Cofactor Activities of Factor VIIIa and A2 Subunit following Cleavage of A1 Subunit at Arg336*

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Factor VIIIa consists of three subunits designated A1, A2, and A3-C1-C2. The isolated A2 subunit possesses limited cofactor activity in stimulating factor IXa-catalyzed activation of factor X. This activity is markedly enhanced by the A1 subunit (inter-subunit $K_D = 1.8 \mu M$). The C-terminal region of A1 subunit (residues 337–372) is thought to represent an A2-interactive site. This region appears critical to factor VIIIa, because proteolysis at Arg336 by activated protein C or factor IXa is inactivating. A truncated A1 (A1336) showed similar affinity for A2 subunit ($K_D = 0.9 \mu M$) and stimulated its cofactor activity to ~50% that observed for native A1. However, A1336 was unable to reconstitute factor VIIIa activity in the presence of A2 and A3-C1-C2 subunits. Fluorescence anisotropy of fluorescein (Fl)-FFR-factor IXa was differentially altered by factor VIIIa trimers containing either A1 or A1336. Fluorescence energy transfer demonstrated that, although Fl-A1336/A3-C1-C2 bound acrylodan-A2 with similar affinity as the native dimer, an increased inter-fluorophore separation was observed. These results indicate that the C-terminal region of A1 appears necessary to properly orient A2 subunit relative to factor IXa in the cofactor rather than directly stimulate A2 and elucidate the mechanism for cofactor inactivation following cleavage at this site.

Under physiological conditions, the $K_D$ for this interaction is ~260 nM (8, 9), however, under slightly acidic, pH and lower ionic strength, A2 and A1/A3C1C2 can be reconstituted to form the trimer with a $K_D$ of ~30 nM (8).

The role of factor VIIIa is to bind factor IXa, generating the phospholipid-dependent intrinsic factor Xase complex, which increases the $k_{cat}$ for factor Xa formation by several orders of magnitude compared with factor IXa alone (10). At least two interactive sites have been identified for the enzyme-cofactor interaction. A high affinity, surface proximal site is likely formed by residues contained in the A3 domain and light chain of factors VIIIa and IXa, respectively, whereas a weaker affinity interaction involves residues localized to the A2 subunit of the cofactor and the serine protease domain (see Ref. 11 for a review). Recent studies have shown that modulation of factor IXa by the isolated A2 subunit enhances the $k_{cat}$ for factor Xa activation by ~100-fold (12) and that the isolated A1 subunit synergizes this effect (13). This A1-dependent increase in A2 cofactor activity (>15-fold) did not result from direct interaction with factor IXa but rather altered the interaction of A2 subunit with the protease.

Little information is available regarding sites of interaction between the A1 and A2 subunits of factor VIIIa. Earlier studies have suggested that the C-terminal acidic region of A1 subunit (residues 337–372) represented an A2-interactive site and was involved in A2 retention following thrombin activation of the cofactor (14). This region is preceded by Arg336, which represents a cleavage site for APC (15) and factor IXa (16, 17). Proteolysis at this site correlates with inactivation of factor VIIIa. The mechanism for this inactivation is poorly understood, but results are consistent with the loss of the C-terminal region reducing the affinity for the A2 subunit (18, 19).

In this study, we examine the interaction of A2 with a truncated A1, cleaved by APC and designated as A1336, as isolated subunits as well as within the reconstituted factor VIIIa trimer. We observed that A1336 showed similar affinity for A2 and exhibited near native-like activity in stimulating activity associated with the isolated A2 subunit. In contrast, the A1336/A3-C1-C2 dimer, formed either by the replacement of A1 with A1336 followed by reassociation with A3-C1-C2 or by APC-catalyzed cleavage of the native A1/A3-C1-C2, yielded a dimer form that was functionally inactive following the addition of the A2 subunit. Fluorescence anisotropy and resonance energy transfer analyses indicated association of A2 with the A1336/
A3-C1-C2 dimer. However, the resultant trimer appeared to possess an altered orientation of A2 relative to the dimer and factor IXa. Therefore, although residues 337–372 are largely dispensable for a functional interaction between the isolated A1 and A2 subunits, this region appears critical for proper orientation of A2 subunit relative to factor IXa in the factor VIIIa trimer. These observations elucidate the mechanism for factor IXa- and APC-catalyzed inactivation of the cofactor.

