Mechanisms of Interactions of Factor X and Factor Xa with the Acidic Region in the Factor VIII A1 Domain*

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The 337–372 sequence of the factor VIIIa A1 subunit contains interactive sites for both zymogen factor X and the active enzyme, factor Xa. Solid phase binding studies indicated that factor Xa possessed a >20-fold higher affinity for the isolated A1 subunit of factor VIIIa compared with factor X. Heparin completely inhibited zero-length cross-linking of the 337–372 peptide to factor Xa but not to factor X. In the presence of calcium, factor Xa showed greater affinity for heparin than factor X. Studies using factor Xa mutants in which heparin-binding exosite residues were individually replaced by Ala showed that the R240A mutant was defective in recognition of the Lys36 cleavage site, generating the A137–372 fragment. These results indicated that factor Xa possesses a unique factor Xa-interactive site within the factor VIII heavy chain (7). Factor Xa also interacts with these residues in the 337–372 acidic region following the A1 domain, in particular a cluster of acidic residues 361–363, contributes to a unique factor Xa-interactive site within the factor VIII heavy chain (17). Factor Xa also interacts with these residues in the A1 subunit, possibly via the heparin-binding exosite of this protease (18), and this binding modulates cleavage at Lys36 (11). We now report on the mechanisms of interaction between the acidic region (residues 337–372) in the factor VIII A1 domain and zymogen factor X or protease factor Xa using a combination of approaches employing synthetic peptides and recombinant factor VIII and factor X mutants. Our results indicate that both zymogen and protease factor Xa have a common interactive site in A1 that overlaps with a cluster of acidic residues at position 361–363. Furthermore, factor Xa but not factor X interacts with high affinity at the 337–372 sequence with participation of residue Arg240 of heparin-binding exosite in the protease.

MATERIALS AND METHODS

Reagents—Purified recombinant factor VIII preparations were generous gifts from Bayer Corp. (Berkeley, CA). The monoclonal antibodies

A1, A2, and heterogeneous fragments of partially proteolysed B domains, together with a light chain consisting of the A3, C1, and C2 domains (2–4).

Factor Xa and thrombin convert factor VIII into an active form, factor VIIIa, following limited proteolysis at Arg722, Arg740, and Arg1689 (5). Proteolysis at Arg722 and Arg1689 are essential for generating factor VIIIa cofactor activity (6). Cleavage at Arg336 likely occurs by altered interaction of the A2 subunit with the truncated A1 (12) coupled with an increase in the Km for substrate factor X (13), the latter reflecting loss of a factor Xa-interactive site within an acidic residue-rich region defined by residues 337–372 (14). Other proteases including activated protein C (5) and factor IXa (15) have been shown also to attack this site. Furthermore, cleavage at Lys36 by factor Xa has been suggested to alter the conformation of A1 limiting productive interaction with the A2 subunit (13). Thus factor Xa represents a unique enzyme relative to factor VIII in that its catalytic mechanisms include both the up- and down-regulation of cofactor function.

An interactive site for factor X in factor VIII necessary for activation has been mapped within the residues 2253–2270 of the C2 domain (16). In addition, we demonstrated recently that the 337–372 acidic region following the A1 domain, in particular a cluster of amino acid residues 361–363, contributes to a unique factor Xa-interactive site within the factor VIII heavy chain (17). Factor Xa also interacts with these residues in the A1 subunit, possibly via the heparin-binding exosite of this protease (18), and this binding modulates cleavage at Lys36 (11). We now report on the mechanisms of interaction between the acidic region (residues 337–372) in the factor VIII A1 domain and zymogen factor X or protease factor Xa using a combination of approaches employing synthetic peptides and recombinant factor VIII and factor X mutants. Our results indicate that both zymogen and protease factor Xa have a common interactive site in A1 that overlaps with a cluster of acidic residues at position 361–363. Furthermore, factor Xa but not factor X interacts with high affinity at the 337–372 sequence with participation of residue Arg240 of heparin-binding exosite in the protease.

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58.12 (19) and C5 (20), recognizing the N-terminal and C-terminal end of A1, were gifts from Drs. Lisa Regan and Zaviero Ruggeri, respectively. The monoclonal antibody RSB12 (19) recognizing the A2 domain was obtained from Green Mountain Antibodies (Burlington, VT). The reagents human α-thrombin, factor IXa, factor X, and factor Xa (Enzyme Research Laboratories, South Bend, IN), chromogenic Xa substrate S-2765 (DiaPharm Group, Westchester, OH), DEGR-factor Xa, RVV-X (Hematologic Technologies Inc., Essex Junction, VT), EDC, and sulfo-NHS-biotin (Pierce, hurinid (Calbiochem), and heparin (Sigma) were purchased from the indicated vendors. Phospholipid vesicles containing 20% phosphatidyserine, 40% phosphatidylcholine, and 40% phosphatidylthanolamine (Sigma) were prepared using N-octyl glucoside (21). The synthetic peptide corresponding to factor VIII residues 357–372 was prepared by Quality Controlled Biochemicals, Inc. (Hopkinton, MA). Biotinylated peptide was prepared using sulfo-NHS-biotin as described previously (17). The B-domainless factor VIII expression construct RNeo factor VIII and baby hamster kidney cells were gifts kindly provided by Dr. Pete Lollar.

