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The A1 and A2 Subunits of Factor VIIIa Synergistically Stimulate Factor IXa Catalytic Activity*

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Factor VIIIa, the protein cofactor for factor IXa, is comprised of A1, A2, and A3-C1-C2 subunits. Recently, we showed that isolated A2 subunit enhanced the k_{cat} for factor IXa-catalyzed activation of factor X by ~100-fold (~1 min^{-1}), whereas isolated A1 or A3-C1-C2 subunits showed no effect on this rate (Fay, P. J., and Koshibu, K. J. (1998) *J. Biol. Chem.* 273, 19049–19054). However, A1 subunit increased the A2-dependent stimulation by ~10-fold. The K_m for factor X in the presence of A2 subunit was unaffected by A1 subunit, whereas the k_{cat} observed in the presence of saturating A1 and A2 subunits (~15 min^{-1}) represented 5–10% of the value observed for native factor VIIIa (~200 min^{-1}). An anti-A1 subunit antibody that blocks the association of A2 eliminated the A1-dependent contribution to factor IXa activity. Inclusion of both A1 and A2 subunits resulted in greater increases in the fluorescence anisotropy of fluorescein-Phe-Phe-Arg factor IXa than that observed for A2 subunit alone and approached values obtained with factor VIIIa. These results indicate that A1 subunit alters the A2 subunit-dependent modulation of the active site of factor IXa to synergistically increase cofactor activity, yielding an overall increase in k_{cat} of over 1000-fold compared with factor IXa alone.

The proteolytically activated form of factor VIII, factor VIIIa, serves as a cofactor for the serine protease factor IXa in the conversion of factor X to factor Xa. This complex of enzyme and cofactor, assembled on an anionic phospholipid surface, is referred to as the intrinsic factor Xase. The role of factor VIIIa is to increase the catalytic rate constant (k_{cat}) by several orders of magnitude. The phospholipid surface is primarily involved in reducing molecular interactions to a two-dimensional space, thereby markedly decreasing the K_m for factor X. The association of factor VIIIa and factor IXa and the mechanism(s) by which factor VIIIa stimulates reaction rate are not fully understood. The importance of this interaction is indicated by defects or deficiency in factor VIII that result in hemophilia A.

Factor VIII circulates as a heavy chain (A1-A2-B domains) and a light chain (A3-C1-C2 domains) associated in a divalent metal ion-dependent heterodimer. Proteolysis by thrombin yields factor VIIIa, a trimer of A1, A2, and A3-C1-C2 subunits[1] (1, 2). The A1 and A3-C1-C2 subunits retain the divalent metal ion-dependent linkage and can be isolated as a stable dimer. Conversely, the A2 subunit is associated with the A1/A3-C1-C2 dimer in a primarily electrostatic interaction and readily dissociates from the dimer at physiological pH and ionic strength. However under appropriate reaction conditions, factor VIIIa can be reconstituted from isolated A1/A3-C1-C2 dimer and A2 subunit (2–4). The affinity of A2 subunit for the A1/A3-C1-C2 has been measured following functional assay (4, 5) as well as physical assay employing surface plasmon resonance (6). In human factor VIIIa, the affinity of A2 subunit for the A1/A3-C1-C2 dimer (K_d ~260 nM at physiological pH) is increased 10-fold under slightly acidic conditions (K_d ~30 nM at pH = 6.0) (5). Little is known about residues in the A2 subunit and A1/A3-C1-C2 dimer that are involved in the intersubunit interaction. Several lines of evidence suggest that the C-terminal acidic region of the A1 subunit (residues 337–372) participates in the retention of A2 subunit following cleavage at the A1-A2 junction. These observations include failure of A2 subunit to bind the A1/A3-C1-C2 dimer in which the A1 subunit has been truncated at residue 336 (6, 7).

At least two subunits of the factor VIIIa heterotrimer have been implicated as possessing factor IXa interactive sites. The factor VIII light chain-derived A3-C1-C2 subunit likely possesses a high affinity site for factor IXa. The free light chain of factor VIII shows similar affinity for factor IXa (K_d ~14 nM, Ref. 8) as is observed for factor VIIIa (K_d ~2–20 nM, Refs. 9 and 10). This suggests little if any of the binding energy for the interaction is contributed by the factor VIII heavy chain-derived subunits, A1 and A2. This interactive site was localized to the A3 domain following studies using inhibition by a monoclonal antibody whose epitope is represented by residues 1778–1840 (8), and was further localized to within residues 1811–1818 based upon studies employing synthetic peptides (11).

Based upon the identification of an activated protein C cleavage site at Arg^{562} in the A2 subunit (7), and the capacity for factor IXa to selectively protect from cleavage at this site (12), a factor IXa interactive site in the A2 subunit was postulated. Synthetic peptides spanning residues 558–565 noncompetitively inhibited factor Xase activity (13). The peptide also blocked the A2 subunit-dependent increase in fluorescence anisotropy of factor IXa labeled in its active site with fluorescein (14). Taken together, these results suggest that residues contained in A2 sequence 558–565 are critical to the interaction between cofactor and enzyme.

