The A2 Subunit of Factor VIIIa Modulates the Active Site of Factor IXa*

(Received for publication, April 14, 1998, and in revised form, May 6, 1998)

Philip J. Fay‡§ and Kyoko Koshibu‡

From the Departments of §Medicine and ¶Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

Factor VIIIa, the protein cofactor for factor IXa, is comprised of A1, A2, and A3-C1-C2 subunits. Isolated subunits of factor VIIIa were examined for their ability to accelerate the factor IXa-catalyzed activation of factor X. The A2 subunit enhanced the $k_{cat}$ for this conversion by 100-fold whereas the $K_m$ for factor X was unaffected. The apparent $K_m$ for the interaction of A2 subunit with factor IXa was ~300 nM. Similar results were obtained using purified 2A expressed as the isoadd domain in Chinese hamster ovary cells, although this material was less stable than the factor VIIIa-derived material. Isolated A1 and A3-C1-C2 subunits showed no effect on the rate of factor X conversion. A2 subunit increased the fluorescence anisotropy of fluorescein-Phe-Phe-Arg-factor IXa ($\Delta r = 0.015$) and markedly increased anisotropy in the presence of factor X ($\Delta r = 0.057$), suggesting that it contributes to the orientation of the factor IXa active site and its relation to substrate. A synthetic peptide to A2 residues 558–565 inhibited the A2-dependent enhancement of factor X activity with an IC$_{50}$ of 40 nM, a value similar to its $K_m$ for inhibition of the intrinsic factor Xase (105 pm). These results indicate that the isolated A2 subunit modulates the active site of factor IXa and identifies a functional role for this subunit in factor VIIIa.

Factors VIII and IX are essential plasma proteins that when deficient or defective result in hemophilia A and B, respectively. The activated forms of these proteins, factor IXa (a serine protease) and factor VIIIa 1 (a protein cofactor), form a Ca$^{2+}$- and anionic phospholipid-dependent complex referred to as the intrinsic factor Xase complex that efficiently converts zymogen factor X to the serine protease, factor Xa (see Ref. 1 for review). The role of the phospholipid surface is to primarily reduce the $K_m$ for substrate, whereas factor VIIIa increases the $k_{cat}$ for this reaction by several orders of magnitude (2).

Factor VIIIa is a non-covalent heterotrimer composed of the A1, A2, and A3-C1-C2 subunits (3, 4). The A1 and A3-C1-C2 subunits retain the divalent metal ion-dependent linkage responsible for the association of the heavy and light chains in heterodimeric factor VIII and can be isolated as a stable dimer (5, 6). The A2 subunit is weakly associated with the dimer ($K_d$ ~260 nM (7, 8)) primarily through electrostatic interaction (4), and at physiologic pH it readily dissociates, resulting in the loss of factor VIIIa activity (4, 7–9). Under the appropriate reaction conditions of slightly acidic pH and low ionic strength, factor VIIIa activity can be efficiently reconstituted from the isolated A2 subunit and the A1/A3-C1-C2 dimer (4, 8–11).

Factor Xase activity is labile and has been referred to as self-damping (12), whereas factor VIIIa-independent conversion of factor X by factor IXa is relatively stable. Association of factor VIIIa with factor IXa in the presence of phospholipid and Ca$^{2+}$ stabilizes cofactor activity (13). Furthermore, reconstitution of heterotrimeric factor VIIIa from the isolated A2 subunit and A1/A3-C1-C2 dimer is enhanced several fold in the presence of factor IXa and phospholipid (10). Two factor IXa-interactive sites have been identified in factor VIIIa (a). One site, localized to residues 1811–1818 in the A3 domain of the factor VIII light chain (14), appears to be the dominant contributor to the interprotein affinity because isolated light chain exhibits a similar affinity for factor IXa ($K_d$ ~15 nM (15)) as is observed for the intact factor VIIIa ($K_d$ ~2 nM (16)). The second site, localized to residues 558–565 of the A2 subunit (domain) (17), is poorly characterized in its interaction with the protease. Thus one mechanism proposed for the factor IXa-dependent stabilization of factor VIIIa is the tethering of these interactive sites by the enzyme (10, 18). The resulting increased interfacial factor VIIIa subunit affinity reduces subunit dissociation, a predominant mode of factor Xase decay (19), effectively promoting substrate turnover. However, prolonged interaction of factor VIIIa with factor IXa results in a loss of cofactor activity because of proteolytic cleavage within the A1 domain (subunit) at Arg$^{336}$ (20, 21). Thus factor IXa modulates factor VIIIa activity, thereby affecting the catalytic efficiency of factor Xase.