**Materials and Methods**

**Reagents—**Recombinant factor VIII preparations were generous gifts from Bayer Corp. and the Genetics Institute. The monoclonal antibody specific for the N terminus of A1 was a gift from Dr. James Brown. The reagents α-thrombin, factor IXa, factor X, and factor Xa (Enzyme Research Laboratories); bovine and human APC (Hematology Technologies); and the chromogenic substrates S-2765 and S-2366 (Kabi-Pharcma) were purchased from the indicated vendors. Fluorescence reagents acrylodan and fluorescein 5-maleimide were obtained from Molecular Probes and Fl-FFR-IXa from Molecular Innovations. Quality Controlled Biochemicals, Inc. synthesized the synthetic peptide corresponding to the A1 subunit region 337–372. All other reagents were purchased from Sigma Chemical Co.

**Isolation of the A1 and A2 Subunits—**The A1 and the A2 subunits were isolated from recombinant factor VIII. Purified heavy chain (~7 mg), prepared as previously described (20), was incubated with thrombin (0.02 μM) for 2.5 h at room temperature in buffer containing 20 mM HEPES, pH 7.2, 0.1 mM NaCl, 2.5 mM CaCl2, and 0.025% Tween 20. The cleaved heavy chain was fractionated by fast-protein liquid chromatography using a Pharmacia Hi-Trap Heparin column. The A1 subunit was collected in the unbound fraction and A2 was eluted at ~0.3 mM NaCl using a linear salt gradient (0.1 mM to 1.0 M). The A1 subunit was further purified by fast-protein liquid chromatography using a Mono-Q column where it eluted at ~0.4 mM NaCl (12). The isolated A1 and the A2 subunits were dialyzed into a low salt buffer and stored at −80°C. Acrylodan-labeled A1 and A2 subunits were fractionated by energy transfer experiments, was prepared as described earlier (21).

**Cleavage of A1 to Form A1336—**Bovine APC was added to the purified A1 in a 1:20 (mol/mol) ratio in a buffer containing 20 mM HEPES, pH 7.2, 0.1 mM NaCl, 5 mM CaCl2, and 0.02% Tween 20, and reacted overnight at 22 °C. A1336 was separated from the cleaved 337–372 peptide and APC as follows. Approximately 100 μl of Q-Sepharose resin, equilibrated in the above buffer, was added to the cleavage reaction and incubated for 30 min. The solution was spun in a microcentrifuge to yield the supernatant containing the truncated A1 subunit. The resin bound the peptide fragment as well as APC as determined by SDS-PAGE of eluted material. A chromogenic assay revealed <0.2% residual APC activity was present in the purified A1336.

**Isolation and Labeling of A1/A3-C1-C2 and A1336/A3-C1-C2—Factor VIII (1 μM) was cleaved with thrombin in 20 mM HEPES, pH 7.2, 0.1 mM NaCl, 2.5 mM CaCl2, and 0.02% Tween 20, and reacted overnight at 22 °C. A1336 was separated from the cleaved 337–372 peptide and APC as follows. Appropriately 100 μl of Q-Sepharose resin, equilibrated in the above buffer, was added to the cleavage reaction and incubated for 30 min. The solution was spun in a microcentrifuge to yield the supernatant containing the truncated A1 subunit. The resin bound the peptide fragment as well as APC as determined by SDS-PAGE of eluted material. A chromogenic assay revealed <0.2% residual APC activity was present in the purified A1336.

**RESULTS**

**Isolation and Purification of Factor VIIIa Subunits—**In earlier studies, the isolated A1 and A2 subunits of factor VIIIa were prepared following proteolysis of recombinant factor VIII by thrombin (12, 13). However, subsequent subunit purification yielded relatively low levels of A2 subunit due to continued association with the A1/A3-C1-C2 dimer. Furthermore, the possibility for contamination of this subunit by low levels of dimer is undesirable due to the potential to regenerate factor VIIIa, thereby yielding significant levels of background activity to that attributed to A2 subunit alone. Recently, we showed that the isolated factor VIIIa heavy chain, which is efficiently cleaved by thrombin to yield A1 and A2 subunits, has no endogenous cofactor activity (20). Thus, purified factor VIIIa heavy chain was employed as a source for the A1 and A2 subunits. Separation of the subunits was facilitated by purification using a heparin chromatographic support, based upon recent identification of a high affinity heparin site within the A1 domain (28).