Recently, we showed that the isolated A2 subunit associates with the protease in the absence of other factor VIIIa subunits (15). The result of this interaction is an approximate 100-fold

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[1] The factor VIIIa nomenclature is based on the domainal structure designated by A1-A2-B-A3-C1-C2. Factor VIIIa subunits are designated as A1 (residues 1–372), A2 (residues 373–740) and A3-C1-C2 (residues 1690–2332) with noncovalent subunit associations denoted by a shill (/) and covalent associations by a hyphen (-).

enhancement in the rate of substrate factor X conversion by a mechanism affecting k_{cat} rather than K_m. This functional effect is unique to A2 and was not observed with the other isolated factor VIIIa subunits. However, the magnitude of this effect is ~1% of that observed for factor VIIIa, indicating contributions of other factor VIIIa subunits to the catalytic rate exist. In this study we show that A1 subunit alters the interaction of A2 subunit with factor IXa to synergistically enhance the A2 effect by ~10-fold, thus yielding a k_{cat} ~10% of that observed for the intact cofactor.

MATERIALS AND METHODS

Reagents—Recombinant factor VIII preparations (Kogenate™) were a gift from James Brown of Bayer Corp. Purified recombinant factor VIII also was a generous gift from Debbie Pittman of the Genetics Institute. The murine monoclonal antibody R8B12, which reacts with the C-terminal region of the factor VIII A2 domain (7), was prepared as described previously (2). Monoclonal antibody specific for the N-terminal region of A1 subunit was also a kind gift from James Brown. Anti-FVIII$^{337–372}$ IgG was obtained from a rabbit immunized with the synthetic peptide consisting of factor VIII residues 337–372 as described previously (16). The reagents α-thrombin, factor IXa$_\beta$, factor X, and factor Xa (Enzyme Research Labs); Fl-FFR-factor IXa[2] (Molecular Innovations), hirudin, and phospholipids (Sigma); and the chromogenic substrate S-2765 (*N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginyl-*p*-nitroanilide-dihydrochloride; Kabi-Pharmacia) were purchased from the indicated vendors.

Proteins—The Kogenate™ concentrate was fractionated to separate factor VIII from albumin following S-Sepharose chromatography (16). Factor VIII was converted to factor VIIIa using thrombin as described (2). Purification of the A2 subunit and A1/A3-C1-C2 dimer by Mono-S chromatography was as described (17). The A1 and A3-C1-C2 were prepared from the A1/A3-C1-C2 dimer following dissociation of the dimer by EDTA and chromatography on Mono-Q (17). In some instances, proteins were concentrated using a MicroCon concentrator (Millipore, 10 kDa cut-off). Factor VIII activity was measured by a one-stage clotting assay using plasma that had been chemically depleted of factor VIII activity as described previously (18). Protein concentrations were determined by the Coomassie Blue dye binding method of Bradford (19).

Factor Xa Generation Assays—The rate of conversion of factor X to factor Xa was monitored in a purified system (20). Factor VIIIa subunits were reacted with factor IXa in 20 mM Hepes, pH 7.2, 100 mM NaCl, 5 mM $CaCl_2$, and 0.01% Tween (Buffer A) in the presence of 200 μg/ml bovine serum albumin and 10 μM PS/PC/PE vesicles. The phospholipid vesicles containing 20% PS, 40% PC, and 40% PE were prepared using octyl glucoside as described previously (21). Time course reactions were initiated with the addition of factor X (see figure legends for reactant concentrations). Aliquots were removed at appropriate times to assess initial rates of product formation and were added to tubes containing EDTA (80 mM final concentration) to stop the reaction. Rates of factor Xa generation were determined by addition of the chromogenic substrate, S-2765 (0.46 mM final concentration). Reactions were read at 405 nm using a V_{max} microtiter plate reader (Molecular Devices).

Data Analysis—The influence of A1 on the affinity of A2 subunit for factor IXa was determined from the rate of factor Xa generation as a function of A2 concentration. Data were fitted to a single site ligand model where, amount bound = capacity × free/K_d + free, using the Marquart algorithm and UltraFit software (BioSoft). Because the concentration of A2 subunit was more than double the concentration of factor IXa for all A2 levels, the value for free A2 used the total A2 concentration. For this reason, the K_d determined is an apparent K_d. Using these conditions, the capacity term reflects the maximal rate enhancement at saturating A2 subunit. Data from initial rate kinetics were fitted to the Michaelis-Menten equation (UltraFit) to determine K_m and k_{cat} values.

Fluorescence Anisotropy—Fluorescence anisotropy measurements were made using a SPEX Fluorolog 212 spectrometer operated in the L format. The excitation wavelength was 495 nm (5-nm band pass), and the emission wavelength was 520 nm (14.4-nm band pass). Reactions (0.2 ml) were carried out at room temperature in Buffer A containing 30 nM Fl-FFR-factor IXa, 50 μM PS/PC/PE vesicles, and the indicated concentrations of factor VIIIa (or factor VIIIa subunits) and factor X in a quartz micro cell. Anisotropy measurements were made by manually rotating the polarizers and monitoring fluorescence for 5 s at each position. Fluorescence intensity determinations (3–5) were made at each position, and the average value was obtained. Blank readings for the buffer containing phospholipid were subtracted from all determinations.