Although interactive sites in the factor VIIIa-factor IXa enzyme complex have been identified, little is known about the contributions made by the factor VIIIa subunits that result in the marked increase in $k_{cat}$ when cofactor is present. Earlier results from our laboratory indicated that the A2 subunit in factor VIII likely interacts with the serine protease domain in factor IXa (18, 22). Studies presented in this report, undertaken to dissect interactions of individual factor VIIIa subunits with factor IXa, show that the isolated A2 subunit associates with the protease in the absence of other factor VIIIa subunits. The result of this interaction is an approximate 100-fold 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 is not observed with the other factor VIIIa subunits. These results support a model in which the A2 subunit of factor VIIIa contributes to catalysis within factor Xase by altering the orientation of the active site of factor IXa relative to substrate.
A2 Subunit Modulates Active Site of Factor IXa

MATERIALS AND METHODS

Reagents—Recombinant factor VIII preparations (Kogenate™) were a gift from Dr. Jim Brown of Bayer Corporation. Purified recombinant factor VIII was also a generous gift from Debbie Pittman of the Genetics Institute. The A2-secreting CHO cell line was a generous gift from Dr. Dorothea Scandella. The murine monoclonal antibody R8B12, which reacts with the C-terminal region of the factor VIII A2 domain (6), was prepared as described previously (4). The synthetic peptide SVDQRGNQ, which corresponds to factor VIII residues 558–565 (FVIII558–565), and a scrambled sequence version of the peptide (QVRSNGQD) were prepared by Quality Controlled Biochemicals. Phospholipid vesicles containing 20% PS, 40% PC, and 40% PE were prepared using octyl glucoside as described previously (23). The reagents—factor IXa, factor IXa, factor Xa, Factor Xa Enzyme Research Laboratories), Fl-FFR-factor IXa (Molecular Innovations), hirudin and phospholipids (Sigma), and the chromogenic substrate S-2765 (N-a-benzoyloxycarbonyl-n-arginyl-l-glycyl-l-argynyl-p-nitroanilide-di- hydrochloride; Kabi-Pharmacia) were purchased from the indicated vendors. The factor IXa incorporated >0.9 mol eq of dansyl-Glu-Gly-Arg chloromethyl ketone, indicating a high specific activity for this preparation of enzyme.

Proteins—The Kogenate™ concentrate was fractionated to separate factor VIII from albumin following S-Sepharose chromatography (24). Factor VIII was converted to factor IXa using thrombin as described (4). Purification of the A2 subunit and A1/A3-C1-C2 dimer by Mono S chromatography was as described (25). The A1 and A3-C1-C2 were separated from the A1/A3-C1-C2 dimer following dissociation of the dimer by EDTA and chromatography on Mono Q (25). A2CHO was purified from serum-free culture medium following application to an R8B12 immunoaffinity column attached in series to an S-Sepharose column as described (26). The purified A2 was essentially homogeneous (<95% pure) as judged by SDS-polyacrylamide gel electrophoresis. Approximately 0.1 mg of A2 was obtained from 200 ml of culture supernatant. 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 (27). Protein concentrations were determined by the Coomassie Blue dye binding method of Bradford (28).

Factor Xa Generation Assays—The rate of conversion of factor X to factor Xa was monitored in a purified system (29). Factor VIIIa subunits were reacted with factor IXa (5 nM) in 20 mM HEPES, pH 7.2, 100 mM NaCl, 5 mM CaCl2, 0.01% Tween (Buffer A) in the presence of 200 μg/ml bovine serum albumin and 10 μM PS-PC-PE vesicles. Time course reactions were initiated with the addition of factor X (see figure legends for reactant concentrations). Aliquots were removed at appropriate times and assayed for factor Xa formation and 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 Vmax microtiter plate reader (Molecular Devices).

