Cleavage at Arg-1689 Influences Heavy Chain Cleavages during Thrombin-catalyzed Activation of Factor VIII*

Jennifer L. Newell and Philip J. Fay
From the Department of Biochemistry and Biophysics, University of Rochester School of Medicine, Rochester, New York 14642

The procofactor, factor VIII, is activated by thrombin or factor Xa-catalyzed cleavage at three P1 residues: Arg-372, Arg-740, and Arg-1689. The catalytic efficiency for thrombin cleavage at Arg-740 is greater than at either Arg-1689 or Arg-372 and influences reaction rates at these sites. Because cleavage at Arg-372 appears rate-limiting and dependent upon initial cleavage at Arg-740, we investigated whether cleavage at Arg-1689 influences catalysis at this step. Recombinant B-domainless factor VIII mutants, R1689H and R1689Q were prepared and stably expressed to slow and eliminate cleavage, respectively. Specific activity values for the His and Gln mutations were ~50 and ~10%, respectively, that of wild type. Thrombin activation of the R1689H variant showed an ~340-fold reduction in the rate of Arg-1689 cleavage, whereas the R1689Q variant was resistant to thrombin cleavage at this site. Examination of heavy chain cleavages showed ~4- and 11-fold reductions in A2 subunit generation and ~3- and 7-fold reductions in A1 subunit generation for the R1689H and R1689Q mutants, respectively. These results suggest a linkage between light chain cleavage and cleavages in heavy chain. Results obtained evaluating proteolysis of the factor VIII mutants by factor Xa revealed modest rate reductions (~5-fold) in generating A2 and A1 subunits and in cleaving light chain at Arg-1721 from either variant, suggesting little dependence upon prior cleavage at residue 1689 as compared with thrombin. Overall, these results are consistent with a competition between heavy and light chains for thrombin exosite binding and subsequent proteolysis with binding of the former chain preferred.

Factor VIII, a plasma protein missing or defective in individuals with hemophilia A, is synthesized as an ~300-kDa single chain polypeptide corresponding to 2332 amino acids. Within the protein are six domains based on internal homologies and ordered as NH2-A1-A2-B-A3-C1-C2-COOH (1, 2). Bordering the A domains are short segments containing high concentrations of acidic residues that follow the A1 and A2 domains and precede the A3 domain and are designated a1 (residues 337–372), a2 (residues 711–740), and a3 (1649–1689). Factor VIII is processed by cleavage at the B-A3 junction to generate a divalent metal ion-dependent heterodimeric protein composed of a heavy chain (A1-a1-A2-a2-B domains) and a light chain (a3-A3-C1-C2 domains) (3).

The activated form of factor VIII, factor VIIIa, functions as a cofactor for factor IXa, increasing its catalytic efficiency by several orders of magnitude in the phospholipid- and Ca2+-dependent conversion of factor X to factor Xa (4). The factor VIII procofactor is converted to factor VIIIa through limited proteolysis catalyzed by thrombin or factor Xa (5, 6). Thrombin is believed to act as the physiological activator of factor VIII, as association of factor VIII with von Willebrand factor impairs the capacity for the membrane-dependent factor Xa to efficiently activate the procofactor (5, 7). Activation of factor VIII occurs through proteolysis by either protease via cleavage of three P1 residues at Arg-740 (A2-B domain junction), Arg-372 (A1-A2 domain junction), and Arg-1689 (a3-A3 junction) (5). After factor VIII activation, there is a weak electrostatic interaction between the A1 and A2 domains of factor VIIIa (8, 9) and spontaneous inactivation of the cofactor occurs through A2 subunit dissociation from the A1/A3-C1-C2 dimer, consequently dampening factor Xase (3).

Thrombin cleavage of factor VIII appears to be an ordered pathway, with relative rates at Arg-740 > Arg-1689 > Arg-372 and the initial proteolysis at Arg-740 facilitating proteolysis at Arg-372 as well as Arg-1689 (10). This latter observation was based upon results showing that mutations at Arg-740, impairing this cleavage, significantly reduced cleavage rates at the two other P1 sites. Thrombin-catalyzed activation of factor VIII is dependent upon interactions involving the anion binding exosites of the proteinase (11, 12). Exosite binding is believed to determine substrate affinity, whereas subsequent active site docking primarily affects V_{max} (13). Furthermore, the complex interactions involving multiple cleavages within a single substrate may utilize a ratcheting mechanism (14) where presentation of the scissile bond is facilitated by a prior cleavage event.