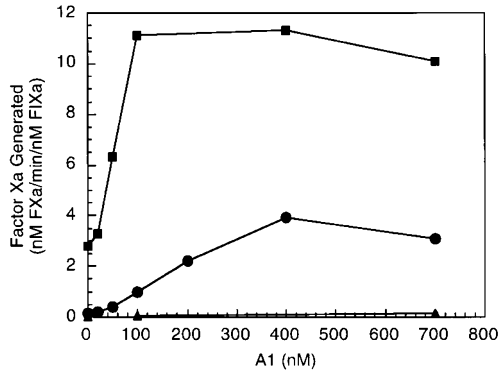

FIG. 1. **A1 subunit stimulates the A2 subunit-dependent enhancement of factor X activation.** Factor Xa generation reactions contained 1 nM factor IXa, 300 nM factor X, 10 μM PS/PC/PE vesicles and were performed as described under "Materials and Methods" in the absence (*triangles*) and presence of 50 nM (*circles*) or 400 nM (*squares*) A2 subunit plus the indicated concentrations of A1 subunit.

Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed using the method of Laemmli (22) with a Bio-Rad minigel electrophoresis system. Electrophoresis was carried out at 200 V for 1 h. Bands were visualized following staining with silver nitrate. Alternatively, the proteins were transferred to polyvinylidene diflouride membranes (Bio-Rad, 0.2 μm) using a Bio-Rad mini-transblot apparatus at 500 mA (constant current) for 30 min in buffer containing 10 mM CAPS, pH 11, and 10% (v/v) methanol. Western blotting was performed using the indicated antibodies followed by a goat anti-mouse horseradish peroxidase-conjugated secondary antibody. The secondary antibody signal was detected using the ECL system (Amersham Pharmacia Biotech) with luminol as the substrate, and the blots were exposed to film for various times.

RESULTS

Synergistic Effect of A1 and A2 Subunits on the Stimulation of Factor X Conversion—In a previous study (15) we showed that isolated A2 subunit increased the rate of factor IXa-catalyzed activation of factor X by ~100-fold. Because this A2-dependent rate increase was fractional compared with that observed for intact factor VIIIa, we examined the effect of A2 in combination with other factor VIIIa subunits on cofactor activity. Titration of A1 subunit in the presence of fixed levels of A2 subunit yielded marked increases in the rate of factor Xa generation relative to A2 subunit alone (Fig. 1). In this experiment, isolated A1 and A2 subunits were recombined in the reaction mixture for approximately 10 min prior to addition of factor IXa, and reactions were initiated with addition of factor X. In the absence of added A2 subunit, the A1 subunit showed no effect on the rate of factor Xa generation, consistent with our earlier observation (15). In the presence of a concentration of A2 subunit (50 nM) that was markedly less than the K_d for the A2-factor IXa interaction (~300 nM, Ref. 15), we observed little stimulation in the absence of A1. However, saturating levels of A1 subunit resulted in an approximate 20-fold stimulation in the rate of substrate conversion. Using a higher level of A2 subunit (400 nM) resulted in a significant stimulation of reaction rate that was further increased ~5-fold in the presence of saturating A1 subunit. Because A1 subunit alone shows no direct factor IXa stimulatory activity, and because the maximal

[2] The abbreviations used are: Fl-FFR-factor IXa, factor IXa modified in its active site with fluorescein-Phe-Phe-Arg-chloromethyl ketone; CAPS, 3-(cyclohexylamino)propanesulfonic acid; PS, phosphotidylserine, PC, phosphotidylcholine; PE, phosphotidylethanolamine; S-2765, Na-α-benzoyloxycarbonyl-D-arginyl-L-glycyl-L-arginyl-*p*-nitroanilide dichloride.

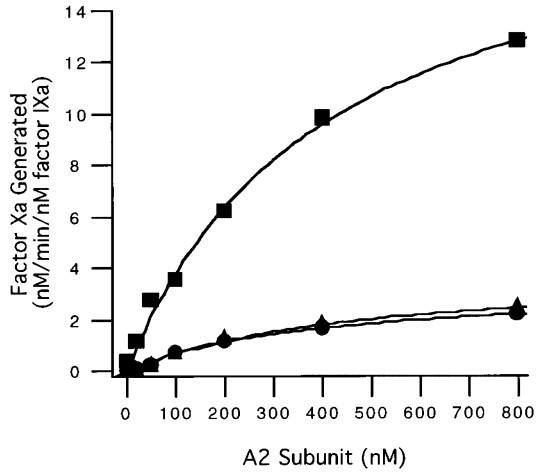

FIG. 2. **Effects of A1 and A3-C1-C2 subunits on the A2 subunit-dependent enhancement of factor IXa-catalyzed activation of factor X.** Factor Xa generation assays were performed as described in the legend to Fig. 1 using the indicated concentrations of A2 subunit alone (*circles*) or in the presence of either 1 μM A1 subunit (*squares*) or 1 μM A3-C1-C2 subunit (*triangles*). Data points were fitted to a single site ligand binding equation as described under "Materials and Methods."