Data Analysis—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/ka on free, using the Marquart algorithm and UltraFit software (BioSoft). Because the concentration of A2 subunit was >> concentration of factor IXa for all A2 levels, the value for free A2 used the total A2 concentration. For this reason the ka on free is determined as an apparent kcat. 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 KeM and kcat parameters.

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 nm band pass). Reactions (0.2 ml) were carried out at room temperature in Buffer A containing 30 mM Fl-FFR-IXa, 50 μM PS-PC-PE vesicles, and the indicated concentrations of A2 subunit and factor X in a quartz microcell. 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 obtained. Blank readings for the buffer containing phospholipid were subtracted from all determinations.

Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed using the method of Laemmli (30) with a Bio-Rad minigel electrophoresis system. Electrophoresis was carried out at 200 V for 45 min. Bands were visualized following staining with silver nitrate. Denitrogenic scans were performed using a Beckman DU 650 spectrophotometer equipped with Gel Scan software.

RESULTS

A2 Subunit Enhances the Factor IXa-catalyzed Conversion of Factor X—Factor VIIIa subunits were isolated as described under “Materials and Methods.” SDS-polyacrylamide gel electrophoresis visualized by silver staining indicated the subunits were of high purity (Fig. 1). One-stage clotting assays using the individual subunits showed no activity indicating the subunit preparations were free of factor VIIIa. The isolated A1, A2, and A3-C1-C2 subunits were separately titrated into reaction mixtures containing factor IXa (5 nM), PS:PC:PE vesicles (10 μM), and factor X (200 nM), and rates of factor Xa generation were determined using a chromogenic assay. Results in Fig. 2 show that inclusion of A2 subunit yielded a dose-dependent increase in the rate of factor X generation, suggesting that association of the isolated A2 subunit with factor IXa altered the catalytic activity of the enzyme. Fitting the data to a single-site ligand binding model yielded an apparent Kd of 314 ± 89 nM. The extent of maximal rate enhancement extracted from the curve (2.6 ± 0.4 nM factor Xa formed per min) indicated an approximate 100-fold increase compared with the rate of substrate conversion in the absence of A2 subunit (0.028 ± 0.005 nM factor Xa formed per min). Neither the A1 nor the A3-C1-C2 subunit affected the rate of factor IXa-catalyzed conversion of factor X over the concentration ranges employed. In fact, increasing levels of A1 subunit actually resulted in a slight (~2-fold) reduction in the rate of factor Xa generation. One explanation for this observation is that the A1 subunit is a substrate for factor IXa cleavage at Arg[396] (21). Thus rate reduction of factor X conversion in the presence of A1 subunit may occur by competition between these factor IXa substrates.

The stimulation of factor IXa-catalyzed conversion of factor X by A2 subunit was further evaluated as a function of substrate concentration. Initial rates of product formation were determined for factor IXa in the absence or presence of A2 subunit (600 nM). This concentration of A2 reflects 2 times the Kd value indicating the majority of enzyme is associated with A2 subunit. Results obtained from this analysis were fitted to the Michaelis-Menten equation, and kinetic parameters were determined. As shown in Table I, near saturating levels of A2 subunit had little if any effect on the Km for the factor IXa-catalyzed conversion of factor X. However, the kcat value was

![Fig. 1. SDS-polyacrylamide gel of purified factor VIIIa subunits. Factor VIIIa subunits were purified as described under "Materials and Methods." Approximately 1 μg of A2 (lane 1), A1 (lane 2), and A3-C1-C2 (lane 3) was applied to the gel, which was stained with silver nitrate.](image-url)
and the indicated concentrations of A3-C1-C2 (described under “Materials and Methods.”) up to 1 h and used to calculate the amount of factor Xa formed. Data points for A2 subunit were fitted to a single-site ligand binding model as described under “Materials and Methods.”