Cleavage at Arg-372 is a critical step in thrombin activation of factor VIII as it exposes a cryptic functional factor IXa-interactive site in the A2 domain (15), whereas cleavage at Arg-1689 liberates factor VIII from von Willebrand factor (16) and contributes to factor VIIIa specific activity (17, 18). Although cleavage at Arg-740 represents a fast step relative to cleavages at other P1 residues in the activation of factor VIII (19), the influence of Arg-1689 cleavage on cleavages in the heavy chain remains unknown. In the present study cleavage at Arg-1689 is examined using recombinant factor VIII variants possessing...
single point mutations of R1689Q and R1689H. Results indicating reduced rates of A1 and A2 subunit generation, which are dependent upon the residue at position 1689, suggest that cleavage at Arg-1689 affects rates of proteolysis at Arg-740 and Arg-372. These observations are consistent with a mechanism whereby heavy chain and light chain compete for a binding thrombin exosite(s), with heavy chain preferred over light chain. In this competition mechanism, cleavage at Arg-740 is favored over Arg-1689. Subsequent cleavage at Arg-372 in heavy chain may involve a ratcheting mechanism after initial cleavage at Arg-740. On the other hand, the mechanism for factor Xa-catalyzed activation of factor VIII appears to be less dependent on cleavage at the Arg-1689 site as compared with thrombin.

MATERIALS AND METHODS

Reagents—The monoclonal antibodies C5 (recognizing the a1 region) and 2D2 (recognizing the A3 domain) were generous gifts from Zaverio Ruggeri and Bayer, respectively. The anti-A2 domain factor VIII monoclonal antibody R8B12 (8) and monoclonal antibody GMA-8003 recognizing the C2 domain were obtained from Green Mountain Antibodies. The reagents human α-thrombin, factor IXa, factor X, and factor Xa were purchased from Enzyme Research Laboratories. Recombinant nonsulfated hirudin and horseradish peroxidase-labeled streptavidin were from Calbiochem. Chromogenic factor Xa substrate Pefa-5523 (Pefachrome® FXa) was purchased from Centerchem. Factor VIII-deficient plasma was prepared as previously described (20). Phospholipid vesicles containing 20% phosphatidylyserine, 40% phosphatidylcholrine, and 40% phosphatidylethanolamine were prepared using N-octyl glucoside as previously described (21). The Bluescript factor VIII vector (pBS factor VIII) and B-domainless factor VIII expression construct RENeo factor VIII were kindly provided by Pete Lollar and John Healey (Emory University).

Construction, Expression, and Purification of Recombinant Factor VIII—B-domainless factor VIII cDNA was restricted from the factor VIII expression construct FVIIHSQ-MSAB-NotI-RENeo using the endonucleases XhoI and NotI and cloned into the pBluescript II K/S-vector. The B-domainless factor VIII cDNA was further restricted using endonucleases SacII/Apal and subcloned into the pBluescript II K/S-vector. The R1689H and R1689Q mutations were introduced into the construct using the Stratagene QuikChange site-directed mutagenesis kit as previously described (22). The presence of only the desired mutation was confirmed using dideoxy sequencing. The mutated factor VIII cDNA was then ligated back into the factor VIII expression construct and subjected to a second round of dideoxy sequencing to confirm that only the desired mutation was present. FuGENE 6 (Roche Applied Science) was used to transfect the factor VIII expression vector into baby hamster kidney cells. The selection, subcloning, and cloning of stable transfectants were performed by standard methods, and the cloned cells were cultured in roller bottles for protein expression (23). The conditioned media was collected daily, and the expressed proteins were purified by SP-Sepharose (Amersham Biosciences) chromatography as previously described (23). A one-stage clotting assay was used to detect active fractions. Yields of purified wild-type and variant factor VIII ranged from 0.08 to 0.4 mg per liter of conditioned media. Resultant factor VIII was >90% pure as judged by SDS-PAGE with the main contaminant being albumin. Factor VIII samples were quick-frozen and stored at −80 °C.