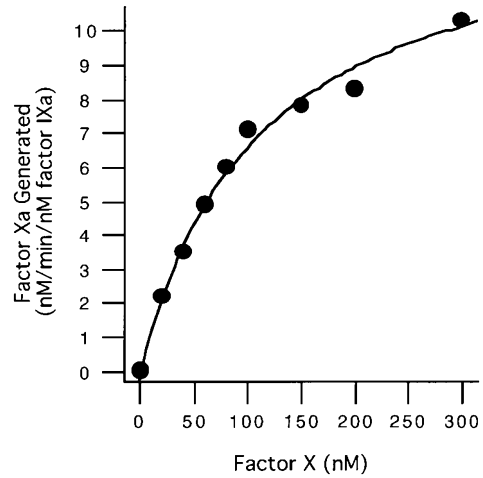

FIG. 3. **Effects of A1 and A2 subunits on the kinetics of factor Xa generation.** Reactions were run as described under "Materials and Methods" using 600 nM each A1 and A2 subunits, 1 nM factor IXa, and the indicated concentrations of factor X. Initial rates of factor Xa generation were plotted as a function of substrate concentration and fitted to a Michaelis-Menton equation using UltraFit software. Extracted values for K_m and k_{cat} were 107.4 ± 27.9 nM and 13.7 ± 1.6 min^{-1}, respectively.

levels of stimulation occur at A1 subunit concentrations in excess of the concentration of the A2 subunit, these results suggest that the stimulation observed in the presence of A1 subunit is mediated through its interaction with the A2 subunit.

A1 Subunit but Not A3-C1-C2 Subunit Stimulates A2-dependent Enhancement of Factor X Conversion—To determine whether this synergy was specific for the interaction of A2 with A1 or if A3-C1-C2 subunit also enhanced the A2 effect, the following experiment was performed. A2 subunit was titrated into the factor Xa generation reaction either alone or in the presence of 1 μM A1 subunit or 1 μM A3-C1-C2 subunit (Fig. 2). In the presence of A1 subunit, an approximate 7-fold increase in the A2 subunit-dependent rate of factor X activation was observed. At 1 μM A1 subunit, the level of stimulation of the A2-dependent effect was similar over the range of A2 concentrations employed in this analysis, suggesting that fold stimulation of factor Xa generation was directly dependent upon A2 subunit concentration. This result also points to the role of A1 in modulating A2 rather than another component(s) of the reaction. Because the A1 subunit likely provides the primary contact for A2 subunit in factor VIIIa (5, 16), we speculate that the affinity for the A1-A2 interaction is approximately that observed for the interaction of A2 with A1/A3-C1-C2 dimer (~260 nM, Refs. 4 and 5). Thus, the concentration of A1 employed in this experiment was consistent with near saturation of A2 subunit at all concentrations. However, titration of A2 subunit in the presence of 1 μM A3-C1-C2 subunit resulted in no incremental increase in the rate of substrate conversion compared with A2 alone. This result indicated that the light chain-derived factor VIIIa subunit was ineffective in modulating the A2-dependent stimulation of factor IXa-catalyzed activation of factor X.

Fitting the rate *versus* concentration data for the three experimental conditions to a single site ligand binding model was performed to evaluate the functional affinity of A2 subunit for factor IXa and the effects of the other factor VIIIa subunits on this parameter. In the absence of other additions, the A2 interaction with factor IXa yielded a functional (apparent) K_d of 377 ± 112 nM, similar to the value of 314 ± 89 nM obtained in our earlier study (15). A2 subunit in the presence of 1 μM A1 or A3-C1-C2 revealed similar affinity values (K_d = 410 ± 130 and 447 ± 194 nM, respectively). Thus the presence of a saturating level of A1 subunit did not significantly alter the affinity of A2 subunit for factor IXa. Interestingly, A3-C1-C2 subunit, which itself possesses relatively high affinity for factor IXa, did not alter the (functional) affinity of A2 for the enzyme. This result is consistent with the failure to observe direct interaction between these two factor VIIIa subunits. Using the capacity term of the ligand binding equation as an indicator of maximal rate enhancement, we obtained an ~7-fold increase in the presence of A1 (19.4 ± 2.9 nM factor Xa/min/nM factor IXa) compared with its absence (3.2 ± 0.4 nM factor Xa/min/nM factor IXa). Taken together, these data suggest that the A1 subunit of factor VIIIa modulates the A2 subunit, altering its interaction, but not its affinity, with factor IXa and resulting in an approximate order of magnitude increase in the rate of substrate conversion.

Effects of A1 Subunit on Kinetic Parameters for A2 Subunit-dependent Factor Xa Generation—In the previous study, we showed an increase in the k_{cat} for factor Xa conversion from 0.013 min^{-1} to 0.98 min^{-1} in the absence and presence of 600 nM A2, respectively, (15). A further increase in this parameter of approximately 10-fold was observed with A2 plus A1 subunits (Fig. 3). The value for k_{cat} obtained from the fitted curve (13.7 ± 1.6 min^{-1}) is approximately 5–10% that obtained with intact factor VIIIa (200 min^{-1}, Ref. 15). Inclusion of A1 subunit had no significant effect on the K_m for factor X (107 ± 28 nM) compared with this value in the absence of A1 subunit (101 ± 28 nM, Ref. 15), consistent with the primary effect of A1 in modulating the activity of the A2 subunit.