Proteins—The factor VIII light and heavy chains were isolated from EDTA-treated factor VIII after chromatography on Sepharose 4B-Sepharose columns (Amersham Biosciences) as described previously (7). The A2 and A1 subunits were purified from thrombin-cleaved factor VIII heavy chain by fast protein liquid chromatography using a Hi-Trap heparin column and a Mono Q column as reported previously (12). SDS-PAGE of the isolated subunits followed by staining with GelCode Blue (Pierce) showed >95% purity. Protein concentrations were determined by the method of Bradford (22).

Recombinant Factor VIII and Xa Preparations—Recombinant wild type factor VIII, as well as three clustered mutants, factor VIII R93A–K96A–R125A–R165A–K169A–K236A–R240A (clustered mutants in which Ala was individually substituted for heparin-binding exosite residues R93A, K96A, R125A, K165A, K169A, K236A, and R240A) (functionally), were constructed, expressed, and purified as described previously (17). Recombinant wild type factor VIII as well as seven mutants in which Ala was individually substituted for heparin-binding exosite residues R93A, K96A, R125A, K165A, K169A, K236A, and R240A (functionally), were constructed, expressed, and purified as described previously (17). Recombinant wild type factor X as well as seven mutants in which Ala was individually substituted for factor Xa were isolated from CHO cells transfected with plasmid containing the factor Xa gene, and the recombinant proteins were expressed, purified, and analyzed as described previously (18, 23). Recombinant factor Xa derivatives were converted to their activated forms (factor Xa) with RVV-X as described previously (24).

Factor Xa Generation Assay—The rate of conversion of factor X to factor Xa was monitored in a purified system (25). 30 nM factor VIII was activated by the addition of 10 nM thrombin in the presence of 10 μM phospholipid vesicles. Thrombin activity was inhibited after 1 min by the addition of 2.5 units/ml hirudin, and the reactions were initiated with the addition of 0.5 mM factor IXa and the indicated amounts of factor X. Aliquots were removed at appropriate times to assess initial rates of product formation and added to tubes containing EDTA (50 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 for 10 min using a Vₘ,ₘicroplate reader (Molecular Devices, Sunnyvale, CA).

Factor X Solid Phase Binding Assay—The direct binding assay was performed using a modification of our previously reported method (14). All reactions were run at 22 °C. The A1 subunit (50 μM) was bound to the microtiter well in 15 mM NaCl, 35 mM NaHCO₃, pH 9.6, overnight at 4 °C. After washing the wells with wash buffer (20 mM HEPES, pH 7.2, 15 mM NaCl, 5 mM CaCl₂, and 0.01% Tween 20), each well was blocked in wash buffer containing 5% bovine serum albumin for 2 h. After blocking times, mixtures of 30 μM factor X and various amounts of DEGR-factor Xa in wash buffer containing 1% bovine serum albumin were added to the wells and incubated for 40 min. After several washings, 30 mM RVV-X activator in 20 mM Tris, pH 7.2, 0.2 mM NaCl, and 5 mM CaCl₂, was added and incubated for 15 min. This was followed by the addition of the chromogenic substrate, S-2765 (0.46 mM final concentration) and the plate was washed at 405 nm for 20 min using a Vₘ,ₘicroplate reader. Control experiments showed that the RVV-X enzyme did not hydrolyze the chromogenic substrate. The amount of nonspecific binding of factor X to the blocked wells containing no A1 subunit was less than 5% of the total signal, and the amount of specific binding was obtained by subtracting nonspecific binding of factor X.

Cross-linking with EDC—Cross-linking reactions containing the indicated concentrations reactants together with biotinylated 357–372 peptide were run in buffer containing 20 mM HEPES, pH 7.2, 50 mM NaCl, 5 mM CaCl₂, and 0.01% Tween 20 at 4 °C for 1 h. Reactions were terminated by the addition of SDS-PAGE sample buffer and boiling.

Binding to Heparin-Sepharose—Factor X or factor Xa (50 μg) in 20 mM Tris-Cl, pH 7.5, 1 mM NaCl, 5 mM CaCl₂, and 0.01% Tween 20 was applied to a 0.5-ml heparin-Sepharose column (Amersham Biosciences) equilibrated in the above buffer. The column was washed and protein eluted with a step gradient of 0.15–0.5 M NaCl using increments of 20 mM NaCl (1 ml/step), and 1-ml fractions were collected. The factor Xa elution position was determined after a 50-fold dilution of each fraction with buffer and addition of the chromogenic substrate, S-2765 (0.46 mM final concentration). Samples were read at 405 nm for 5 min using a Vₘ,ₘicroplate reader. The elution position of factor X was determined using a similar method after the reaction of each diluted fraction with 30 mM RVV-X activator for 15 min to convert thezymogen factor X to active enzyme.