Enzyme-linked Immunosorbent Assay—The concentration of purified factor VIII proteins was determined by a sandwich enzyme-linked immunosorbent assay. GMA-8003 was used as the capture antibody, and biotinylated R8B12 was used as the detection antibody. The amount of factor VIII bound to the plate was determined as previously described (22) using a chromogenic assay utilizing streptavidin-linked horseradish peroxidase (Calbiochem) with the chromogen o-phenylenediamine dihydrochloride (Sigma). Purified commercial recombinant factor VIII (Kogenate; Bayer Corp.) was used as the standard. Factor VIII-specific activity values were determined using one-stage clotting and enzyme-linked immunosorbent assay (22).

Factor Xa Generation Assay—The rate of conversion of factor X to factor Xa was monitored in a purified system (24). For the factor VIII activation time course after thrombin addition, factor VIII (1 nM) was reacted with thrombin (0.05 nM) in the presence of phospholipid vesicles (10 μM) at 22 °C. Samples were removed at indicated times, and thrombin activity was inhibited by the addition of hirudin (0.1 units/ml). Factor Xa generation was initiated by the addition of factor IXa (20 nM) and factor X (300 nM). The reactions were terminated with EDTA (50 mM) at the indicated times. Rates of factor Xa generation were determined by the addition of the chromogenic substrate Pefa-5523 (0.46 μM final concentration). Reactions were read at 405 nm for 5 min using a Vmax microtiter plate reader (GE Healthcare). To assess the Km (apparent), various concentrations of wild-type and mutant factor VIII (0–45 nM) were reacted with thrombin (0.05 nM) for 15 s. Thrombin was inactivated by the addition of hirudin (0.1 units/ml) in the presence of phospholipid vesicles (10 μM), and each sample was reacted with factor IXa (20 nM) and factor X (300 nM). Aliquots were removed at appropriate times to assess initial rates of product formation, added to tubes containing EDTA (50 mM final concentration), and processed as described above. To assess the Km (apparent), various concentrations of the R1689Q factor VIII (0–60 nM) were added to a reaction containing wild-type factor VIII (5 nM) and thrombin (0.05 nM) in the presence of phospholipids (10 μM) for 1 min. Thrombin was inactivated by the addition of hirudin (0.1 units/ml), and each sample was reacted with factor IXa (20 nM) and factor X (300 nM) as described above.

Cleavage of Factor VIII by Thrombin or Factor Xa—Factor VIII (100 nM) was reacted with 2.5 nM thrombin in a buffer containing 20 mM HEPES (pH 7.2), 0.14 M NaCl, 5 mM CaCl2, and 0.01% Tween 20. Alternatively, factor VIII (100 nM) was reacted with factor Xa (2.5 nM) in the presence of phospholipid vesicles (10 μM) in the above buffer. Reactions were run at 22 °C, and samples were taken at indicated time points during the time course. The reactions were terminated by the addition of SDS-PAGE sample buffer and boiling for 3 min.

Electrophoresis and Western Blotting—Samples were run by SDS-PAGE on 8% polyacrylamide gels. Electrophoresis was carried out using a Bio-Rad mini gel apparatus at 175 V for 1 h.
Proteins were transferred to polyvinylidene fluoride membrane for Western blotting. Blots were probed using the anti-factor VIII monoclonal antibodies indicated in the figure legends followed by reaction with a goat anti-mouse alkaline phosphatase-linked secondary antibody (Sigma). The signal was detected using the ECF® (enhanced chemifluorescence) system (Amersham Biosciences), and the blots were scanned at 570 nm using a Storm 860 (GE Healthcare). Densitometric scans were quantitated from linear density regions of the blots using ImageQuant software (GE Healthcare).

Data Analysis—All experiments were performed at least three separate times, and the average values with standard deviations are shown. Western blot analysis determining the rates of subunit generation after thrombin cleavage of wild type and mutants were calculated based upon summing all factor VIII band density values. The intensity of each band was determined by volume integration and corrected for variability in background staining by using densities in the areas immediately adjacent to each band. The concentration of A1, A2, and A3-C1-C2 subunits was normalized to the total density in each lane to correct for slight differences in loading. Initial time points were fitted using non-linear least squares regression to the second order polynomial equation,

\[
[FVIII] = A + Bt + Ct^2
\]  
(Eq. 1)

where \([FVIII]\) is the concentration of A1, A2, or A3-C1-C2 in nm, \(t\) is the time in minutes, and \(A\), \(B\), and \(C\) are coefficients of the quadratic equation. \(A\) is the initial concentration of factor VIII in nm. \(B\) corresponds to the slope value when the time is 0 min and \(C\) represents the non-zero quadratic coefficient. The absolute value of \(B\) represents the rate of the A1, A2, or A3-C1-C2 subunit generation that was normalized by the thrombin concentration and expressed in nm A1, A2, or A3-C1-C2 subunit/min/nM thrombin, respectively.