Table I

| Sample | $K_m$ (μM) | $k_{cat}$ (min⁻¹) | $k_{cat}/K_m$ (μM⁻¹ min⁻¹) | Fold |
|--------|------------|-------------------|-----------------------------|------|
| Factor IXa | 0.121 ± 0.027 | 0.013 ± 0.001 | 0.107 (1) |
| + A2 | 0.101 ± 0.028 | 0.082 ± 0.07 | 9.8 (92) |
| + FVIIIa | 0.033 ± 0.010 | 202 ± 21 | 6121 | 57,200 |

Reactions were run in buffer A containing 10 μM PS-PC-PE vesicles as described under “Materials and Methods.” Reactions run in the absence of other additions contained 20 nM factor IXa. Reactions run in the presence of A2 subunit (600 nM) contained 5 nM factor IXa, and those run in the presence of factor VIIIa (10 nM) contained 0.5 nM factor IXa. Values represent the average of at least two separate experiments.

Further evidence that the enhancement of factor Xa generation was A2-dependent and not the result of contaminating factor VIIIa and/or subunits was obtained following analysis of A2 expressed as an isolated domain in CHO cells. This A2 form, designated A2CHO, was purified as described under “Materials and Methods” to >95% pure as judged by SDS-polyacrylamide gels. Similar to the A2 subunit derived from factor VIIIa, the A2CHO enhanced the factor IXa-dependent conversion of factor X in a dose-dependent manner (Fig. 4). Data points were fitted to a single-site ligand binding model. The derived value for $K_d$ (1.46 ± 0.53 μM) indicated a severalfold weaker affinity for A2CHO compared with A2 subunit derived from factor VIIIa. Furthermore, the maximal rate enhancement for this form of A2 was approximately 30% that observed for the factor VIIIa-derived material. These disparities may be attributed to the apparent instability of the A2CHO form. We observed that $\sim$50% of the factor IXa-enhancing activity of A2CHO was lost following 48 h at 4 °C compared with no significant loss of activity of the factor VIIIa-derived material under the same conditions. In fact, the latter was stable for over 2 weeks under these conditions. For this reason, the results presented above were obtained within 24 h of purification of the A2CHO. Similar to the -fold stimulation observed with the two A2 forms in the factor IXa-dependent conversion of factor X, factor VIIIa reconstitution using either A2 form (40 nM) plus the A1/A3-C1-C2 dimer (20 nM) revealed the factor VIIIa-derived A2 yielded $\sim$4-fold more factor VIIIa activity than the A2CHO (110 versus 25 units/ml, respectively). SDS-polyacrylamide gel electrophoresis of reduced and non-reduced samples indicated that the instability of A2CHO did not result from proteolysis or dimerization of the A2 by formation of a disulfide bond between free Cys residues in the subunit (data not shown). Taken together, these results indicate that factor IXa-catalyzed conversion of factor X to factor Xa is enhanced by the presence of the isolated A2 subunit, and the extent of this enhancement appears dependent upon the intrinsic stability (conformation) of the A2.

Fluorescence Analysis of the A2-Factor IXa Interaction—To determine whether isolated A2 subunit interacted similarly with the Fl-FFR-factor IXa, this active site-modified factor IXa was used as a competitor of native factor IXa (fixed at 5 nM) in the factor Xa generation assay. Titration with Fl-FFR-factor IXa resulted in the dose-dependent inhibition of the A2-dependent enhancement effect (results not shown). Fifty percent inhibition of factor Xa generation was obtained at $\sim$6 nM Fl-FFR-factor IXa, indicating that A2 interacted similarly with...
the native and active site-modified enzymes. Earlier, we showed that the anisotropy value for F1-FFR-factor IXa in the presence of factor VIIIa could be achieved following addition of the A2 subunit to a mixture consisting of A1/A3-C1-C2 dimer plus F1-FFR-factor IXa, suggesting that A2 altered the factor IXa active site in the presence of the complementary factor VIIIa subunits (22). This active site modulating activity of A2 was further investigated following analysis of the isolated subunit on the fluorescence anisotropy of F1-FFR-factor IXa. For these experiments, F1-FFR-factor IXa (30 nm) in the presence of PS-PC-PE vesicles (50 μM) was reacted with 600 nM A2 subunit, and fluorescence measurements were determined as described under “Materials and Methods.” This concentration of A2 subunit was calculated to yield nearly 70% saturation of the labeled factor IXa. The presence of A2 subunit resulted in a modest increase in the fluorescence anisotropy of F1-FFR-factor IXa (Δr = 0.015), suggesting that A2 subunit alters the orientation of the factor IXa active site (Table II). When F1-FFR-factor IXa was reacted with substrate factor X (300 nM), we observed an increase in anisotropy likely reflecting constraints on the rotational motion of the fluorophore in the enzyme-substrate complex. This factor X-dependent effect on F1-FFR-factor IXa was markedly increased in the presence of A2 subunit (Δr = 0.057), suggesting that this factor VIIIa subunit made a significant contribution to the orientation of the active site of the enzyme relative to the substrate.