Cleavage of the A1 or Isolated Heavy Chain Subunit by Recombinant Factor Xa—Recombinant factor Xa derivatives were added to the isolated A1 or heavy chain subunit at the indicated concentration in a buffer containing 20 mM HEPES, pH 7.2, 0.1 mM NaCl, 5 mM CaCl₂, and 0.01% Tween 20, and reactions were run at 22 °C. Samples were taken at the indicated times, and reactions were immediately terminated and prepared for SDS-PAGE and boiling for 3 min.

Electrophoresis and Western Blotting—SDS-PAGE was performed using 8 or 13% gels according to the procedure of Laemmli (26). Electrophoresis was carried out using a Bio-Rad mini gel apparatus at 150 V for 1 h. The protein was transferred to a polyvinylidene difluoride membrane and probed using the factor VIII monoclonal antibody followed by anti-mouse alkaline phosphatase-linked secondary antibody. The signal was detected using the enhanced chemiluminescence system (Amersham Biosciences), and blots were scanned at 570 nm using Storm 860 (Molecular Devices). In addition, the biotinylated peptide was probed using peroxidase-conjugated streptavidin. The signal was detected using the enhanced chemiluminescence system (Amersham Biosciences), and blots were exposed to film for various times. Densitometric scans were quantitated using ImageQuant software (Molecular Devices).

Data Analysis—All experiments were performed at least three separate times, and average values are shown. The Kᵢₗ and Kᵢ₀ values for factor VIIa/Ixa-catalyzed activation of factor X and factor Xa-catalyzed cleavage of A1 subunit were calculated from the Michaelis-Menten equation, with catalytic efficiencies expressed as the ratio of Kᵢₒ/Kᵢₗ. Interprotein binding energy was calculated by Equation 1.

\[ \Delta G^\circ = -RT \ln K_d \]  

(Eq. 1)

To evaluate the inhibition of the interaction between factor X and A1 subunit by DEGR-factor Xa, nonlinear least squares regression analysis was performed according to a competition inhibition model using Equation 2,

\[ \% \text{binding} = \frac{B_{\text{max}} \cdot [\text{factor X}]}{K_d + \frac{[\text{DEGR-factor Xa}] + [\text{factor X}]}{K_i}} \]  

(Eq. 2)

where Bₘₜₜ represents maximum binding, Kᵢ is the dissociation constant for factor X and A1 subunit, and Kᵢₒ is the inhibition constant for DEGR-factor Xa interaction with A1 subunit.

The apparent rate constants (kₗ) for cleavage of isolated A1 subunit substrates by factor Xa were analyzed as reported previously (11).

The apparent rate constant values (kₗₜₜ, kₗₑ, kₗₜₑ, and kₗₑ) in Scheme 1 are based on the parallel and series reactions for intact A1 subunit cleavage by factor Xa and were estimated by nonlinear least squares regression using multiple equations as described previously (11). D1–D4 represent low levels of small degradation products that derive from the A1 substrates.

RESULTS

Relationship of the 337–372 Acidic Region Interaction with Factor X and Factor Xa—The C-terminal acidic region separating A1 and A2 domains in the factor VIII heavy chain represents a common interactive site for factor X (14) and factor Xa (17). To investigate mechanisms employed by thezymogen and proteinase in interacting with this region, we first tested the
capacity of DEGR-factor Xa to inhibit binding of factor X to the A1 subunit in a solid phase assay. The assay employed measures factor X bound to immobilized A1 subunit after its conversion to factor Xa by RVV-X and subsequent quantitation of the protease concentration by a chromogenic substrate (14). Control experiments showed that the binding of factor X to A1 subunit was of affinity ($K_D \sim 600 \text{ nM}$) similar to that reported previously (14). Mixtures with various concentrations of DEGR-factor Xa and factor X (300 nM) were reacted with 50 nM A1 subunit as described under “Materials and Methods.” DEGR-factor Xa inhibited the binding of factor X to A1 by $\sim 50\%$ in a dose-dependent manner (Fig. 1). Because this is a nonequilibrium assay, the apparent $K_i$ value calculated from fitting the curve (18.0 $\pm$ 2.0 nM) suggested that the interaction of A1 subunit with factor Xa occurs with an affinity value that was $\sim 20$-fold that of factor X.