Factor Xa cleavage of the factor VIII light chain was assessed by Western blot analysis as described above and fitting the data to the second order polynomial equation (Equation 1) using non-linear least squares regression. \(A\) is the initial concentration of factor VIII in nm. \(B\) corresponds to the slope value when the time is 0 min. The absolute value of \(B\) represents the rate of light chain loss that was normalized by the factor Xa concentration and expressed in nm light chain/min/nM factor Xa, respectively.

Kinetic parameters were determined by Xa generation analysis. \(K_m\) and \(V_{max}\) were calculated from initial rate data by fitting the data using non-linear least-squares regression analysis to the Michaelis-Menten equation,

\[
v_o = \frac{V_{max} \times [S]}{K_m + [S]} + \frac{[S]}{Ki}
\]  
(Eq. 2)

where \(v_o\) is the initial velocity in (nm/min), and \([S]\) is the concentration of wild-type or mutant factor VIII in nm. Using these conditions, the \(V_{max}\) recorded is for factor Xa generation and relates directly to the amount of factor VIIIa formed, whereas \(K_m\) is a measure of the concentration of substrate factor VIII acted upon by thrombin.

The \(K_i\) for R1689Q factor VIII was calculated by fitting the data to the competitive inhibition equation by nonlinear least squares regression analysis,
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The generation of A3-C1-C2 subunit after thrombin-catalyzed cleavage of recombinant factor VIII mutants. Panels A and B, recombinant factor VIII R1689H and R1689Q (100 nM) were reacted with thrombin (10 nM) for 120 min and subjected to SDS-PAGE and blotting using an anti-A3 antibody. Also in panels A and B, wild type (WT, 100 nM) was reacted with 2.5 nM thrombin for 25 min. In panel C, initial time points, shown as continuous lines, were fitted to the second-order polynomial equation (Equation 1) using non-linear least squares regression. The inset shows results obtained using recombinant factor VIII wild type (100 nM) reacted with a lower thrombin concentration (0.25 nM). The abbreviations WT, SC, a3-A3-C1-C2, and A3-C1-C2 represent wild type, single chain, intact light chain, and thrombin-cleaved light chain, respectively.

Table 1

Rates of subunit generation during factor VIII activation by thrombin

| Factor VIII | Rate of A1 subunit generation | Rate of A2 subunit generation | Rate of Arg-1689 subunit generation |
|-------------|-------------------------------|-------------------------------|-------------------------------------|
| Wild type   | 38.2 ± 2.7                    | 27.4 ± 1.5                    | 136 ± 11                            |
| R1689H      | 11.8 ± 1.4                    | 7.9 ± 1.5                     | 0.4 ± 0.03                          |
| R1689Q      | 5.7 ± 0.4                     | 2.6 ± 0.3                     | N/A                                 |

Note: Table 1 provides the rates of subunit generation for A1, A2, and A3-C1-C2 subunits by thrombin cleavage of wild-type and mutant factor VIII, estimated by non-linear regression analysis of the data shown in Figs. 2 and 3 and supplemental Fig. 1.

The generation of wild-type factor VIII (100 nM) with thrombin (2.5 nM) showed efficient cleavage of both the single chain and the light chain, resulting in the rapid generation of the A3-C1-C2 subunit. These blots were quantitated by scanning densitometry and subjected to nonlinear least squares regression analysis (Fig. 2C) to calculate the rates of A3-C1-C2 subunit generation (Table 1). Because generation of A3-C1-C2 is derived from a single cleavage at Arg-1689, rates for cleavage and subunit generation are equivalent. The cleavage rate for thrombin at this site was reduced ~340-fold for the R1689H variant compared with wild type (Table 1). This value reflects a greater reduction in cleavage rate than the ~80-fold reduction that was observed in a prior study when His replaced Arg at the 372 site (25). However, this result is consistent with accommodation of His in the specificity binding pocket of the enzyme, allowing for scissile bond cleavage. Alternatively, we observed no detectable light chain cleavage in the R1689Q mutant (Fig. 2, B and C), consistent with this mutation precluding active site docking. We also observed that prior to the reaction with thrombin, this variant possessed a low level (~10% of 2D2-reactive material).
of a high molecular band running just below the single chain material and consistent with a contiguous A2-light chain intermediate. This band was also visualized when blotting with the anti-A2 antibody (see supplemental Fig. 1) supporting this contention. The reason for its presence in the untreated sample is unclear; however, its disappearance after thrombin addition indicates further processing at the Arg-740 site.

