

The results obtained from the energy transfer studies comparing Ac-A2 association with Fl-A1 in the absence or presence of A3-C1-C2 suggest that ~90% of the binding energy for A2 association within the factor VIIIa heterotrimer is derived from direct interaction with the A1 subunit. This result is consistent with earlier studies demonstrating a lack of competition of isolated factor VIII light chain (A3-C1-C2) with the A1/A3-C1-C2 dimer for A2 subunit in a functional, factor VIIIa reconstitution assay (8, 17). This value of 90% may represent a minimum value, because A3-C1-C2 appears to alter the conformation of A1 so as to enhance its interaction with A2. This observation was suggested by the weak (undetermined) affinity of isolated A137–336 for A2 subunit as assessed by the energy transfer assay, whereas association of this truncated subunit with A3-C1-C2 restored a high affinity interaction of A2 with the dimer.

Although the molecular basis for the loss of functional interaction between the isolated A1 and A2 subunits with cleavage at the N-terminal site is not well understood, examination of the homology model (38) indicates that A1 residues Pro35–Phe39 juxtapose the A2 domain and that this interaction may

### Table III

| A1 form | Ac-A2 fluorescence quenching |
|---------|------------------------------|
|         | $K_d$ (nM) | $\%$ Quenching |
| A1      | 432 ± 66 | 72.2 ± 7.8 | 46.1 ± 3.0 | 55.6 ± 2.5 |
| A137–336| >1500    | 85.2 ± 10.8| -7         | 29.9 ± 1.6 |

**Fig. 7.** Energy transfer between Fl-A1 form/A3-C1-C2 dimer and Ac-A2. Fl-A1/A3-C1-C2 or Fl-A137–336/A3-C1-C2 dimer was reconstituted by reacting 2 μM A3-C1-C2 and 2 μM Fl-A1 (open circles) or Fl-A137–336 (closed circles). Ac-A2 (100 nM) was reacted with varying amounts of each dimer for 30 min as described under “Materials and Methods.” % Donor Quenching refers to the fluorescence intensity of Ac-A2 reconstituted with the dimer relative to that of Ac-A2 alone. The data were fitted as described above.
be significant. Specifically, Val$^{26}$ and Asp$^{27}$ are separated from A2 residues 537–541 by −2.5–5 Å. These potential contacts in A2 include Arg$^{31}$ and Val$^{35}$, and point mutations at these two sites have been shown to result in hemophilia A (HAMSTEK data base; europium.csc.mrc.ac.uk). However, no point mutations within this sequence in A1 have been identified to yield a hemophilia phenotype. Thus, we speculate that the first 36 residues of A1 may either directly contact A2 and/or promote a conformation of A1 important to its physical interaction with A2. Interestingly, this effect is somewhat ameliorated in the presence of the A3-C1-C2 subunit. Reconstitution of factor VIIIa using the A1$^{37–336}$ form yielded ~30% of the activity of the cofactor formed with native A1 subunit and ~60% of the activity of the cofactor formed with A1$^{336}$ truncated only at its C terminus. These results suggested that any putative conformation defect attributed to cleavage at Lys$^{36}$ is somewhat compensated for following the reassociation of A1 with the A3-C1-C2 dimer. This restoration of cofactor function in the presence of A3-C1-C2 was expected based upon earlier observations employing phospholipid-independent, factor Xa generation assay indicating that A1-A3 contacts subsequently positively influence the interaction of A1 with A2 subunits (39). However, the disparate extents of fluorescence quenching we observed for the acrylodan-labeled A2 subunit by the Fl-A1 and Fl-A1$^{37–336}$ forms in the presence of A3-C1-C2 suggested an altered (increased) interspatial separation of fluorophores in the factor VIIIa form possessing the cleaved A1. We suggest that these changes in conformation are responsible for reduced cofactor activity and reflective of altered interaction between the A1 and A2 subunits. Although factor Xa generation assays showed the truncated A1 forms retained significant activity following reconstitution reactions to yield factor VIIIa, the one-stage clotting assay failed to report significant activity using these forms of the cofactor. We observed a similar result with the one-stage clotting assay using factor VIIIa reconstituted from the A1$^{336}$ form in an earlier report (16). We now identify a factor contributing to this assay discrepancy. Kinetic analyses using factor Xa generation show a $K_n$ for factor X of ~40 nM using factor Xase composed of native factor VIIIa, whereas this value was increased 5-fold for factor Xase comprised of the C-terminal truncated A1. Because the typical factor Xa generation assay uses concentrations of substrate that yield (near) $V_{\max}$ reaction rates (29), the rates are independent of the concentration of factor X. However, because the plasma concentration of factor X is ~120 nM and the plasma is diluted 4-fold in the one-stage assay, the limiting amount of substrate factor X (~20% the $K_m$ value for factor Xase comprised of the cleaved A1 forms) would markedly depress the rate of factor Xase activity and subsequent clot formation for reactions run using these A1 forms. These results contribute to understanding the basis for cofactor inactivation following cleavage at Arg$^{336}$ by enzymes such as activated protein C. The kinetic studies ascribe a role for the A1 C terminus 337–372 in the interaction of factor Xase with substrate factor X. This functional effect is consistent with earlier studies suggesting that this sequence represents a factor X interactive site (18). In a complementary study, zero length cross-linking employing either factor VIIIa or the A1/A3-C1-C2 showed a critical salt bridge between the C-terminal region of A1 and the protease domain of factor X (40). The requirement for the A1 C-terminal region was based upon absence of a cross-linked product using the A1$^{336}$/A3-C1-C2 dimer form. Interestingly, a region in the protease domain distinct from the activation peptide sequence was identified based upon persistence of the linkage following zymogen activation by the X activator in Russel’s viper venom. Thus, we speculate that factor Xa may also interact with the C-terminal region of the A1 subunit. Indeed, attack at Lys$^{36}$ appears dependent upon an intact C terminus of the A1 subunit, indicating an ordered reaction pathway in factor Xa-catalyzed inactivation of factor VIIIa.3 The study by Parker et al. (41) provides a detailed analysis of the proteolytic activation of porcine factor VIII by factor Xa. In addition to cleavage at the thrombin-susceptible sites Arg$^{372}$, Arg$^{270}$, and Arg$^{1689}$, factor Xa also cleaves at Arg$^{336}$ within A1 and Arg$^{390}$ within A2 to yield a pentameric factor VIIIa. Although inactivation of cofactor was not studied in that report, no cleavage at Arg$^{336}$ was noted. Interestingly, Arg$^{336}$ in A1 is unique to the pig protein and is a Glu residue at the homologous site in the human, dog, and mouse proteins (42–44). Conversely, the Lys$^{36}$ cleavage site we report in this study is replaced by Gly (pig and dog) and Thr (mouse) in the animal proteins, indicating that this factor Xa site is restricted to the human factor VIII.