We observed that DEGR-factor Xa failed to yield complete inhibition of factor X binding to the immobilized A1 subunit. The reason for this partial ($\sim 50\%$) level of inhibition is not known but may reflect the presence of another factor X-interacting site in addition to the common binding site. Alternatively, this observation may reflect a limitation of the solid phase binding assay resulting from altered conformations in the immobilized A1 subunit and/or the nonequilibrium conditions employed. To address these issues, an alternate form of analysis was used to characterize the competition between zymogen and protease for interaction with the A1 subunit. Previous results from our laboratory demonstrated that the zero-length cross-linking reagent, EDC, mediated covalent linkage between a peptide corresponding to residues 337–372 of the A1 subunit and the heavy chains of factor X (14) or factor Xa (17), suggesting that both proteins form salt bridge(s) with the peptide sequence. Thus, EDC was utilized as a complementary solution phase approach to study direct interaction of these proteins with this factor X/xa-interactive site. Mixtures containing equivalent concentrations of factor X and DEGR-factor Xa were combined with a fixed concentration (40 M) of biotinylated 337–372 peptide and reacted with 300 M EDC as described under “Materials and Methods.” Samples of the reaction mixtures were subjected to SDS-PAGE and immunoblotting, and bands representing cross-linked products were quantitated using densitometry scanning (Fig. 2A). Cross-linked products of 337–372 peptide with DEGR-factor Xa showed significantly greater density compared with factor X. At the maximum protein concentration employed (1.6 $\mu$M), reaction products containing DEGR-factor Xa were $\sim 10$-fold greater than factor X-containing products. These observations were consistent with the results from the solid binding assay using the intact A1 subunit. Furthermore, the EDC-mediated cross-linking procedure was modified to a competition format to assess relative inhibitory activities of DEGR-factor Xa (or factor X) on the formation of cross-linked products of peptide with factor X (or DEGR-factor Xa). 500 nM factor X or factor Xa and 40 $\mu$M biotinylated 337–372 peptide were reacted with 300 $\mu$M EDC in the presence of various concentrations of DEGR-factor Xa or factor X, respectively. DEGR-factor Xa completely inhibited the formation of cross-linked product of factor X and 337–372 peptide (Fig. 2B, left panel). However, factor X showed little if any inhibition for DEGR-factor Xa and 337–372 peptide cross-linking (Fig. 2B, right panel). These data, taken together with results from the solid phase assay, suggest that both factor X and factor Xa interact with common region within the 337–372 region of A1 subunit, that this interaction is mutually exclusive, and that the protease form of the substrate possesses a markedly greater affinity for this site compared with the zymogen.

**Effect of Heparin on Factor X or Factor Xa and 337–372 Peptide Binding with EDC—** We speculated recently that factor Xa may interact with the A1 337–372 region via its heparin-binding exosite (11). To test this hypothesis, we examined the effect of heparin on the EDC-mediated cross-linking of the 337–372 peptide to factor X and factor Xa. 500 nM factor X or factor Xa was reacted with 40 $\mu$M biotinylated 337–372 peptide followed by the addition of 300 $\mu$M EDC in the presence of various concentrations of heparin as described under “Materials and Methods.” Products of the reactions were detected by immunoblotting (Fig. 3A), and the density of cross-linked products observed was quantitated using densitometry (Fig. 3B). Heparin completely blocked the formation of cross-linked product comprised of 337–372 peptide and factor Xa in a dose-dependent manner ($IC_{50} = 1.4 \pm 0.2$ units/ml). In contrast, heparin-dependent inhibition of the cross-linking between 337–372 peptide and factor Xa was marginal, yielding $\sim 65\%$ of the control value for cross-linked product at the highest concentration of heparin employed (10 units/ml). These results

**Fig. 1. Inhibition of factor X binding to A1 subunit by DEGR-factor Xa.** Mixtures of 300 nM factor X and varying amounts of DEGR-factor Xa were reacted with the A1 subunit (50 nM) that had been immobilized onto microtiter wells. Bound factor X was detected after the addition of 30 nM RVV-X and chromogenic substrate as described under “Materials and Methods.” The amount of factor X binding to A1 in the absence of DEGR-factor Xa (100% level) was $\sim 0.5$ nM. The percentage of factor X binding was plotted as an inhibitory function of DEGR-factor Xa concentration, and the data were fitted by nonlinear least squares regression using Equation 2.
support the hypothesis that factor Xa interacts directly with the 337–372 region via its heparin-binding exosite of the proteinase. The negative results observed with factor X also serve as a control for the lack of interaction of the peptide with heparin. The failure of heparin to alter the interaction of peptide with factor X would be consistent with a putative heparin proexosite in the zymogen. Alternatively, factor X may bind peptide by an unrelated mechanism.

The disparate effects of heparin on interactions involving factor Xa with the 337–372 segment of A1 were consistent with differential affinities of the zymogen and proteinase for the proteoglycan. The affinities of factors X and Xa for heparin were evaluated by a NaCl gradient elution of the protein from a heparin-Sepharose column (Fig. 4). Factor Xa elution was monitored by amidolytic activity, whereas column fractions containing factor X were first converted to factor Xa prior to running the amidolytic assay. Factor Xa was eluted at a NaCl concentration of 0.22 M, whereas factor X failed to bind the column at 0.1 M NaCl and was recovered in the flow-through fraction. These observations indicated a modest affinity for factor Xa for heparin, whereas little if any affinity of factor X for heparin was determined under these reaction conditions. These results are consistent with heparin binding of factor Xa mediated through the heparin-binding exosite, whereas this site is not formed in the zymogen.