Inhibition of the A2 Effect by FVIII558–565 Peptide—Previous work from our laboratory suggested that the sequence in and around residues 558–565 in the A2 subunit represented a factor IXa interactive site (17). Several lines of evidence supported this contention including selective factor IXa-dependent protection from activated protein C-catalyzed cleavage at Arg562 (33) and the capacity for a peptide prepared to this sequence to non-competitively inhibit factor Xase (17). Direct evidence that this peptide inhibited the A2-factor IXa interaction was obtained following an experiment to determine the peptide’s effect on the A2-dependent enhancement of factor X activation by factor IXa (Fig. 5). Inclusion of the FVIII558–565 peptide reduced this enhancement to activity levels approaching that of factor IXa alone. Determination of the IC50 indicated a value of 40 μM. This concentration compares with the Kd determined for the non-competitive inhibition of intrinsic factor Xase by the peptide (105 μM (17)). Furthermore, a scrambled sequence version of the peptide had minimal inhibitory effect on the A2-dependent enhancement of the reaction. This result indicated an A2 sequence-specific interactive site on factor IXa and was consistent with earlier data showing a lack of effect of the scrambled sequence peptide in inhibiting factor Xase (17). In the absence of A2, the peptide did not effect the factor IXa-catalyzed conversion of factor X, indicating that occupancy of this site in factor IXa is not sufficient to modulate the active site of the enzyme.

DISCUSSION

Factor VIIIa increases the kcat of factor IXa-dependent conversion of factor X by several orders of magnitude. The mechanism by which this occurs is largely unknown. In this report we show that the isolated A2 subunit of factor VIIIa markedly contributes to this catalytic activity by enhancing the reaction rate ~100-fold. This effect does not result from reducing the Km for substrate factor X but rather reflects an A2 subunit-dependent alteration of the factor IXa active site. This property of catalytic enhancement is unique to the A2 subunit in that neither the isolated A1 nor the A3-C1-C2 subunit of factor VIIIa affected the rate of factor X conversion. Based upon these observations we suggest that the A2 subunit, which is essential for factor VIIIa activity (4, 5), serves a primary role in modulating the active site of factor IXa in the intrinsic factor Xase complex.

The degree by which A2 subunit increases the rate of substrate conversion is fractional compared with that observed for intact factor VIIIa. Original studies using the bovine system indicated that kcat was increased by 5 orders of magnitude in the presence of factor VIIIa compared with its absence (2). This effect is somewhat lessened in the human system with increases in kcat ranging from ~500 (34) to ~30,000–60,000 (Refs. 31 and 32 and this study). This variability likely reflects the extent of saturation of factor IXa with factor VIIIa. In addition, human factor VIII appears to reduce the Km for sub-
strate by severalfold (Refs. 31 and 32 and this study).