Inhibition of the A1-dependent Enhancement by Anti-factor VIII$^{337–372}$ Antibody—The association of A2 subunit with A1 appears to be mediated through the A1 C-terminal acidic region. In an earlier study, we showed that a polyclonal antibody prepared to this sequence (residues 337–372) inhibited the reassociation of A2 subunit with the A1/A3-C1-C2 dimer (16). An experiment was performed to determine whether this reagent affected the A1-dependent enhancement of the A2 effect. In the absence of antibody, we observed an ~3-fold rate increase for the reaction mixture supplemented with A1 subunit (Fig. 4). Inclusion of the IgG resulted in a dose-dependent elimination of this A1-dependent enhancement of factor Xa generation in the presence of A2 subunit. In the absence of A1

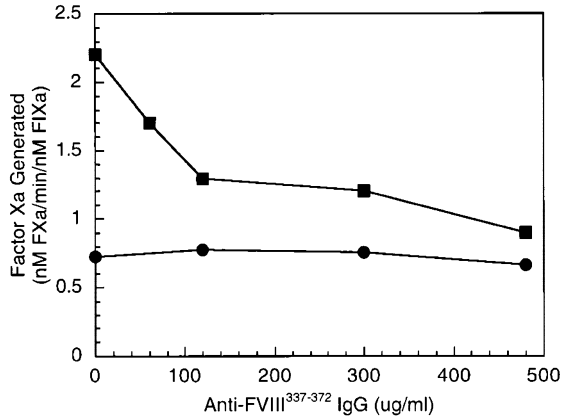

FIG. 4. **Effect of anti-FVIII$^{337–372}$ IgG on factor Xa generation in the presence of A1 and A2 subunits.** Indicated levels of the IgG were reacted with A2 subunit (120 nM) in the absence (*circles*) and presence (*squares*) of A1 subunit (70 nM) for 2 h at room temperature. Factor Xa generations were performed following addition of factor IXa (1 nM) and factor X (300 nM).

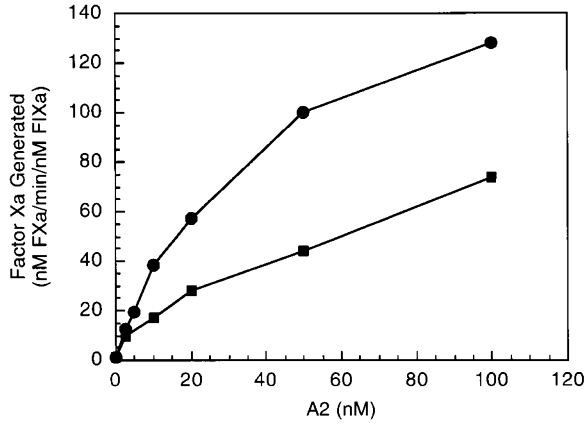

FIG. 5. **Reconstitution of factor VIIIa from isolated A1, A3-C1-C2, and A2 subunits in the absence and presence of Cu(II).** The A1/A3-C1-C2 dimer was prepared following reconstitution of A1 and A3-C1-C2 (500 nM each) overnight at 4 °C in Buffer A supplemented with 10 mM $CaCl_2$ in the absence (*squares*) or presence (*circles*) of 10 μM $CuCl_2$. Factor VIIIa was reconstituted in a reaction containing 50 nM of either dimer preparation plus indicated levels of A2 subunit. Factor Xa generation assays were performed as described under "Materials and Methods."

subunit, the IgG showed no effect on A2 stimulation of factor Xa generation. This result indicated that disruption of the interaction between A1 and A2 subunits eliminated the synergy observed in the presence of the two subunits and supported the notion that the effect of A1 subunit in this assay is to directly modulate A2 subunit.

Reconstitution of Factor VIIIa from Isolated Subunits—Although A2 in the presence of A1 subunit results in a significant increase in k_{cat} for factor Xa generation, this value reflects ~10% of the increase observed with factor VIIIa. To ensure that the reagents employed retained the potential to yield native cofactor-like activity, factor VIIIa was reconstituted from the three isolated subunits in a two-step reaction and evaluated in a factor Xa generation assay. In the first reconstitution step, the A1/A3-C1-C2 dimer was prepared following overnight reaction of the individual subunits in either a Ca(II) or Ca(II)/Cu(II) buffer. The products of these reactions were then mixed with A2 subunit prior to addition of factor IXa, and the reactions were initiated following addition of the substrate factor X. As shown in Fig. 5, authentic factor VIIIa-like activity (>100 nM factor Xa/min/nM factor IX) required reassociation of the three individual subunits. Although Ca(II) alone was sufficient to promote formation of significant factor VIIIa activity, the presence of Cu(II) during the association of A1 and A3-C1-C2 subunits enhanced the specific activity of the cofactor by ~2-fold, consistent with our earlier findings (23). The factor VIIIa reconstituted under these conditions showed a similar k_{cat} (~160 nM factor Xa/min/nM factor IXa) as observed earlier for native factor VIIIa (~200 nM factor Xa/min/nM factor IXa, Ref. 15). This result indicated that (i) the subunits employed in this study were functionally capable of generating the native cofactor, and (ii) A2 and A1 subunits are insufficient to yield the maximal k_{cat} effect, identifying an essential role for the A3-C1-C2 in contributing to this kinetic parameter.

