Localization of a Factor X Interactive Site in the A1 Subunit of Factor VIIIa*

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The protein cofactor, factor (F) VIIIa, is required for the efficient conversion of the substrate FX to FXa by the serine protease FIXa. The interaction between human FVIII (and its constituent subunits) and FX was characterized using a solid phase binding assay performed in the absence of phospholipid and FIXa. Saturable binding of FX to heterodimeric FVIII, the FVIII heavy chain (contiguous A1-A2 domains), the FVIIIa-derived A1/A3-C1-C2 dimer, and the isolated A1 subunit was observed with estimated $K_d$ values ranging from approximately 1 to 3 $\mu M$. The interaction of FX with FVIII was inhibited by moderate ionic strength and was Ca$^{2+}$-dependent, consistent with the salt sensitivity observed in a phospholipid-independent FXa generation assay. Negligible binding to FX was observed for the isolated A2 and A3-C1-C2 subunits of FVIIIa, suggesting that the A1 subunit of FVIII contains a primary binding site for FX. A synthetic peptide to the COOH-terminal acidic region of the A1 subunit, designated FVIII(337–372), bound FX and effectively competed with A1 for FX binding ($K_i = 16 \mu M$). Cross-linking between the FVIII(337–372) peptide and the FX heavy chain was observed following reaction with 1-ethyl-3-[(diethylamino)propyl]carbodiimide. The presence of FX reduced the rate of activated protein C-catalyzed cleavage at Arg336 by approximately 5-fold. These results identify a primary FX interactive site on the cofactor of the intrinsic FXase.

The plasma protein FVIII plays a central role in the formation and activity of the intrinsic FXase complex. This surface-dependent complex, consisting of the serine protease FXa and protein cofactor FVIIIa, efficiently converts substrate FX to FXa (1). Kinetically, the role of FVIIIa in the intrinsic FXase complex is to amplify the rate of FIXa-catalyzed proteolysis of FX by several orders of magnitude (2). The exact mechanism by which FVIIIa functions is not known. However, FVIIIa likely increases the catalytic efficiency of FXa through both cofactor-enzyme and cofactor-substrate interactions, which in turn alter the enzyme-substrate interaction accelerating the reaction rate. Indeed, several binding or structural sites have been identified on the FVIII molecule that have implications for its function and regulation. The fact that FVIII deficiency or dysfunction results in hemophilia A, the most common of the severe bleeding disorders, underscores its importance in the blood coagulation cascade.

FVIII is synthesized as a single chain (3, 4) of domainal structure A1-A2-B-A3-C1-C2 and circulates in the plasma as a two-chain species of approximately 300 kDa (5). Short regions (~40 residues) rich in acidic residues separate the A1-A2, A2-B, and B-A3 domains. The heavy chain (A1-A2-B domains) and light chain (A3-C1-C2 domains) are noncovalently linked by divalent metal ion(s) at residues localized to the A1 and A3 domains (6–8). Metal ion chelation disrupts the heterodimeric structure, which subsequently can be reconstituted by addition of Ca$^{2+}$ or Mg$^{2+}$ (9). FVIII is activated by specific proteolytic cleavages generated by thrombin (10), to form the heterotrimer FVIIIa (A1/A2/A3-C1-C2) (11, 12). The A1 and A3 subunits retain the noncovalent divalent ion linkage to form the stable A1/A3-C1-C2 dimer (12, 13), whereas the A2 subunit is weakly associated with this dimer through electrostatic forces (13, 14).

Previous results from our laboratory suggested that the FVIII A1 subunit may contain the interactive site for FX. APC-catalyzed cleavage of the A1 subunit at position Arg336 in the FVIIIa-derived A1/A3-C1-C2 dimer, markedly reduced its interaction with FX as judged by fluorescence anisotropy and solid phase binding assay (15). The aim of the present study was to further characterize the interaction between FVIII and FX and to localize the region of FVIII, which is primarily involved in this binary complex. This interaction was assessed by employing solid phase binding experiments with immobilized proteins, solution phase chemical cross-linking, and functional assays. Results from this study show that the COOH-terminal region of the A1 domain/subunit contains a primary binding site for FX that is exposed in both the unactivated and activated molecules.