**Proteolysis of A1 Subunit after Reaction with Factor Xa Heparin-binding Exosite Mutants**—Contribution of the heparin-binding exosite of factor Xa to its catalytic mechanisms involving the 337–372 sequence was evaluated further using two factor VIII-derived substrates, purified A1 subunit and the isolated factor VIII heavy chain. Factor Xa proteolytically inactivates factor VIIIa, cleaving the A1 subunit to a terminal product designated A137–336. This product may be derived via two pathways as identified by the intermediates, A11–336 and

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Fig. 2. **EDC cross-linking of factor X or DEGR-factor Xa and 337–372 peptide binding.** A, equivalent concentrations of factor X (closed circles) and DEGR-factor Xa (open circles) were reacted with 40 μM biotinylated 337–372 peptide in the presence of 300 μM EDC for 1 h followed by immunoblotting using streptavidin as described under "Materials and Methods." Bands representing cross-linked products were quantitated by densitometry scanning. B, 500 nM factor X (left panel) or 500 nM DEGR-factor Xa (right panel) and 40 μM biotinylated 337–372 peptide were reacted with 300 μM EDC in the presence of various concentrations of DEGR-factor Xa or factor X, respectively, and immunoblotted. Densitometry was used to quantitate the extent of inhibition.

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**Factors X and Xa Interaction with Factor VIII A1 Domain**

(A)

![Diagram](image)

(B)

![Diagram](image)
Factors X and Xa Interaction with Factor VIII A1 Domain

Fig. 3. Effect of heparin on factor X or factor Xa and 337–372 peptide cross-linking by EDC. A, 500 nM factor X or factor Xa was reacted with 40 µM biotinylated 337–372 peptide with 300 µM EDC in the presence of heparin (lanes 1–8; 0, 0.6, 1.2, 2.5, 3.7, 5, 7.5, and 10 unit/ml, respectively) for 1 h followed by immunoblotting using streptavidin as described under “Materials and Methods.” B, densitometry was used to quantitative inhibition of reactions run in A with factor X (open circles) and factor Xa (closed circles). Band density of the cross-linked product of factor X (or Xa) and 337–372 peptide in the absence of heparin was defined as 100%.

A1α37–372, generated by initial cleavage at Argα336 and Lysα36, respectively (11), as shown in Scheme 1. Results from a prior study (11) indicated that the association of factor Xa with the C-terminal region of A1 subunit (residues 337–372) was required for selective proteolysis of A1 subunit at Lysα36, based upon the observation that A1α1–336 was a poor substrate for further proteolysis. Recently, the heparin-binding exosite of factor Xa has been identified, and site-directed mutagenesis has been used to generate a panel of factor X (and factor Xa) reagents possessing point mutations in candidate residues (18). Thus, we could predict that factor Xa heparin-binding exosite mutants wherein basic residues are replaced by alanine would show selectively reduced activity in the cleavage of A1 subunit at Lysα36.

To determine the contribution of individual residues comprising the heparin-binding exosite of factor Xa for catalysis of the A1 substrate, we examined the proteolytic cleavage of A1 subunit by recombinant factor Xa exosite mutants in time course assays. The A1 subunit (2.4 µM) was reacted with wild type or each one of seven exosite mutants (R93A, K96A, R125A, R165A, K169A, K236A, and R240A) of 0.24 µM factor Xa as described under “Materials and Methods.” Densitometry scanning of the stained gels was employed to quantitative band densities of the substrate A1 and each cleavage product. Estimation of apparent rate constants for each cleavage reaction was derived from fitting the band density data to the equations for the parallel series reaction as described under “Materials and Methods.” Control experiments showed that the amidolytic activities, as monitored by factor Xa-catalyzed hydrolysis of the chromogenic substrate S-2765 for all recombinant factor Xa mutants, were similar to that of wild type as determined previously (18). Compared with factor Xa wild type, proteolytic cleavage of A1 subunit by factor Xa R240A mutant showed a marked diminution of the A1α37–372 intermediate during the reaction time course (Fig. 5, A and B, insets). The apparent cleavage rate at Lysα36 in the intact A1 subunit (k11) catalyzed by the R240A mutant was ∼20% of that observed with wild type factor Xa (Table I). Furthermore, the apparent cleavage rate at Argα336 in the intact A1 subunit (k12) catalyzed by this mutant was nominally reduced to ∼70% of that for wild type. Because these reaction rates define the initial interactions of the mutant factor Xa with the intact A1 substrate, the observation that both were reduced may suggest the presence of a defect in substrate recognition attributable to the R240A mutation. Furthermore, the observation that conversion of intermediate A1α1–336 to the terminal product (k22) relative to the rate cleavage of the Argα37–372 (k12) for the mutant was ∼40% that of wild type factor Xa (0.53 versus 1.34, Table I) supports a role for the acidic C-terminal region of A1 in the interaction with this heparin-binding exosite residue. However, the other six factor Xa mutants evaluated catalyzed cleavage of the A1 subunit to the terminal product, A1α37–336, with rates similar to those observed for wild type factor Xa (data not shown). Of note is the observation that Argα240 of factor Xa appears to make the largest contribution of all the basic residues within the exosite to affinity for heparin (18).