Fluorescence Analysis of the Factor VIIIa Subunit-Factor IXa Interaction—This active site modulating activity of A1 in combination with A2 was further investigated for effects on the fluorescence anisotropy of Fl-FFR-factor IXa (Table I). In the previous report we observed that the presence of A2 subunit resulted in a modest increase in the fluorescence anisotropy of Fl-FFR-factor IXa (Δr = 0.015), suggesting that A2 subunit effects the orientation of the factor IXa active site (15). Furthermore, the increase in anisotropy of Fl-FFR-factor IXa plus A2 in the presence of saturating factor X (Δr = 0.086) was significantly greater than the factor X-dependent effect observed for factor IXa alone (Δr = 0.044), suggesting that this factor VIIIa subunit made a significant contribution to the orientation of the active site of the enzyme relative to the substrate. We now show that inclusion of both A1 and A2 subunits yielded further increases in anisotropy values obtained in the absence (Δr = 0.041) and presence (Δr = 0.073) of factor X. These results suggested that the increased rotational constraints imposed in the presence of both factor VIIIa subunits reflects the functional synergy observed in factor Xa generation rates. Furthermore, these values approach those observed with the native factor VIIIa (Ref. 14; Table I), suggesting a gradient to the factor VIIIa subunit-dependent increases in anisotropy that parallels the subunit-dependent effects on catalytic function.

TABLE I
Fluorescence anisotropy of F1-FFR-factor IXa

Sample[a]	Anisotropy[b]	
	Minus factor X	Plus factor X
F1-FFR-FIXa	0.193 ± 0.002[c]	0.237 ± 0.011[c]
F1-FFR-FIXa + A2 subunit	0.208 ± 0.005[c]	0.294 ± 0.010[c]
F1-FFR-FIXa + A2 + A1 subunits	0.234 ± 0.010 (5)	0.310 ± 0.009 (4)
F1-FFR-FIXa + factor VIIIa	0.277 ± 0.008 (3)	0.341 ± 0.013 (4)

[a] Reactions were performed as described under "Materials and Methods" and contained F1-FFR-FIXa (30 nM), PS/PC/PE (50 μM) and factor VIIIa subunit (600 nM each) or factor VIIIa (100 nM) and factor X (300 nM) where indicated.

[b] Values represent the mean ± S.D. for the number of determinations indicated in parentheses.

[c] Values are from Ref.

Isolated A1 Subunit Is Not a Substrate for Cleavage by Factor IXa—The above data demonstrate the A1 subunit contributes to cofactor activity when combined with A2. However, unlike A2 subunit, the A1 subunit is also a substrate for factor IXa cleavage at Arg^{336} (24, 25). The A1 subunit is cleaved by factor IXa at an appreciable rate (~0.2–0.5 min^{-1}, Ref. 24) in the A1/A3-C1-C2 dimer and in a phospholipid-dependent reaction. This rate is increased ~3-fold for the factor VIIIa substrate, suggesting a contribution of A2 subunit to this reaction (24). The following experiment was performed to determine whether the isolated A1 subunit remains a substrate for factor IXa and, if so, whether cleavage rate is influenced by the presence of A2