**Purified heavy chain was assessed to prepare the isolated subunits as described under “Materials and Methods.” As shown by gel electrophoresis, both subunits exhibited high degrees of purity (Fig. 1A) and were obtained in reasonable yield (~700 μg and ~300 μg of A1 and A2 subunits, respectively). Compared with earlier preparations of the subunits, yields were two to three times higher using the heavy chain as a starting material.
starting material. Subsequent reaction of isolated A1 subunit with bovine APC yielded the A1336 truncation. Western blot analysis using an anti-A1 domainal monoclonal antibody specific for the N-terminal sequence revealed quantitative cleavage at Arg336 (Fig. 1B). The cleaved A1 subunit was purified using Q-Sepharose resin as described under “Materials and Methods.” The A1336 failed to bind to the resin and was recovered in the supernatant following centrifugation, whereas the acidic C-terminal peptide product as well as APC were adsorbed by the resin (Fig. 1C). Residual APC activity in the purified A1336 sample was less than 0.2% of that present in the unfractionated reaction.

Stimulation of A2 Activity by A1 and A1336—In earlier reports, we showed that A2 subunit markedly increased the rate of factor IXa-catalyzed conversion of factor X to Xa (12) and that addition of the isolated A1 subunit further increased this activity by >10-fold (13). Comparison of the capacity for native and truncated A1 subunits to stimulate cofactor activity associated with A2 subunit is shown in Fig. 2. Reactions contained 400 nM A2 subunit and indicated levels of the A1 subunit forms, which were preincubated in the presence of factor IXa prior to initiation of the reaction with factor X. Increasing concentrations of either form of A1 stimulated A2 activity and this effect was saturable with respect to added A1. Maximal rates of factor Xa generation of ~15 and ~8 min⁻¹ were obtained at saturating levels of A1 and A1336, respectively. Negligible levels of activity were obtained in the absence of A2 subunit (data not shown) and were corrected for prior to curve fitting. The value obtained at saturating A1 was equivalent to an earlier reported value (13), although the twofold stimulation compared with A2 activity alone is several times greater. This effect resulted from the somewhat lower factor Xa-generating activity observed with the isolated A2 alone (~0.1 min⁻¹ using 400 nM A2) compared with the value obtained for an equivalent A2 concentration observed in an earlier report (~0.4 min⁻¹ (12)), possibly reflecting a more highly purified preparation of the subunit. Interestingly, the calculated affinities for the inter-subunit interactions (Kₐ values of 1.8 and 0.9 µM for A2 with native and truncated A1 forms, respectively) were similar, representing binding energy values of 7.8 and 8.2 kcal mol⁻¹, respectively. Taken together, these kinetic and binding parameters indicate that the C-terminal region of A1 is not necessary for either its association with A2 subunit or significant stimulation of A2 activity when examined using the isolated subunits.

Based on the observation that the truncated A1 yielded ~50% the A2 stimulatory activity of the intact A1 subunit, an experiment was performed to determine whether residues 337–372 directly contributed to the activity generated. This analysis was undertaken, in part, based on earlier results from our laboratory suggesting this peptide as interacting with A2 subunit (14, 23). A2 subunit-dependent stimulation of factor IXa was examined in the absence and presence of a synthetic peptide representing A1 residues 337–372 (Fig. 3). Results from this analysis showed no enhancement of activity by the peptide, but rather showed that high concentration of the peptide inhibited the rate of factor Xa generation. Similarly, peptide inhibited A2-dependent activity in the presence of A1 and A1336 subunits. These results indicated that the C-terminal A1 acidic peptide neither directly stimulates A2 nor synergistically enhances the activity of A1336 directed toward the A2 subunit. The mechanism for the observed inhibition of this peptide is not currently understood but does appear to be dependent upon the presence of the A2 subunit, consistent with an interaction between the peptide and A2 subunit (14, 23). Control experiments showed that the peptide had no effect on factor Xa activity directed toward the chromogenic substrate (data not shown).

Reconstitution of Factor VIIIa from Isolated Subunits—Although cleavage of the A1 subunit at Arg336 has minimal effects on the A1-dependent stimulation of A2 subunit activity, cleavage of factor VIIIa at this site by APC (29–31) or factor IXa (16, 17) abolishes activity. Therefore, we investigated this apparent disparity following reconstitution of factor VIIIa with either native or truncated A1 forms. The A1(A1336)3/C3-C1-C2 dimer was formed following reaction of the A3-C1-C2 subunit (500 nM) and varying amounts of either A1 or A1336 run in the presence of Ca²⁺ (Fig. 4). After an overnight incubation at 4 °C, the dimer solution was diluted 10-fold and reacted with A2 subunit, and reconstituted factor VIIIa activity was determined using a one-stage clotting assay.