Although the effect of isolated A2 subunit on the active site of factor IXα, as judged by activity assays, appears significant, it is not equivalent to that observed for factor VIIIα. Further, A2 subunit induced a modest increase in the fluorescence anisotropy of Fl-FFR-factor IXα (Δr = 0.015) compared with a substantially greater value obtained with saturating factor VIIIα (Δr = 0.069) (18). Similar to earlier results (35), we observed that factor X resulted in an increase in anisotropy of Fl-FFR-factor IXα. Inclusion of A2 subunit resulted in a markedly greater increase in anisotropy observed in the presence of Fl-FFR-factor IXα plus factor X (Δr = 0.057). This effect did not result from a direct interaction of A2 with factor X based upon earlier results showing no interaction between the two proteins (25). Thus A2 subunit likely induced a conformation in the enzyme, thereby altering its interaction with substrate to more closely resemble the interaction of factor X with intrinsic factor Xase. Taken together, the activity and fluorescence data support the hypothesis that a synergism involving other factor VIIIα subunits is required for optimal cofactor-dependent modulation of the factor IXα active site (22). Interestingly, A2 subunit also enhances factor IXα activity directed toward another macromolecular substrate, the A1/A3-C1-C2 dimer. Comparison of cleavage rates of Arg336 in the A1/A3-C1-C2 with the intact factor VIIIα heterotrimer revealed that the latter was cleaved severalfold faster (21), suggesting a stimulation by the presence of A2 subunit.

Results from this study show that the affinity of A2 subunit for factor IXα (≈300 nM) is markedly weaker that the affinity of factor VIIIα for the enzyme. Values for the latter interaction are variable, ranging from ~70 pm (36) to ~2 nM (16). The reasons(s) for this variability (is) are not entirely clear but may reflect assays used for quantitation, with those based upon activity yielding higher affinity values than those employing physical methods to assess binding. The recent observation that factor X increases the affinity of enzyme and cofactor by 10–15-fold (36) would contribute to values derived using a functional assay. Thus it appears that the majority of binding energy is derived from interaction of factor IXα with subunits other than A2. Solid phase binding studies indicated that the factor VIII light chain binds factor IXα with a Kd of ~15 nM (15). A similar value was obtained for the A1/A3-C1-C2 dimer in solution phase using fluorescence anisotropy (18). However, the capacity for factor IXα to promote the interaction of A1/A3-C1-C2 dimer with the A2 subunit, as judged by enhancement in the reconstitution factor VIIIα activity (10), suggested multiple interactive sites between cofactor and enzyme. The above results indicate that the A2 subunit-factor IXα interaction is at least 100-fold weaker than the interaction of factor IXα with intact cofactor and supports the hypothesis that the light chain (A3-C1-C2 domains) of factor VIII provides for the primary interaction with factor IXα.

The presence of low levels of factor VIIIα contaminating the purified factor VIIIα subunits would markedly increase rates of factor X activation. However, several lines of evidence indicate that observed activities were not derived from low levels of the intact cofactor. First, neither isolated A1 nor A3-C1-C2 demonstrated the capacity to accelerate substrate conversion, indicating that the subunits were free from intact cofactor. Second, the catalytic efficiency (kcat/Km) at near saturating A2 (9.8 μM⁻¹ min⁻¹) was ~600-fold less than the value at saturating factor VIIIα (6121 μM⁻¹ min⁻¹). Third, isolated A2 domain expressed in a CHO cell line also demonstrated the capacity to stimulate the factor IXα-dependent conversion of factor X. The magnitude of this effect using the CHO-expressed material was severalfold less than that observed with material derived from factor VIIIα and appeared to reflect the intrinsic instability of the former. The reason(s) for this instability is (are) not known. One possibility reflects incorrect folding of the individual domain during its synthesis/secretion. Alternatively, this observed instability may reflect the cell type used for expression. For example, expression of factor VIII in CHO cells is dependent upon co-expression of von Willebrand factor to stabilize the heterodimer structure (37). Expression from these cells in the absence of von Willebrand factor yields a large proportion of factor VIII as separated subunits. In contrast, expression of factor VIII in baby hamster kidney cells does not require co-expression with von Willebrand factor to yield the intact heterodimer (38).