MATERIALS AND METHODS

Reagents—Bis-aminoanilinonaptholsulfonic acid (Molecular Probes), EDC (Pierce), PPACK (Calbiochem), S-2765 (KabiChromogenix) and Hirudin (Sigma) were purchased from the indicated vendors. The ECL Western detection system was purchased from Amersham Corp. Several monoclonal antibodies were employed in this study. ESH-8, which recognizes residues 2248–2285 in the C2 domain of FVIII light chain (16), and the anti-FX heavy chain monoclonal antibody were obtained from American Diagnostica. RSB12 recognizes an epitope near the COOH terminus of the A2 domain of the FVIII heavy chain and was described previously (12). An antibody specific for the NH2-terminal sequence of the A1 subunit was kindly provided by Dr. Jim Brown of...
buffer containing 20 mM Hepes, pH 7.2, 50 mM NaCl, 5 mM CaCl₂, 0.1% Tween 20, and 100 µg/ml BSA. The FXVIII was converted to FXIIIa with the addition of 20 nM thrombin for 3 min, after which hirudin (100 nM) was added. FXa generation was initiated with the addition of 500 nM FX, and the reaction was incubated at 37 °C. Aliquots were removed at various intervals up to 20 min, diluted into EDTA (50 mM), and quenched by the addition of 0.5 M EDTA. For reactions containing FIXa only, aliquots were removed at intervals up to 100 min. A portion of each aliquot was diluted into buffer (20 mM Tris, pH 7.2, 100 mM NaCl; final volume, 44 µl), and 6 µl of S-2765 (4 mM stock) was added to the wells. The FXa was generated by incubating the wells at 405 nm for 20 min using a Vmax plate reader. The amount of FXa was determined by comparison with a standard generated from known amounts of FXa.

**ECD Cross-linking of FXVIII**

Reactions contained 1 µM FX and 5 µM FXVIII**V**-**V** and were incubated for 1 h in buffer containing 20 mM Hepes, pH 6.5, 50 mM NaCl, 5 mM CaCl₂, and 0.1% Tween 20. EDC was added to a final concentration of 2 mM, and cross-linking was allowed to proceed for 1.5 h. Control reactions contained FX or the FXVIII**V**-**V** peptide alone in the presence of EDC. Cross-linking was terminated by the addition of SDS-polyacrylamide gel electrophoresis sample buffer, and the samples were boiled for 3 min in preparation for electrophoresis.

**APC Cleavage Assay—Cleavage at the Arg**

The assay was performed in reactions containing 300 nM A1/A3-C1-C2 dimer, 70,000 cpm of tritiated FX (0.1% BSA), and 100 µg of monoclonal antibody, R8B12 to capture heavy chain on the well surface. Bound FX was quantitated following the addition of coating buffer. The assay was then performed in a similar manner (Fig. 1). Binding isotherms for the two dimeric forms varied from 10–30% and was somewhat greater (up to 40%) for the R8B12 antibody.

**Electrophoresis and Western Blotting—SDS-polyacrylamide gel electrophoresis** was performed using the method of Laemmli (19) with a Bio-Rad minigel system. Electrophoresis was carried out at 150 V for 1 h. The proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad, 0.2 µm) using a Bio-Rad mini transblot apparatus at 100 V for 1.5 h in buffer containing 192 mM glycine, 25 mM Tris, and 20% (v/v) methanol.

Cross-linked product was detected by Western blotting using the anti-FXVIII**V**-**V** antibody, followed by a goat anti-rabbit antibody horseradish peroxidase conjugate. The same blot was stripped and incubated with rabbit anti-human Factor VIII (Dienes) (generously provided by Bayer Corporation). The anti-Factor VIII polyclonal antibody has been described previously (17). Factor VIII heavy and light chains were prepared using the method of Bradford (18) with BSA as the standard. They yield confidence level of 95–100%.