Results from this experiment show high levels (~200 units/
Cofactor Activities following Cleavage of A1 Subunit

Effects of factor VIIIa subunits on the fluorescence anisotropy of F1-FFR-IXa in the absence and presence of factor X

| Sample | Minus factor X | Plus factor X |
|--------|---------------|---------------|
| F1-FFR-IXa | 0.225 ± 0.006 | 0.241 ± 0.007 |
| F1-FFR-IXa + A1/A3-C1-C2 | 0.256 ± 0.004 | 0.269 ± 0.004 |
| F1-FFR-IXa + A1/A3-C1-C2 + A2 | 0.281 ± 0.005 | 0.304 ± 0.006 |
| F1-FFR-IXa + A1336/A3-C1-C2 | 0.241 ± 0.004 | 0.250 ± 0.004 |
| F1-FFR-IXa + A1336/A3-C1-C2 + A2 | 0.270 ± 0.005 | 0.300 ± 0.006 |

* Reactions were conducted as described under "Materials and Methods" and contained 50 µM phospholipid vesicles, 100 nM F1-FFR-IXa, 600 nM peptide 337-372 on A2 subunit-dependent activity in the presence and absence of A1 or A1336. Reactions containing A2 (400 nM) alone (circles), A2 plus 400 nM A1 (triangles), or A2 plus 400 nM A1336 (squares) were preincubated at room temperature for 10 min in the presence of 10 µg/ml PSPCPE vesicles and 5 nM factor IXa. Reactions were initiated with 300 nM factor X, and the rate of factor Xa generation was determined as described under "Materials and Methods."
Ac-A2 subunit (fluorescence donor) overlaps with the excitation spectrum of the Fl-A1/A3-C1-C2 dimer (fluorescence acceptor). Upon binding of Ac-A2 to the Fl-dimer, 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 (33).

Reconstitution reactions were run using Ac-A2 (100 nM) and increasing amounts of either the Fl-A1/A3-C1-C2 or Fl-A1336/A3-C1-C2 dimer (Fig. 5). Reactions were run at pH 6.0 which enhances the inter-subunit affinity (8), to obtain near complete association of A2 subunit with the dimer forms. The relative fluorescence intensities were recorded at 472 nm, and the percentage donor quenching was calculated based on the intensity of Ac-A2 in the absence and presence of the Fl-dimers. Donor quenching was observed for either dimer form, indicative of association of Ac-A2 subunit. The Kq values calculated for interaction of Ac-A2 subunit with Fl-A1/A3-C1-C2 and Fl-A1336/A3-C1-C2 dimers were 102 and 50 nM, respectively, and were consistent with the inter-subunit affinity previously determined using a functional assay (Kq = 30 nM (8)). At near-saturating levels of acceptor (~800 nM), greater levels of quenching were observed with native A1-containing dimer (~55%) compared with the dimer possessing the truncated A1 (~40%), indicating a closer inter-fluorophore separation in the former pairing. Calculation of exact distances using this parameter plus other spectral data is not meaningful given the presence of acceptor fluorophores in both the A1 and A3-C1-C2 subunits of the dimer forms (21). However, because identical residues in the dimer forms are labeled with the fluorophore, these results suggest an altered orientation of A2 subunit relative to the dimer, which is dependent upon the presence of the C-terminal region of the A1 subunit.

In an additional series of experiments, the capacity of peptide 337–372 to interfere with the energy transfer between Ac-A2 (100 nM) and Fl-A1/A3-C1-C2 (400 nM) was assessed. Peptide (500 μM) had little if any effect on energy transfer, yet this level of peptide yielded greater than 50% inhibition of the reconstituted cofactor activity (data not shown). The extent of inhibition observed in this series of experiments was somewhat reduced compared with an earlier study (14) and likely reflects the greater specific activity of reconstituted factor VIIIa in the present report. These results suggest that the peptide-dependent inhibition did not result from blocking the association of A2 with the C-terminal region of the A1 subunit, consistent with alternative A2-interactive regions in the A1 subunit.

**FIG. 5.** Energy transfer between Ac-A2 and Fl-A1/A3-C1-C2 or Fl-A1336/A3-C1-C2. The emission intensity of Ac-A2 was measured at 472 nm and recorded in the absence and presence of the indicated levels of the Fl-labeled dimer forms. Fl-A1/A3-C1-C2 (squares) or Fl-A1336/A3-C1-C2 (circles) and Ac-A2 were incubated together for 30 min at room temperature prior to fluorescence readings. Percent donor quenching refers to the fluorescence intensity of Ac-A2 reconstituted with the dimer relative to that of Ac-A2 alone. Unlabeled dimer had no effect on the fluorescence emission of Ac-A2 (21). Low levels of the fluorescence acceptors (<50 nm) failed to yield reliable data due to the high relative background readings, and these data were not incorporated into the affinity calculations.