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To examine the effects of cleavage at residue 1689 on other activating cleavages in factor VIII, reactions were initiated using catalytic levels of thrombin (2.5 nM) relative to factor VIII (100 nM) and run over a 25-min time course. A similar approach was used to assess rates of A1 subunit generation using the anti-A1 domain-specific monoclonal antibody, C5. When examining thrombin cleavage of the factor VIII mutants, results revealed overall reduced rates of cleavage at Arg-372 as seen by retention of factor VIII heavy chain (contiguous A1-A2) with a reduction in A1 subunit generation as compared with wild-type protein (Fig. 3). Because the A1 subunit is derived after a single cleavage at Arg-372, its generation reflects the rate of cleavage at that site. Results from the blots were quantitated, and rates of A1 subunit generation are shown in Table 1. These results suggest that slowing thrombin-catalyzed proteolysis by incorporating a non-optimal P1 His at position 1689 yields a modest
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FIGURE 4. Activation of wild-type and mutant factor VIII by thrombin. Recombinant factor VIII wild-type, R1689H, and R1689Q (1 nM) were reacted with thrombin (0.05 nM) for the indicated times. Thrombin was inactivated by the addition of hirudin (0.1 units/ml), and factor VIIIa was reacted with factor IXa (20 nM), phospholipid vesicles (10 μM), and factor X (300 nM) as described under "Materials and Methods." ▲, wild type; ■, R1689H; ●, R1689Q. Experiments were performed at least three separate times, and average values with error bars are shown.

(−3-fold) reduction in Arg-372 cleavage (Fig. 3, A and C), whereas replacement of the P1 residue with the non-cleavable Gln yield a further rate reduction of A1 subunit generation of −7-fold (Fig. 3, B and C). Thus, stalling cleavage within light chain effectively stalls the rate-limiting cleavage at Arg-372 in the heavy chain.

Generation of A2 Subunit by Thrombin Cleavage of the Factor VIII Variants—Rates of A2 subunit generation were evaluated using the anti-A2 domain-specific antibody R8B12. A2 subunit is derived from cleavages at Arg-372 and Arg-740, the latter at the a2-B junction. In the B-domainless factor VIII heterodimer, a remnant (−14 residues) of the B domain remains C-terminal to the a2 segment, and its presence or absence cannot be discriminated by SDS-PAGE. Thus, the band visualized as A2 subunit requires only the single cleavage at Arg-372 to be released from the heavy chain of the heterodimer, whereas cleavages at both Arg-372 and Arg-740 are necessary to excise this subunit from single chain factor VIII. For these reasons, results presented for rates of A2 subunit generation give limited information on actual cleavage rates at Arg-740 but are useful in assessing rates of A2 subunit generation in the variants relative to wild-type factor VIII.

A2 subunit generation data show that rates for cleavages at all thrombin-sensitive sites in the mutants were reduced as seen by retention of significant amounts of single chain factor VIII over a 25-min time course (see supplemental Fig. 1). Furthermore, the contiguous A2-light chain intermediate, noted above in the R1689Q variant was also observed in the His variant, likely a result of the denser staining of single chain material in this blot compared with the 2D2 (light chain) blot. Although the intermediate appeared to follow a similar rate of loss of single chain material in the His variant, this band persisted similar to that of the single chain band in the Gln variant. Using a 120-min time course, the rate of A2 subunit generation for the R1689H and R1689Q mutants were reduced ~4- and ~11-fold, respectively, compared with wild-type factor VIII (see supplemental Fig. 1 and Table 1). The rates of A1 subunit generation were greater than those of A2 subunit generation, suggesting that the mutations at Arg-1689 affect cleavage at both Arg-372 and Arg-740. Taken together with the other blotting data, these results show that slowing the cleavage rate of Arg-1689 slows procofactor activation by reducing the rates of thrombin-catalyzed cleavages at both Arg-740 and Arg-372 within factor VIII heavy chain.