We considered that an increase in Km might be observed with alteration of a heparin-binding exosite that interacted with substrate A1 subunit. Therefore, to confirm further that residue Argα240 of factor Xa participates in the interaction with A1 subunit, Km values for the cleavage of isolated A1 subunit by wild type and R240A factor Xa were determined. Various concentrations of A1 subunit were reacted with a constant concentration of factor Xa as described under “Materials and Methods.” After SDS-PAGE and Western blotting, band densities were measured using densitometry scanning to quantitate the degradation rate of A1. Results from these studies are shown in Fig. 6. The apparent kcat value for A1 cleavage obtained from...
the fitted curve for the R240A mutant was similar to that of wild type \(0.0197 \pm 0.0016\) and \(0.0201 \pm 0.0012\) min\(^{-1}\), respectively. However, the apparent \(K_m\) value obtained with R240A was 4-fold greater compared with wild type \((267 \pm 61\) nM, respectively). This increased \(K_m\) for R240A resulted in the mutation possessing 25% catalytic efficiency \((k_{cat}/K_m)\) of wild type factor Xa and indicated a contribution of Arg240 in mediating the binding of factor Xa to the A1 subunit.

Effect of the 337–372 Peptide on Isolated Heavy Chain Cleavage by Factor Xa R240A Exosite Mutant—Factor Xa also catalyzes activation of factor VIII, and interaction of the proteinase with heavy chain residues 337–372 may facilitate cleavage at Arg372 separating the A1 and A2 domains. An experiment was designed to assess the contribution of this factor VIII segment relative to cleavage of the isolated heavy chain substrate by the wild type and R240A enzymes. The isolated heavy chain \((100 \) nM) was reacted with 20 nM wild type or 50 nM R240A factor Xa in the presence of various concentrations of the 337–372 peptide for 5 h as described under “Materials and Methods.” Reaction products were separated by SDS-PAGE, and Western blotting was used to identify generation of the A2 subunit from the heavy chain. A control experiment showed complete con-

![Figure 5](http://www.jbc.org/).
version of heavy chain to A1 plus A2 subunits required reaction with 20 nM wild type and 50 nM R240A for this time course (data not shown), suggesting that although the enzymes possessed equivalent amidolytic activity, the mutant was somewhat deficient in cleaving the natural substrate. Cleavage at the A1-A2 domain junction of isolated heavy chain catalyzed by the wild type enzyme was inhibited \(90\%\) by the peptide in a dose-dependent manner. The calculated \(IC_{50}\) value was \(90\ \mu M\). In contrast, this cleavage catalyzed by R240A factor Xa was inhibited \(20\%\) at the maximum concentration of peptide employed (400 \(\mu M\)) (Fig. 7). These data provide further support for a direct interaction between Arg\(^{240}\) of the heparin-binding exosite and the acidic region linking A1 and A2 domains.

Identification of Factor X-interactive Site Using the Clustered Mutants within 337–372 Residues in A1 Subunit—A hallmark of the interdomain linking region (residues 337–372) is the high content of acidic residues (13 Glu or Asp residues of 36). We recently evaluated the effects of mutations where clustered acidic residues were replaced \textit{en bloc} by Ala (17). Results of that study suggested the involvement of a cluster comprised of residues Asp\(^{361}\)-Asp\(^{362}\)-Asp\(^{363}\) in facilitating factor Xa-catalyzed proteolysis on factor VIII substrates, suggesting that this region may serve as an interactive site for the enzyme. Therefore, to investigate the contribution of acidic residues within sequence 337–372 to the factor X-interactive site, we evaluated the cofactor properties of three factor VIIIa mutants containing Ala substitutions at clustered acidic regions including, 341–345 (Glu-Glu-Ala-Glu-Asp), 347–349 (Asp-Asp-Asp), and the 361–363 segments (17).

We hypothesized that an increase in \(K_m\) may be observed with alteration of an acidic cluster that participates in an interaction with substrate factor X. For this analysis, 30 nM factor VIII wild type and mutant forms were activated by thrombin, reacted with 0.5 nM factor IXa, and 10 \(\mu M\) phosphatidylserine-phosphatidylcholine-phosphatidylethanolamine vesicles, and reactions were initiated with varying amounts of factor X as described under “Materials and Methods.” Results from these studies are shown in Fig. 8 and Table II. The \(k_{cat}\) values obtained from the fitted curves for factor Xase consisting of factor VIIIa forms composed of the three individual cluster mutants were similar to that of wild type. However, the \(K_m\) value obtained with factor Xase comprising the 361–363Ala mutant was 1.6-fold greater compared with those composed of wild type or the 341–345 and 347–349 cluster mutants. This increased \(K_m\) resulted in a \(-45\%\) reduction in catalytic efficiency (\(k_{cat}/K_m\)) for factor Xase composed of 361–363Ala mutation, compared with the other factor VIIIa forms. Using the \(K_m\) parameter as an indicator for the thermodynamic stability...
of the enzyme-cofactor-substrate complex, the calculated binding energy values indicated a small but appreciable contribution (0.28 kcal mol\(^{-1}\), ~3%) for 361–363 residues in the association of factor X and factor VIIIa within factor Xase (Table II).