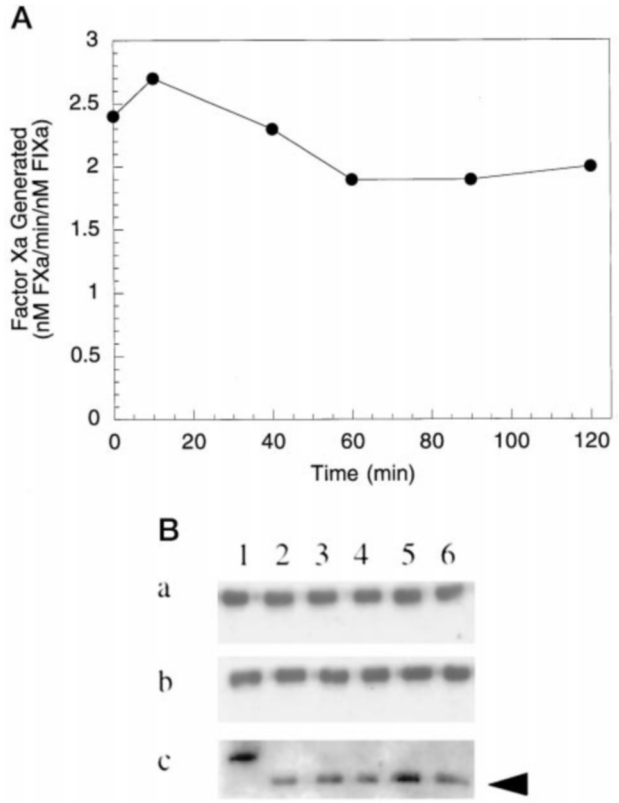

FIG. 6. *A*, stability of the A1-dependent stimulation of A2 cofactor activity. The A1 and A2 subunits (300 nM each) were reacted with 20 nM factor IXa in buffer A containing 200 μg/ml bovine serum albumin and 100 μM PS/PC/PE vesicles. At the indicated times, aliquots were removed and residual factor Xa generating activity was determined following addition of factor X to 300 nM. *B*, A1 subunit as a substrate for factor IXa. Western blotting using the 58.12 anti-A1 N terminus monoclonal antibody was performed as described under "Materials and Methods." Blot *a* shows samples obtained from the reaction described in *panel A* above. Blot *b* is a similar reaction run in the absence of A2 subunit. Blot *c* is a reaction containing 100 nM A1/A3-C1-C2 dimer in place of the isolated factor VIIIa subunits and under the conditions described above. *Arrowhead* identifies the A1 subunit cleaved at Arg^{336}. *Lanes 1–6* indicate time points at 0, 10, 40, 60, 90, and 120 min, respectively.

subunit. A1 subunit (300 nM) in the absence or presence of A2 subunit (300 nM) was reacted with factor IXa (20 nM) in the presence of PS/PC/PE vesicles. Samples were removed during the time course and assayed for both factor Xa generating activity and A1 subunit composition, the latter by blotting with an antibody that detects both intact and cleaved A1 subunit. The results of this experiment, shown in Fig. 6, indicate that the A1 contribution to A2-dependent activity remained relatively stable during the course of the reaction, retaining >80% of the initial activity level after a 2-h reaction with factor IXa. Consistent with this retention of activity, no detectable proteolysis of A1 was observed either in the absence or presence of A2 subunit. Alternatively, near total cleavage of A1 subunit in the A1/A3-C1-C2 dimer was observed following a 10-min reaction under near identical reaction conditions. These results show a dissociation of the cofactor from substrate activities of A1 relative to factor IXa and indicate the importance of A3-C1-C2 subunit in defining A1 as substrate. Thus, whereas A3-C1-C2 subunit is needed for maximal cofactor activity, this subunit appears essential for factor IXa to recognize A1 as substrate.

DISCUSSION

In the present study we demonstrate a significant contribution of A1 subunit to the A2-dependent stimulation of the catalytic activity of factor IXa toward factor X. The role of A1 appears to modulate the A2 subunit which in turn alters the interaction of enzyme with substrate. This effect is suggested by the ~10-fold increase in k_{cat} for the A2-dependent effect in factor Xa generation, whereas little if any change in A2 affinity for factor IXa is observed in the presence of A1 subunit. That A1 modulates A2 rather than factor IXa and/or factor X is suggested by several observations including (i) the lack of any contribution to catalytic rate in the presence of A1 subunit alone, (ii) a requirement for a stoichiometric excess of A1 relative to A2 subunit, and (iii) the inhibition of the A1-dependent contribution to activity by an antibody that disrupts the interaction between the two factor VIIIa subunits. These results implicate the A1 subunit as contributing to the cofactor function of factor VIIIa through an indirect mechanism relative to enzyme in the intrinsic factor Xase.

Relatively little information is available on the interactions between A2 and other factor VIIIa subunits. The affinity of A2 for A1/A3-C1-C2 is both pH (5, 26) and ionic strength (2) sensitive, with slightly acidic conditions increasing affinity by ~10-fold (5). The dissociation rate constant (~0.35 min^{-1}) is about 3-fold faster for the human protein than the porcine material (4). A2 subunit exhibits little if any affinity for the A3-C1-C2 subunit based upon failure of the latter subunit to inhibit reassociation of A2 with A1/A3-C1-C2 in a functional assay. This result is supported by the present study showing lack of effect of A3-C1-C2 on the A2-dependent stimulation of factor IXa activity even though the A3-C1-C2 possesses high affinity for the enzyme (8). Conversely, A1 subunit was demonstrated to effectively inhibit the association of A2 subunit with the A1/A3-C1-C2 dimer in that near equivalent concentrations of A1 and dimer yielded 50% inhibition of factor VIIIa reconstitution (5). Thus A2 likely interacts primarily with A1 subunit in the factor VIIIa heterotrimer.

The C-terminal region of A1 subunit appears critical for the association of A2 subunit. In an early experiment, it was observed that activated protein C-catalyzed cleavage of the A1/A3-C1-C2 dimer at Arg^{336} yielded a truncated version of the dimer ($A1^{336}$/A3-C1-C2), which was ineffective in competing with the native dimer for A2 association (7). Similarly, the truncated dimer failed to bind A2 subunit as judged by surface plasmon resonance (6). Subsequent studies showed direct interaction of the A2 subunit with a fluorescently labeled peptide corresponding to A1 C-terminal residues 337–372 (16) and localized a covalent linkage formed by the zero-length cross-linker, ethyl-dimethylaminopropylcarbodiimide, between the C-terminal region of A1 and the N-terminal half of A2 (27). Finally, antibody prepared to a synthetic peptide corresponding to the acidic residues 337–372 blocked the reconstitution of factor VIIIa activity from A2 plus dimer, presumably by interfering with the inter-subunit interaction (16). This immunologic reagent also blocked the contribution of A1 subunit to the A2-dependent stimulation of factor IXa activity, consistent with the role of the A1 C-terminal region in representing an A2 interactive site.