Thrombin Activation of Factor VIII Proteins—Thrombin proteolysis of the factor VIII variants was analyzed using a factor Xa generation assay, as described under "Materials and Methods," to assess the generation of cofactor activity (Fig. 4). Wild-type and mutant factor VIII (1 nM) were reacted for the indicated times with thrombin (0.05 nM) in a time course assay monitored by Xa generation. Analysis of wild-type protein showed that activity increased rapidly, peaking at ∼2 min, after which cofactor activity dropped to ∼10% peak activity at 30 min (Fig. 4). This activity loss over the time course results from factor VIIIa inactivation by A2 dissociation from the A1/A3-C1-C2 dimer leading to dampening of factor Xase (26). In comparison to wild-type factor VIII, the R1689H variant showed an ∼2-fold reduction in peak activation, suggesting less efficient activation of the procofactor (Fig. 4). The R1689Q mutant had a significantly altered activation profile with the thrombin addition, resulting in slowly increasing cofactor activity, reaching a maximal activity level that represented ∼10% the level observed for wild type (Fig. 4). The activity profile of the R1689Q mutant varied from those seen with wild type and R1689H as there was a plateau of activation from 1 to 7 min that gave the appearance of R1689Q factor VIIIa being more stable over the time course. However, the rate of procofactor activation is likely balanced by the rate of cofactor inactivation (27), suggesting that the rate of thrombin activation of the procofactor is reduced. These data are consistent with the specific activity values for the variants along with the Western blot analyses, suggesting that cleavage at Arg-1689 influences observed rates involved in procofactor activation.

Kinetic Parameters for Thrombin Activation for Wild-type and R1689Q Factor VIII—A factor Xa generation assay as described under "Materials and Methods" was used to indirectly measure the kinetic parameters for thrombin activation of factor VIII. A catalytic concentration of thrombin (0.05 nM) was reacted with variable amounts of wild-type factor VIII (0–45 nM) for 15 s to ensure initial rate conditions. Subsequent addition of phospholipids (10 μM) and a high concentration of factor IXa (20 nM) was used to ensure formation of the factor Xase complex, and factor Xa generation reactions were initiated by the addition of factor X (300 nM). Under these conditions the rate of factor Xa formation is proportional to the con-
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FIGURE 5. Kinetics of thrombin activation of recombinant wild-type and R1689Q factor VIII. In panel A variable amounts of wild-type (WT) factor VIII (0–45 nM) were activated with thrombin (0.05 nM) for 15 s. Thrombin was inactivated by the addition of hirudin (0.1 units/ml) in the presence of phospholipids (10 μM). The addition of factor IXa (20 nM) and factor X (300 nM) initiated factor Xa generation as described under "Materials and Methods." Rates of factor Xa generation are plotted as a function of wild-type factor VIII concentration and fitted to the Michaelis-Menten equation (Equation 2) by non-linear least squares regression. Experiments were performed at least three separate times, and average values are shown. In Panel B, various concentrations of recombinant factor VIII R1689Q (0–60 nM) and wild-type factor VIII (5 nM) were reacted with thrombin (0.05 nM) for 15 s. Thrombin was inactivated by addition of hirudin (0.1 units/ml) in the presence of phospholipids (10 μM). Factor Xa generation was initiated by addition of factor X (300 nM) and factor IXa (20 nM) as described under "Materials and Methods." The 100% value obtained in the absence of R1689Q was ~90 nM/min. Data were corrected by the amount of factor Xa generated by R1689Q factor VIII. Rates of factor Xa generation are plotted as a function of R1689Q factor VIII concentration and fitted to the competitive inhibition equation (Equation 3) by non-linear least squares regression. Experiments were performed at least three separate times and average values are shown.

Table 2

| Factor VIII | Rate of A1 subunit generation | Rate of A2 subunit generation | Rate of light chain loss | Rate of Arg-1721-A3-C1-C2 subunit generation |
|-------------|-------------------------------|-------------------------------|-------------------------|-----------------------------------------------|
| Wild type   | 27.9 ± 1.3                    | 12.4 ± 1.9                    | 14.3 ± 1.2              | 6.6 ± 0.9                                     |
| R1689H      | 8.7 ± 1.0                     | 5.3 ± 0.9                     | 4.2 ± 0.3               | 4.2 ± 0.6                                     |
| R1689Q      | 5.9 ± 0.8                     | 4.0 ± 0.5                     | 3.4 ± 0.3               | 3.4 ± 0.4                                     |