**Effect of Factor VIII361–363Ala Mutant on the \(K_m\) for Factor X R240A—The above results show that the 337–372 region of factor VIII comprises a factor Xa exosite-interactive site and further show the acidic segment 361–363 modulates the cofactor-factor X interaction. Because mutation of factor Xa at Arg\(^{240}\) reduces exosite interaction with the complementary site in factor VIIIa, an experiment was performed to examine the effects of mutation at Arg\(^{240}\) on the zymogen-factor VIII interaction. The R240A factor X was used as substrate in factor Xa generation assays using factor Xase comprising wild type factor VIII or the 361–363 triple mutant of factor VIIIa. Results from this experiment are shown in Fig. 9 and Table III. The \(k_{cat}\) values obtained from the fitted curves for the two factor VIIIa forms were similar, independent of the factor X substrate.

These values were ~35% lower than the \(k_{cat}\) values obtained using plasma-derived factor X (Table II), which also yielded modestly reduced \(K_m\) values. The reason for the ~2-fold increase in catalytic efficiency observed for the plasma-derived compared with recombinant factor X substrates is not known; however, these results are consistent with the literature (23).

Similar to our results with the plasma-derived factor X, the \(K_m\) value for wild type factor X obtained with factor Xase comprising the 361–363Ala mutant was ~1.8-fold greater than wild type factor Xase. However, although \(K_m\) values were dependent on the factor VIII form, they were independent of the factor X substrate form, as evidenced by similar \(k_{cat}\) values using either wild type or R240A factor X. These results suggested that the R240A mutation in zymogen factor X showed no apparent affect on interaction with factor Xase.

**DISCUSSION**

Zymogen factor X and the active enzyme, factor Xa, both interact with factor VIIIa. Although the enzyme regulates factor VIII activity, either by proteolytically activating the procofactor form or inactivating the active cofactor; the zymogen associates with the cofactor, contributing to the efficient conversion of zymogen to active enzyme by the intrinsic factor Xase. Results of this study show that the association of factor X and factor Xa with the 337–372 sequence of factor VIIIa is mutually exclusive, both utilizing a common interactive site, with the active enzyme exhibiting a markedly higher affinity interaction than the zymogen. This site localizes to the C-terminal region of the A1 subunit because the rate of cleavage at Lys\(^{36}\), but not at Arg\(^{336}\), was blocked by heparin. It is of interest to note that cleavage at the heparin-exosite point mutant of factor Xa was defective in cleaving Lys\(^{36}\) in A1, exhibiting a cleavage rate that was only ~8-fold using an A1 substrate lacking this region (A1\(^{336}\)) (11). In agreement with previous result, in the present study, heparin effectively blocked EDC-mediated cross-linking between the 337–372 peptide and factor Xa. In addition, a heparin-exosite point mutant of factor Xa was defective in cleaving Lys\(^{36}\) in A1, exhibiting a cleavage rate that was only ~20% of that observed with wild type factor Xa. This defect derived primarily from a ~4-fold increase in \(K_m\) for the A1 substrate.

A series of basic residues have been ascribed to the heparin-binding exosite of factor Xa (18). Identification of these residues was inferred from conservation of 7 of 11 basic residues comprising the heparin-binding exosite of thrombin. The role of these residues was verified after construction of point mutations replacing these residues individually with alanine (18). Based upon parameters such as altered affinity for heparin-Sepharose and rate constants for heparin-dependent inhibition of factor Xa by antithrombin, Arg\(^{240}\) was identified as the most critical heparin-binding residue in this exosite (18). Our studies evaluating action of factor Xa on factor VIII subunit substrates indicated little contribution of the exosite residues with

### Table II

**Kinetic parameters for activation of factor X by factor Xase in complex with recombinant factor VIIIa A1 cluster mutants**

The values were calculated by nonlinear least squares regression from the data shown in Fig. 8 using Equation 2. WT, wild type.

| Factor VIII    | \(K_m\) (nM) | \(k_{cat}\) (min\(^{-1}\)) | \(k_{cat}/K_m\) (nM \(\cdot\) min\(^{-1}\)) | Binding energy (kcal mol\(^{-1}\)) |
|---------------|--------------|----------------------------|------------------------------------------|-------------------------------|
| WT            | 46.6 ± 3.3   | 276 ± 6                    | 5.92                                     | 9.94 ± 0.7                    |
| 341–345Ala    | 44.7 ± 4.4   | 263 ± 8                    | 5.88                                     | 9.95 ± 0.97                   |
| 347–349Ala    | 53.4 ± 2.4   | 268 ± 4                    | 5.02                                     | 9.86 ± 0.44                   |
| 361–363Ala    | 74.1 ± 4.5   | 254 ± 6                    | 3.42                                     | 9.68 ± 0.58                   |

**FIG. 9. Effects of factor VIIIa361–363Ala on the kinetics of recombinant factor X wild type and R240A activation.** 30 nM recombinant factor VIII wild type (open symbols) or 361–363Ala (closed symbols) was activated by 10 nM thrombin in the presence of 10 μM phospholipid vesicles. Factor Xa generation assays were initiated with 30 nM factor Xase and varying amounts of recombinant factor X wild type (circles) or R240A (squares) as described under “Materials and Methods.” Initial rates of factor Xa generation were plotted as a function of factor X concentration and fitted to the Michaelis-Menten equation by nonlinear least squares regression.
exception of Arg240, which when substituted with Ala yielded markedly reduced rates of proteolysis. These results tend to support a role for the heparin-binding exosite of factor Xa in both procofactor activation and cofactor inactivation, based upon effects observed in cleavage of both factor VIII heavy chain and the factor VIIIa A1 subunit, respectively. Furthermore, the relatively lower affinity of factor X observed for the A1-containing substrates supports the contention that this exosite is not formed in the zymogen.