**Results**

The interaction between FX and FXVIII was assessed by immobilizing the cofactor protein forms in microtiter wells directly or through a capture antibody. Bound FX was quantitated following its conversion to FXa by the FX activator in RVV and subsequent reaction with a FXa-specific chromogenic substrate. FX bound to FXVIII and A1/A3-C1-C2 (immobilized to ESH-8 via the light chain) in a saturable and dose-dependent manner (Fig. 1). Binding isotherms for the two dimeric forms were similar, suggesting that thrombin activation was not a prerequisite for recognition of the substrate interactive site on the cofactor. A similar result was obtained with FXVIII heavy chain (sandwich A1-A2-B detection) using the anti-A2 monoclonal antibody, R8B12 to capture heavy chain on the well surface (data not shown). Furthermore, FX binding to the A3-C1-C2 light chain was negligible compared with intact FXVIII and the A1/A3-C1-C2 dimer. This result was not due to reduced levels of A3-C1-C2 bound to the wells, because control experiments (not shown) indicated equivalent amounts of all light chain-
containing forms adsorbed to the capture antibody. These results suggested that the heavy chain-derived A1 (domain) subunit was essential for FX interaction with the cofactor.

To determine whether the association of FXIII forms with the ESH-8 capture antibody affected the interaction of cofactor with FX, we utilized a phospholipid-independent FXa generation assay. Recent data have shown that FXIIIa markedly increases the catalytic efficiency of FX conversion by FIXa in the absence of a surface (20). Rates of FX conversion by 50 nM FIXa in the absence (0.273 ± 0.005 pmol/min) or the presence (76.9 ± 0.01 pmol/min) of 100 nM FXIIIa showed that in the absence of phospholipid, low levels of FXa were formed by FIXa alone, and this rate was increased nearly 300-fold by the cofactor (data not shown). Preincubation of FXIIIa with ESH-8 (145 nM) prior to the reaction had no effect on the FXIIIa-dependent enhancement of FXa generation (67.7 ± 0.75 pmol/min). This result indicated that the capture antibody did not perturb the interaction of cofactor with substrate.

Estimated $K_d$ values derived from the binding curves are presented in Table I. It is important to note that these values are estimates derived from nonequilibrium conditions in that washing of the microtiter wells following interaction of FX resulted in dissociation of FX. For this reason we performed this step rapidly (<1 min total) to minimize loss of bound material. Experiments (not shown) to assess the effect of duration of the wash step on loss of bound FX indicated that minimally 15% of material was lost under the conditions we employed. Thus the values obtained are subject to this limitation of the assay.

Characterization of the FX-FVIII Interaction—The effect of pH, ionic strength, and Ca$^{2+}$ were examined to further characterize the interaction of FX with FVIII. FX (300 nM) was incubated with ESH-8 immunobilized FVIII at varying concentrations of NaCl. Fig. 2 shows that FX binding to FVIII was enhanced at low ionic strength. A slightly acidic pH (6.5) resulted in a 3-fold increase in bound FX compared with experiments performed at pH 7.2, and both conditions showed similar salt sensitivity (data not shown). Control experiments (not shown) to detect the amount of FVIII bound to ESH-8 using a biotinylated anti-R8B12 antibody showed that the ESH-8 antibody-FVIII interaction and the FVIII interchain association were not affected by the ionic strength of the wash buffer nor the duration of the wash and incubation steps subsequent to FVIII binding.

The effect of ionic strength on the interaction of FX with FVIII was also assessed using the phospholipid-independent FXa generation assay. FIXa in the absence or the presence of FXIIIa was incubated with FX under conditions that varied from 15 to 150 mM NaCl. Fig. 2 shows that FXa generation in the presence of FXIIIa was markedly affected by increasing ionic strength compared with FIXa alone. The rate of FXa generated in the presence of FXIIIa was nearly 30-fold greater at 15 mM than at 100 mM NaCl, whereas in the absence of FXIIIa this difference was less than 2-fold. The FXIIIa-dependent salt effects observed in this functional assay parallel the effects observed in the solid phase binding assay and are consistent with the cofactor-substrate interaction being sensitive to ionic strength.