The above data support the notion that A1 stimulation is mediated through its interaction with A2. However, the mechanism by which A1 modulates the "cofactor" activity of A2 subunit toward factor IXa remains to be determined. One possibility is that A1 alters A2 conformation, and this alteration potentiates the cofactor role without altering affinity for the enzyme. Support for such a conformation change is suggested by changes in the affinity of the apolar dye, bisanilinonapthalsulfonic acid, for a surface-exposed hydrophobic pocket in the free A2 subunit compared with the A2 domain/subunit in factor VIII/factor VIIIa (28). In addition, thrombin cleavage of the

factor VIII heavy chain results in the formation of a new salt linkage between A1 and A2 subunits (27) that could yield a conformation change in the latter.

An alternative mechanism of A1 subunit directly modulating substrate factor X appears unlikely, but cannot be discounted. The anisotropy of Fl-FFR-factor IXa was increased in the presence of the A1/A3-C1-C2 dimer, and a further increase was observed in the presence of factor X (29). However, these effects may result in part from contribution of the A3-C1-C2 to factor IXa binding. Recently we showed that A1 subunit possesses an interactive site for factor X that is also contained within the C-terminal acidic region (17). The affinity for this interaction (K_d ~1–3 μM, (17)) is markedly lower than the concentration of factor X used in the factor Xa generation reactions performed in the present study. Furthermore, the degree of stimulation of the A2 effect by A1 subunit was independent of factor X concentration. Finally, the presence of A1 subunit did not affect the K_m for factor X. Taken together with the inability of isolated A1 subunit alone to enhance the rate of factor X conversion, these observations argue against this factor VIIIa subunit substantially affecting the enzyme-substrate interaction by an A2-independent mechanism.

The cofactor activity resulting from the presence of both A1 and A2 subunits yields an ~1000-fold increase in the k_{cat} for factor IXa-catalyzed conversion of factor X compared with factor IXa alone. This magnitude increase is ~10% of that observed in the presence of intact factor VIIIa. The reason for the disparity does not appear to reflect inactive/defective subunits resulting from the isolation procedures because native-like factor VIIIa activity can be reconstituted from the isolated A1, A3-C1-C2, and A2 subunits. Thus the difference in k_{cat} values obtained for A1 plus A2 *versus* intact factor VIIIa may be attributed to contributions by the A3-C1-C2 subunit. This result is consistent with observations showing that thrombin cleavage of the light chain actually contributes to the specific activity of factor VIIIa (30, 31).

The A3-C1-C2 subunit also appears to be responsible for the factor VIIIa-dependent reduction in K_m for factor X. K_m values for factor X are similar for reactions run with factor IXa alone (121 nM; Ref. 15), factor IXa plus A2 (101 nM; Ref. 15) and factor IXa plus A2 and A1 subunits (107 nM; this study). However, the K_m for factor X interactions run with intact factor VIIIa (33 nM; Ref. 15) is reduced by severalfold. This effect is likely dependent upon the phospholipid surface binding properties localized within the C2 domain of this subunit (32).

Finally, A3-C1-C2 appears to be critical for physiological interactions of factor VIIIa with factor IXa. First, this subunit contains a high affinity site for factor IXa (K_d~14 nM; Ref. 8) that approaches the affinity of factor VIIIa for the enzyme (K_d ~2 nM; Ref. 9). As noted earlier, the affinity of A2 subunit for factor IXa is markedly weaker (K_d ~300 nM; Ref. 15), and as determined in the present report, this value is unchanged by the presence of the A1 subunit. Second, the capacity for factor VIIIa to be recognized as substrate for factor IXa (that is, cleaved at Arg^{336} in the A1 subunit) appears dependent upon A3-C1-C2 and influenced by A2. Earlier studies showed that factor IXa cleaved intact factor VIIIa ~3-fold faster than A1/A3-C1-C2, implying a role for A2 in the rate increase (24). This result is not surprising given the recent observation of A2 enhancing factor IXa-catalyzed cleavage of substrate factor X. However, in the absence of A3-C1-C2, we observe no detectable cleavage of A1 subunit independent of the presence of A2 subunit and at concentrations that approach near maximal levels for the effects of these isolated subunits on factor Xa generating activity. This observation suggests A3-C1-C2 is likely to be required for proper orientation of A1 subunit for cleavage by factor IXa in the surface-dependent reaction. This orientation of A1 imposed by A3-C1-C2 may also be required for maximal cofactor activity. Thus the restriction in factor IXa proteolytic activity toward A1 subunit may also account for the sub-maximal rates of factor X conversion in the presence of A1 and A2 factor VIIIa subunits compared with the intact cofactor.

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