Parker et al. (41) also noted that the catalytic efficiency ($k_{cat}/K_m$ for factor Xase comprised of factor Xa-activated cofactor is several fold lower than that for factor Xase comprised of thrombin-activated factor VIIIa, a result consistent with an earlier study from that laboratory (35). The reason for this is the combination of an ~3-fold increase in the $K_m$ for factor X plus a similar fold decrease in $V_{\max}$. These parameters approach the deficiencies in $k_{cat}$ and $K_m$ as well as the overall reduced catalytic efficiency (~15-fold) we observed for factor Xase wherein the reconstituted cofactor was altered solely within its A1 subunit. Thus, it is tempting to speculate that cleavage of porcine factor VIIIa at Arg$^{336}$ in the A1 subunit is primarily responsible for the reduced catalytic efficiency relative to thrombin-activated factor VIIIa as observed by these investigators.

In summary, we show that cleavage of the A1 subunit by factor Xa occurs at two distinct sites, Lys$^{36}$ and Arg$^{336}$, which are localized on opposite faces of the A1 domain according the homology model of Pemberton et al. (38). This spatial orientation suggests a complex interaction of protease with this subunit in attack of the scissile bonds. Both cleavages reduce, by degrees, the intrinsic capacity of A1 subunit to stimulate the activity of the cofactor. This effect is augmented in the plasma is diluted 4-fold in the one-stage assay employing factor Xase activity in plasma. Conversely, the Lys$^{36}$ cleavage site we report in this study is replaced by Gly (pig and dog) and Thr (mouse) in the animal proteins, indicating that this factor Xa site is restricted to the human factor VIII.

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