The effect of Ca$^{2+}$ on the FX-FVIII interaction was examined, because this ion is required for both structural and functional considerations related to efficient FXa generation. FX (300 nM) was incubated in wells containing FVIII captured via the ESH-8 antibody. The binding of FX was carried out in buffer containing varying amounts of CaCl$_2$, and the total ionic strength of the buffer was kept constant with the addition of NaCl. The interaction was determined to be Ca$^{2+}$-dependent.

### Table I

| FXVIII form | $K_d$ (nM) |
|-------------|------------|
| FXVIII$^b$  | 2.61 ± 0.72 |
| A1/A3-C1-C2$^b$ | 1.83 ± 0.49 |
| FXVIII heavy chain$^c$ | 1.94 ± 0.60 |
| A1$^d$ | 0.89 ± 0.33 |
| FXVIII$^{337–773}$ | 0.97 ± 0.10 |

*a* Affinity values obtained from the solid phase assay are estimates resulting from limitations in the assay.

*b* Protein immobilized via ESH-8 capture antibody.

*c* Protein immobilized via R8B12 antibody.

*d* Protein bound to wells directly.
because we observed no binding in the absence of Ca\(^{2+}\) and a linear increase in FX binding up to 5 mM CaCl\(_2\) (data not shown). Therefore, although Ca\(^{2+}\) appears to be essential for the cofactor-substrate interaction, it is not possible to distinguish between a direct or indirect role for Ca\(^{2+}\) in mediating this effect.

An additional experiment assessed the potential contribution of exposed hydrophobic sites on FVIII to FX binding. Binding was measured in the presence of increasing amounts of the apolar compound bis-anilinonaptholsulfonic acid. This reagent binds to exposed hydrophobic sites on the FVIII subunits with high (\(\leq \mu M\)) affinity (21). Occupancy of these hydrophobic areas on FVIII by bis-anilinonaptholsulfonic acid did not alter the binding of FX (data not shown). Collectively, these data indicate that electrostatic forces are important and probably predominate in the FX-FVIII interaction.

**Localization of the FX Site to the A1 Subunit**—The binding of FX to isolated A1 or A2 subunits directly immobilized to the microtiter wells is shown in Fig. 3. The A1 subunit supported appreciable binding of FX, whereas the A2 subunit did not. Control experiments (not shown) using biotinylated antibodies specific for each of the two subunits indicated that similar amounts of A1 and A2 subunits were bound to the wells and that 50 nM protein essentially saturated the binding sites on the well. The binding of FX to the A1 subunit was Ca\(^{2+}\)-dependent and could be reversed by the addition of EDTA or high ionic strength (results not shown). The apparent \(K_d\) value estimated for the FX-A1 interaction (Table I) was similar to those observed for the higher order FVIII structures, consistent with the notion that sequences and/or structures present on the A1 subunit constitute the FX interactive site on the cofactor.

**The COOH Terminus Region of A1 Represents a FX Interactive Site**—In a recent report from our laboratory, we showed that cleavage of the A1 subunit in the A1/A3-C1-C2 dimer by APC reduced the ability of that molecule to bind FX (15). This loss of FX binding activity in the truncated dimer was tentatively attributed to altered conformation rather than loss of an interactive site. We have reinvestigated the role of the acidic COOH-terminal region of A1 subunit (residues 337–372), which is removed by APC cleavage, in the interaction with FX. Fig. 4 shows the binding of FX to this immobilized synthetic peptide (designated FVIII\(^{337-372}\)). The estimated affinity of FX for this peptide was equivalent to that estimated for the A1 subunit. Further characterization of this interaction suggested that the highly acidic nature of the peptide was not responsible for FX binding, because synthetic polymers of aspartic acid or glutamic acid were not functional in this assay.