Homology modeling of factor VIII A domains based on the ceruloplasmin (39) or nitrude reductase (40) crystal structures identifies a loop structure at the junction of the d3 and d4 subdomains of the A2 domain. Arg⁶⁶⁶ contained within this loop is cleaved by activated protein C resulting in loss of cofactor activity (6). Early evidence that the sequence in and around this scissile bond represented a factor IXα interactive site was based upon the observation that factor IXα selectively protected this site from activated protein C-catalyzed cleavage although not affecting the rate of cleavage at Arg⁶⁶⁶ (33). The capacity for the synthetic peptide, FVIII⁶⁶⁶–⁶⁶⁵, to non-competitively inhibit the intrinsic factor Xase (17), block the factor IXα-dependent enhancement of factor VIIIα reconstitution from isolated subunits (17), as well as inhibit the A2-dependent increase in Fl-FFR-IXα anisotropy by factor VIIIα (18) led us to suggest that this central region of A2 interacted with factor IXα. Several missense mutations within this region yielding CRM⁺ hemophilia A have been attributed to a defect in the interaction of cofactor with enzyme (39). Indeed, in a recent study Amano et al. (41) prepared recombinant factor VIII molecules bearing point mutations in this region that result in CRM⁺ hemophilia A. IC₅₀ levels for the 558–565 peptide were reduced for these molecules compared with wild type factor VIII, providing indirect evidence that the mutant molecules more weakly interacted with factor IXα.

Results from this study showed that the FVIII⁶⁶⁶–⁶⁶⁵ peptide did not affect the activity of factor IXα-catalyzed conversion of factor X. This result suggested the peptide interacts with a site on the protease that is not an allosteric region or at the active site of the enzyme. The latter contention is supported by failure to observe an effect of the peptide on the fluorescence anisotropy of Fl-FFR-IXα (18). However, this peptide inhibited the A2-factor IXα interaction with an IC₅₀ of ~40 μM. This value is similar to the Kd determined for the non-competitive inhibition of intrinsic factor Xase by the peptide (105 μM (17)) and supports the hypothesis that inhibition of the enzyme complex occurred by perturbation of the interaction of this subunit with factor IXα. The IC₅₀ value is ~100-fold greater than the Kd value obtained for the A2 subunit-factor IXα interaction (300 nM). Thus, the peptide may represent a portion of the interactive site. Alternatively, the FVIII⁶⁶⁶–⁶⁶⁵ peptide may exist in an ensemble of conformations of which the inhibitory conformation represents a fraction of the total structures.

Acknowledgments—We thank Dr. Jim Brown of Bayer Corporation and Debbie Pittman of Genetics Institute for the gifts of recombinant factor VIII and Dr. Dorothea Scandella for the A2-expressing CHO cell line. We also thank K. Lapan for preparation of the A1 and A3-C1-C2 subunits.

REFERENCES
1. Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P., and Krishnaswamy, S. (1990) Blood 76, 1–16
2. van Diejen, G., Tans, G., Rosing, J., and Hemker, H. C. (1981) J. Biol. Chem. 256, 3433–3442
3. Lollar, P., and Parker, C. G. (1989) Biochemistry 28, 666–674
4. Fay, P. J., Haidaris, P. J., and Smulski, T. M. (1991) J. Biol. Chem. 266,
A2 Subunit Modulates Active Site of Factor IXa