One explanation for the observed disparity between the affinities for factor Xa and factor X for this site may reflect different conformations for these residues in exosite and proexosite forms, respectively. Thus this relationship may parallel the observations of Anderson et al. (28) who characterized proexosite 1 in prothrombin. These investigators demonstrated that the zymogen prothrombin bound the C-terminal peptide derived from hirudin with a 130-fold higher 

\[ K_d \]

than that observed for binding of the peptide to thrombin. Formation of the exosite from proexosite was shown to result as a consequence of zymogen activation yielding a change in conformation from the low affinity state. Factor X binds A1 subunit with low affinity (\( K_d \approx 600 \text{ nM} \)). Furthermore, factor X was shown to interact with the 337–372 peptide, which appears to represent an exosite-specific ligand. This affinity was fractional compared with the peptide interaction with factor Xa, as judged by competition analysis as monitored by EDC-mediated cross-linking. These results support the presence of a proexosite in factor X which, upon activation, yields enhanced reactivity with the C-terminal region of the A1 subunit.

Consistent with the above hypothesis were the observed affinities for factor X and Xa using heparin-Sepharose chromatography. Although factor Xa bound the resin and was eluted at moderate salt (0.22 M NaCl), factor X failed to bind at 0.1 M NaCl, consistent with a significantly reduced affinity for the zymogen. Interestingly, the NaCl concentration that eluted factor Xa was significantly lower than that observed by Rezaie (18) (0.4 M). However, that study evaluated heparin binding using a Gla-domainless factor Xa form, which may possess enhanced affinity for heparin as a result of the elimination of inhibitory interactions between factor Xa and heparin, which are mediated by the anionic Gla domain (29).

Acidic residues within the C-terminal region of the factor VIIIa A1 subunit may contribute to the complementary factor Xa interactive site. Using a series of acidic cluster-to-Ala mutations, we recently showed that a segment of three Asp residues (residues 361–363), when replaced by Ala en bloc, yielded values for the rate of factor Xa-catalyzed activation of factor VIII and peak factor VIIIa activity that were \( \approx 30 \) and \( \approx 50\% \), respectively, the values obtained with wild type factor VIII (17). In the present study, we used these factor VIII mutants to assess the kinetics of factor Xa generation, thereby gaining insights into the role of these clustered acidic residues during the activation of factor X by the intrinsic factor Xase. Although \( k_{\text{cat}} \) values were not affected by the mutations, we observed a modest (\( \approx 60\% \)) increase in the \( K_m \) only with factor Xase comprising factor VIIIa possessing the 361–363 cluster substitution. These results suggested that both factor Xa and factor X utilize the 361–363 sequence during their interactions with factor VIIIa. However, the similarity of kinetic parameters determined for the activation of wild type factor X and factor X possessing the R240A mutation with the 361–363 factor VIIIa cluster mutation suggested that this Arg residue does not likely tether an Asp residue within this acidic cluster. Comparison of the \( K_m \) value obtained for factor X activation with the 361–363 cluster mutant (74 nM) with the \( K_m \) obtained from an earlier study employing a factor VIIIa truncated at both Lys337 and Arg338 (A177–336) (210 nM (13)) is consistent with residues other than the 361–363 segment within the factor X-interactive region defined by residues 337–372 contributing to binding interactions.

One possible role for residues 361–363 in the catalytic mechanism represents electrostatic steering of substrate factor Xa. This hypothesis is suggested by the minimal contribution of this segment to the thermodynamic stability of factor Xa interaction with factor Xase. Using \( K_m \) as an indicator of substrate affinity, the substitution of Asp residues to Ala in cluster 361–363 results in a reduction in binding energy of \( -0.3 \text{ kcal/mol} \), a value significantly less than the binding energy attributed to a salt bridge (ion pair). This observation is reminiscent of earlier studies examining the contribution of clustered acidic residues on the C-terminal tail of hirudin into interaction with anion-binding exosite 1 of thrombin (30, 31). Those studies showed at least two specific acidic residues that did not make direct interaction with thrombin but contributed between \( -0.25 \) and \( 0.5 \text{ kcal/mol} \) to the interprotein binding energy. More recently, a number of basic residues contained within exosite 1 of thrombin were suggested to contribute to the positive electrostatic field generated by the exosite, and this field was necessary for productive interaction, by electrostatic steering, with the complementary negative field generated by hirudin (32). Thus our future studies will focus on determination of contributions of specific residues to steering and ionic tethering interactions involved in the mechanisms of factor Xa interactions with factor VIIIa.

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