In addition to supporting direct binding of FX, the FVIII\(^{337-372}\) peptide effectively competed with the A1 subunit for FX (Fig. 5). In this experiment, immobilized A1 subunit (50 nM) was reacted with FX (300 nM) in the absence or the presence of increasing concentrations of the peptide. The apparent \(K_d\) calculated (15.9 \(\mu M\)), a measure of the affinity of peptide for FX in the solution phase, was an order of magnitude greater than the \(K_d\) value estimated from the direct plate binding assay. The reason for this disparity is not known but likely reflects the uncertainty in the value obtained from the direct binding assay resulting from nonequilibrium conditions. For this reason the \(K_d\) value obtained from the competition assay may be a more accurate representation of the intermolecular affinity.

To complement the data obtained using solid phase assays, the interaction of FX and the FVIII\(^{337-372}\) peptide was further demonstrated in solution phase. Association of the two components was assessed following cross-linking using EDC, a zero-length cross-linking reagent reactive toward residues containing primary amines and carboxyl groups (22). Formation of covalent cross-links implies direct contact between these residues. Fig. 6 shows that this reaction results in the covalent attachment of the peptide to the heavy chain of FX. Western blotting using the anti-FVIII\(^{337-372}\) antibody (left panel) recognized a product that migrated at a position on the gel slightly higher than the FX heavy chain only in the presence of FX and peptide. The peptide alone was not detected in this blot. The companion blot (right panel) assessed the same reactants using an anti-FX heavy chain antibody. EDC-treatment of FX (right panel, lane 2) shows the FX heavy chain and low levels of a higher molecular weight band of a size consistent with formation of a cross-link between the FX heavy and light chains. In the presence of the FVIII\(^{337-372}\) peptide (right panel, lane 3), we observed the FX heavy chain band and a product of slightly lower mobility with similar staining intensity. The latter product represents the cross-linked FX heavy chain-peptide adduct based upon its reactivity with both anti-peptide and anti-FX antibodies. As judged by silver staining (data not shown), the mobility of FX light chain was unaffected by inclusion of the FVIII\(^{337-372}\) peptide in the presence of EDC. This result, coupled with the failure to observe a FX light chain-peptide adduct with the anti-peptide antibody, suggested the heavy chain of FX contains the FVIII\(_a\) interactive site.

**FX Protects A1/A3-C1-C2 from APC Cleavage**—Because FX binds within the COOH-terminal region of the A1 subunit, one might predict that its binding would affect APC-catalyzed cleavage at the nearby Arg\(^{336}\)-Met\(^{337}\) bond. Rates of cleavage of the A1/A3-C1-C2 substrate were determined by densitometry following SDS-polyacrylamide gel electrophoresis and Western blotting using a monoclonal antibody specific for the NH\(_2\) terminus of the A1 subunit. We observed that the presence of FX reduced the rate of cleavage at the Arg\(^{336}\) bond by \(\sim 5\)-fold (Fig. 7). This result was consistent with FX sterically blocking the protease and/or masking the scissile bond.

**DISCUSSION**

In this report we investigated the interaction between the cofactor FVIII and the substrate FX of intrinsic FXase. Direct binding of FX to immobilized FVIII and its derived subunits was evaluated in the absence of FXa and phospholipid using an assay system capable of detecting subnanomolar amounts of FX following its conversion to FXa. These analyses were complemented by solution phase assays utilizing chemical cross-linking and protease protection. Results from this study indi-
cate that the FX interactive site is contained within the A1 subunit of FVIIIa and is localized to the COOH-terminal acidic region. Interestingly, activation of FVIII does not appear to be a prerequisite for exposure of the FX site, as suggested by the similar affinity of FX for FVIII (FVIII heavy chain) and the A1 subunit. This result is consistent with the early observation that immobilized FX could be used in a purification scheme for FVIII (23).