8957–8962
5. Lollar, P., and Parker, C. G. (1990) J. Biol. Chem. 265, 1688–1692
6. Fay, P. J., Smudzin, T. M., and Parker, F. J. (1991) J. Biol. Chem. 266, 20139–20145
7. Fay, P. J., and Smudzin, T. M. (1992) J. Biol. Chem. 267, 13246–13250
8. Lollar, P., Parker, E. T., and Fay, P. J. (1992) J. Biol. Chem. 267, 12841–12846
9. Lamphear, B. J., and Fay, P. J. (1992) J. Biol. Chem. 267, 3725–3730
10. Pittman, D. D., Millenson, M., Marquette, K., Bauer, K., and Kaufman, R. J. (1992) Blood 79, 399–397
11. Jensen, Y. (1990) J. Biol. Chem. 265, 17539–17544
12. Pittman, D. D., Millenson, M., Marquette, K., Bauer, K., and Kaufman, R. J. (1992) Blood 79, 389–397
13. Lollar, P., Knutson, G. J., and Fass, D. N. (1984) Blood 63, 1303–1308
14. Lenting, P. J., van de Loo, J. W., Donath, M. J., van Mourik, J. A., and Mertens, K. (1996) J. Biol. Chem. 271, 1935–1940
15. Lenting, P. J., Donath, M. J., van Mourik, J. A., and Mertens, K. (1994) J. Biol. Chem. 269, 7150–7155
16. Duffy, E. J., Parker, E. T., Mutucumarana, V. P., Johnson, A. E., and Lollar, P. (1992) J. Biol. Chem. 267, 17006–17011
17. Fay, P. J., Beattie, T., Huggins, C. F., and Regan, L. M. (1994) J. Biol. Chem. 269, 20552–20557
18. O’Brien, L. M., Medved, L. V., and Fay, P. J. (1995) J. Biol. Chem. 270, 27087–27092
19. Fay, P. J., Beattie, T. L., Regan, L. M., O’Brien, L. M., and Kaufman, R. J. (1996) J. Biol. Chem. 271, 6027–6032
20. O’Brien, D. P., Johnson, D., Byfield, P., and Tuddenham, E. G. (1992) Biochemistry 31, 2805–2812
21. Lamphear, B. J., and Fay, P. J. (1992) Blood 80, 3120–3126
22. Regan, L. M., O’Brien, L. M., Beattie, T. L., Sudhakar, K., Walker, F. J., and Fay, P. J. (1996) J. Biol. Chem. 271, 3982–3987
23. Mimms, L. T., Zampighi, G., Nozaki, Y., Tanford, C., and Reynolds, J. A. (1981) Biochemistry 20, 833–840
24. Fay, P. J., Haidaris, P. J., and Huggins, C. F. (1993) J. Biol. Chem. 268, 17861–17866
25. Lapan, K. A., and Fay, P. J. (1997) J. Biol. Chem. 272, 2082–2088
26. O’Brien, L. M., Huggins, C. F., and Fay, P. J. (1997) Blood 90, 3943–3950
27. Casillas, G., Simonetti, C., and Pavlovsky, A. (1971) Congrulation 4, 107–111
28. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
29. Lollar, P., Fay, P. J., and Fass, D. N. (1993) Methods Enzymol. 222, 128–143
30. Laemmli, U. K. (1970) Nature 227, 680–685
31. Rawala-Sheikh, R., Ahmad, S. S., Ashby, B., and Walsh, P. N. (1990) Biochemistry 29, 2606–2611
32. Gilbert, G. E., and Arena, A. A. (1997) Biochemistry 36, 10768–10776
33. Regan, L. M., Lamphear, B. J., Huggins, C. F., Walker, F. J., and Fay, P. J. (1994) J. Biol. Chem. 269, 9445–9452
34. Mertens, K., van Wijngaarden, A., and Bertina, R. M. (1985) Thromb. Haemostasis 54, 654–660
35. Lollar, P., Parker, E. T., Curtis, J. E., Helgerson, S. L., Hoyer, L. W., Scott, M. E., and Scandella, D. (1994) J. Clin. Invest. 93, 2497–2504
36. Mathur, A., Zhong, D., Sahaarawal, A. K., Smith, K. J., and Bajaj, S. P. (1997) J. Biol. Chem. 272, 23418–23426
37. Kaufman, R. J., Walser, L. C., and Dorner, A. J. (1988) J. Biol. Chem. 263, 6352–6362
38. Boeker, B. G. (1992) Transfus. Med. Rev. 6, 256–260
39. Pemberton, S., Lindley, P., Zaitsev, V., Card, G., Tuddenham, E. G. D., and Kemball-Cook, G. (1997) Blood 89, 2413–2421
40. Pan, Y., DeFay, T., Gitschier, J., and Cohen, F. E. (1995) Nat. Struct. Biol. 2, 740–744
41. Amano, K., Sarkar, R., Pemberton, S., Kemball-Cook, G., Kazazian, H. H., Jr., and Kaufman, R. J. (1998) Blood 91, 538–548