**DISCUSSION**

In earlier reports we showed that the isolated A2 subunit stimulated factor IXa-catalyzed conversion of factor X (12), and this effect was further enhanced in the presence of isolated A1 (13). Little information is known on the inter-subunit contacts between A1 and A2 subunits and how this association stimulates the cofactor activity associated with A2. An earlier series of studies examining cofactor function suggested that the C-terminal acidic region of A1 represented an A2-interactive site important for A2 retention following factor VIII activation (14). Thus one might predict that this region contributes to the enhancement of cofactor activity attributed to the isolated A2 subunit. Comparison of the capacity of A1 and A1336, the latter obtained after treatment of the isolated subunit with APC, to stimulate A2 subunit-dependent factor Xa generation revealed an ~50% reduction (kcat ~ 15 min⁻¹ and ~ 8 min⁻¹, respectively) when the C-terminal region of A1 was absent. Thus, although this region is not essential for the A1-dependent enhancement of A2 activity as measured using the isolated subunits, its presence is necessary for maximal stimulation of this effect.

On the other hand, we show that A1336 does not functionally replace native A1 subunit in factor VIIIa reconstitution studies. This observation is consistent with a number of studies showing that factor VIIIa cleaved at Arg336 by either APC (29–31) or factor IXa (16, 17) yields inactive factor VIIIa. Studies using the former protease show two sites of attack in factor VIIIa, Arg336 and Arg562 (18). Cleavage at the latter site destroys a factor IXa-interactive site (34), and this site is protected in the presence of factor IXa (35). The site at Arg336 reacts more rapidly in the presence of human APC (29, 36). Studies using recombinant factor VIII, where Arg562 is mutated so that the only susceptible bond is Arg336, still show inactivation by APC (31). Factor IXa catalyzes relatively slow inactivation of factor VIIIa, which correlates with cleavage at a single site (Arg336) within the heavy chain-derived A1 and A2 subunits (16, 17). The mechanism by which cleavage at this site disrupts cofactor activity is not well understood but has been attributed to enhanced dissociation of A2 subunit and/or reduction in the affinity or functional interaction of factor X (18, 19, 30).

Thus, the persistence of significant function attributed to the truncated A1336 subunit when assessed following the activity of isolated A2 compared with a lack of activity when assessed within the heterotrimer led us to investigate the mechanisms by which these disparate results may be explained. Clearly, the retention of A2 within factor VIIIa is a critical determinant to factor Xase stability in that dissociation of the A2 subunit represents the primary mechanism for factor Xase decay (6, 7). Results presented in this report show that this C-terminal region of A1 subunit contributes little if any to the inter-subunit affinity. Data using a functional assay that monitors stimulation of A2 activity by A1 subunit indicated similar inter-subunit affinity values (1–2 μM) for A2 with either native or truncated A1 forms. Although the extent of stimulation was partially reduced using the truncated A1 subunit, inclusion of peptide 337–372 failed to stimulate A2 or synergistically stimulate the effect observed with A1336. Taken together, these results suggest that residues other than the C-terminal tail of A1 are responsible for the inter-subunit affinity and directly contribute to the A2-dependent cofactor activity.

The basis for a role of residues 337–372 in the retention of A2 subunit was derived, in part, from an earlier study (14) show-
ing that the peptide blocked factor VIIa reconstitution in a functional assay. That study pre-dated subsequent observations showing cofactor activity associated with the isolated A2 subunit (12) and development of a fluorescence energy transfer-based assay to assess physical association of the A2 subunit with the A1/A3-C1-C2 dimer (21). Although the exact mechanism for peptide-dependent inhibition is unclear, our current results suggest that this peptide appears to inhibit a step in the activity assay probably unrelated to blocking the association of A2 with dimer. One possible mechanism for the observed inhibition is the peptide binds clustered basic residues in A2 subunit (21) that may indirectly affect subsequent interaction with factor IXa, thereby limiting cofactor activity. Alternatively, the peptide, which was also shown to interact with factor X (22) within the serine protease domain (37), may bind a site in the substrate thereby interfering with the generation of factor Xa.

However, other existing evidence for an association between residues 337–372 of A1 and the A2 subunit is suggestive of a functional interaction. Zero-length cross-linking studies indicated a salt bridge between the A1 and A2 subunits of factor VIIIa that was not present between these domains in the unactivated molecule (23). Furthermore, this cross-link did not form when A1 was truncated at Arg336, whereas the 337–372 VIa that was not present between these domains in the unactivated molecule appears to eliminate cofactor function by altering the orientation of A2 subunit within the trimer rather than by promoting its dissociation.

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