Several of our findings parallel the data that have been obtained for the FVa-prothrombin interaction. The heavy chain of FVa (contiguous A1-A2 domains) contains the major binding site for prothrombin (24, 25), although a more localized site has not been identified. The affinity for this interaction (~10 μM) has been determined by solution phase condition (24–26). This value is similar to the Kᵢ observed for the inhibition of the A1-FX interaction by FVIII³³⁷–³⁷² peptide, which is likely a more accurate estimation of the intermolecular affinity than results directly obtained with the solid phase binding assay. The latter values, which range from ~1 to 3 μM should be considered estimates based upon limitations of the assay. Additionally, APC cleavage of analogous sites in FVa and FVIIIa results in a decreased affinity for prothrombin (24, 25) and FX (15), respectively. In contrast to the calcium dependence of the FVIII-FX interaction, the association of FV with prothrombin is calcium-independent (25). Fluorescence studies have also revealed that FVa increased both the emission intensity and polarization of membrane-bound DEGR-meizothrombin, as well as reduced the distance of closest approach between the active site probe and the phospholipid surface (27). From that study it was concluded that one role for FVa in prothrombinase complex is to modulate the conformation and orientation of substrate. Based upon the similarity in location and affinity of the substrate binding sites, FVIIIa may possess a similar role relative to substrate in the intrinsic FXase complex.

Lollar et al. (28) have shown that FVIIIa supported a FX-dependent increase in the fluorescence anisotropy of an active site-labeled FIXa, suggesting that the cofactor and substrate may interact directly. Recently, Regan et al. (15) demonstrated a similar effect, but to a lesser extent, for the FVIIIa-derived A1/A3-C1-C2 dimer, and this property was abolished following APC-catalyzed cleavage at Arg³³⁶ in the A1 subunit. The truncated A1³³⁶/A3-C1-C2 dimer also showed a markedly reduced affinity for FX as measured by a solid phase binding assay. Inclusion of an anti-FVIII³³⁷–³⁷² polyclonal antibody did not...

FIG. 4. Localization of the FX binding site to FVIII³³⁷–³⁷². FX (0–3 μM) was incubated with FVIII³³⁷–³⁷² peptide (△), polyaspartic acid (○), and polyglutamic acid (□) coated directly to microtiter wells (100 nM/well). The quantitation of bound FX is described under “Materials and Methods.” Data points for the FVIII³³⁷–³⁷² peptide were fitted to a single site ligand binding curve, whereas points for the two synthetic acidic polymers were fitted to straight lines.

FIG. 5. Inhibition of FX binding to A1 by FVIII³³⁷–³⁷². FX (300 nM) was incubated with A1-coated wells (50 nM/well) in the absence or the presence of increasing amounts of FVIII³³⁷–³⁷² peptide as a solution phase competitor. The assay was performed as described above. The calculated Kᵢ for FVIII³³⁷–³⁷² was 15.9 μM.

FIG. 6. Cross-linking of the FVIII³³⁷–³⁷² peptide to FX. FX (1 μM) was incubated in the absence and the presence of increasing amounts of FVIII³³⁷–³⁷² (5 μM) for 1 h in buffer containing 20 mM Mes, pH 6.0, 50 mM NaCl, 5 mM CaCl₂, 0.1% Tween 20. EDC was added to a final concentration of 2 mM, and the cross-linking reaction was run for 1.5 h at room temperature. The reaction was terminated, and the proteins were resolved on a 12% gel. Material was detected following Western blotting using the anti-FVIII³³⁷–³⁷² antibody (left panel) and anti-FX heavy chain antibody (right panel) as described under “Materials and Methods.” Lanes 1–3 represent the peptide alone, FX alone, and FX plus the peptide, respectively. Positions of molecular mass standards (in kDa) are shown on the left.
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A 25% reduction in the rate of catalysis at 37°C, and aliquots were removed at the indicated times. Subunits were resolved by electrophoresis on a 6–15% gel, and the cleaved and intact A1 subunit was identified and quantitated following Western blotting with the anti-A1 monoclonal antibody as described under “Materials and Methods.” The results are presented as a plot of log% versus time. Slopes of the lines from linear regression are −0.066 (correlation, 0.91) and −0.031 (correlation, 0.99) for reactions performed in the presence and the absence of FX, respectively.

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 affect the binding of FX to the A1/A3-C1-C2, suggesting that the A1 subunit COOH-terminal acidic residues, removed by APC cleavage, alone did not constitute the FX binding site. However, that conclusion was tentative and based upon the premise that binding of FX and the antibody to the FVIII segment delineated by residues 337–372 was mutually exclusive. We now show that the antibody detects the cross-linked peptide-FX complex, indicating that the premise was incorrect.

Results from the present study demonstrate the FVIII FX interaction possesses a high sensitivity to ionic strength. Both the solid phase binding assay and the lipid-independent FXa generation assay show salt optima at less than physiologic, suggesting that this interaction is primarily electrostatic. This conclusion is supported by the formation of a covalent cross-link between FVIII(C37–372) and FX by EDC. This reagent initially reacts with an acidic residue to form an unstable O-acetylisourea adduct and subsequently reacts with a closely associated nucleophilic side chain, thereby covalently preserving the original salt bridge (22). Low (µM) concentrations of peptide and FX resulted in significant complex formation with the amount of FX driven into complex by 5 µM peptide approaching 50%. Thus, these results suggest the presence of a salt bridge(s) involving presumably acidic residue(s) the COOH-terminal region of A1 subunit of FVIIIa and basic residue(s) in the heavy chain of FX.

Both APC (10, 29) and FXa (30, 31) cleave FVIIIa at the Arg600-Met601 bond, which proceeds the proposed interactive site for FX and which results in the inactivation of the cofactor. Recently, our laboratory showed that the presence of FX reduced the rate at which FXa cleaved this site by ~10-fold (32). Results from the current study show that FX similarly protects the Arg600 site from APC cleavage. Operationally, the protection of cofactor by substrate could help to sustain maximal FXase activity until substrate becomes limiting. Although the mechanism for this effect remains to be determined, the proximity of the FX binding site and scissile bond is consistent with FX masking the scissile bond and/or sterically precluding the enzymes from efficient catalysis.

Advances in A2 subunit retention following thrombin cleavage at the A1-A2 junction (14, 17). This interaction was determined to be essentially electrostatic and pH-dependent. However, the ionic strength sensitivity of FVIIIa reconstituted is significantly reduced with the salt sensitivity of the FVIII FX interaction. The acidic character of this sequence plays an important role in the inter-FVIIIa subunit interaction, and polymers of aspartic acid or glutamic acid also inhibited the reconstitution of FVIIIa from the A1/A3-C1-C2 dimer and A2 subunit (17). The interaction of this region with FX appears less dependent upon the charge of the peptide in that the synthetic acidic polymers do not support FX binding. Although the affinity values obtained from the solid phase binding assays are equivocal, the similarity in values obtained for the intact A1 and FVIII(C37–372) peptide make it tempting to speculate that the majority of binding energy for the FX interaction is contained within this sequence. Comparison of the human (5) and murine (33) cDNA sequences for FVIII show marked homologies in this region as well as a region of identity at the end of the segment.

Based upon electron microscopy (34, 35) and hydrodynamic (8) analyses, the A domains (subunits) of FVIII (FVIIIa) form a globular structure. Modeling by homology mapping with nitrite reductase, a protein containing A domains homologous to those of FVIII, suggests the three A domains are arranged such that each is similarly exposed to solvent (36). The recent determination of the x-ray structure of porcine FIXa suggests an interesting model for the membrane-bound FXase (37). The model depicts the tulip-shaped FIXa and an equivalently arranged FX arching across the cofactor from opposite sides, such that the FIXa active site is juxtaposed with the scissile bond in FX. This arrangement is compatible with predictions of the various contacts that are formed within the FXase complex. FIXa possesses a high affinity site in the A3 subunit (38) and a weaker affinity site in the A2 subunit (39), the latter of which directly modulates the active site. Results from the current study indicate that the A1 subunit possesses a primary FX binding site, which contacts the FX heavy chain. Thus all three A subunits participate in protein-protein interactions, providing essential contacts between enzyme and substrate, which manifest in an accelerated rate of catalysis.

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