To investigate potential differences in thrombin binding to wild-type and the R1689Q factor VIII, the variant was used as a competitor of wild-type factor VIII activation by thrombin (Fig. 5B). Wild-type factor VIII (5 nM) was reacted with thrombin (0.05 nM) for 15 s in the absence or presence of variable concentrations of R1689Q factor VIII (0–60 nM). After the addition of hirudin, the resultant factor VIIIa was reacted with factor IXa (20 nM) and factor X (300 nM) in the presence of phospholipid vesicles (10 μM), and the rates of factor Xa generation were determined. Rates of factor Xa generation decreased in a dose-dependent manner after the addition of the variant, with a 10-fold excess of R1689Q factor VIII yielding ~60% inhibition of thrombin activation of wild-type factor VIII after correcting for the contribution of activated R1689Q variant to product formation. The apparent Kᵢ value for R1689Q factor VIII (6.4 ± 2.4 nM) was similar to the Kᵢ (apparent) of wild-type factor VIII. These results indicate that although alternate P1 residues exist in the R1689Q mutant, the P1 residue at 1689 does not impair thrombin binding. This result supports the contention that exosite binding is a significant contributor to thrombin binding to factor VIII (12, 28).

Factor Xa-catalyzed Cleavage of Heavy Chain in Wild-type and Mutant Recombinant Factor VIII—In contrast to thrombin, proteolytic activation of factor VIII by factor Xa is membrane-dependent. Proteolysis of factor VIII wild type and variants (100 nM) by factor Xa (2.5 nM) were performed in the presence of phospholipid vesicles (10 μM), and products were evaluated after SDS-PAGE and Western blotting using C5 antibody to assess generation of the A1 subunit and R8B12 antibody to evaluate A2 subunit generation (supplemental Fig. 2). Although factor Xa cleavage of the wild-type factor VIII single chain and heavy chain yielded a similar pattern of reaction products as with thrombin, the rates of factor VIII A1 and A2 subunits formed were ~2-fold slower than those observed for thrombin (supplemental Fig. 2 and Table 2). Results evaluating Arg-372 proteolysis of R1689H and R1689Q by factor Xa revealed faster rates of generation of A1 subunit compared with those observed using thrombin such that the rate reductions were 3- and 5-fold, respectively, compared with wild-type factor VIII (supplemental Fig. 2 and Table 2). In addition to generation of the A1 subunit, we noted low rates of generation of an A1³⁸⁶ subunit derived from factor Xa cleavage of the A1 subunit at Arg-336 (29). Rates of generation of this fragment were independent upon point mutation at Arg-1689. Similar to the generation of A1 subunit, rates for A2 subunit generation after cleavage of the R1689H and R1689Q variants were reduced by 2- and 3-fold. These results suggest that there is an effect of Arg-1689 cleavage on factor Xa cleavage rates at both heavy chain sites, albeit to a lesser extent than observed for thrombin.
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Cleavage of Light Chain in Wild-type and Mutant Factor VIII Forms by Factor Xa—In addition to proteolysis of factor VIII light chain at Arg-1689, factor Xa cleaves at Arg-1721 in the light chain. The role of this cleavage is not clear as it appears to be benign with respect to activity in the cofactor (6, 27). Factor Xa cleavage and Western blotting of wild-type protein revealed the two light chain products using the anti-A3 domain (supplemental Fig. 3). On the other hand, proteolysis of either mutant lacked an Arg-1689 cleavage-derived light chain yielding only the Arg-1721-cleavage-derived subunit (supplemental Fig. 3). This result suggested that cleavage at Arg-1721 by factor Xa does not require initial processing at the Arg-1689 site and that when cleavage at the 1689 site is encumbered by mutation, reactions proceed at the 1721 site. Quantification of blotting data (supplemental Fig. 3) indicated that R1689H and R1689Q factor VIII mutants yielded a slower rate ∼3- and 4-fold, respectively, of light chain loss compared with wild-type protein over the time course (Table 2). Also, in comparison to wild-type factor VIII, there was an ∼2-fold reduction in factor Xa cleavage at Arg-1721 in both the R1689H and R1689Q factor VIII mutants (supplemental Fig. 3). The resultant reduction in the rate of light chain cleavage in the mutants suggests that reducing the cleavage rate at the 1689 site modestly slows the rate at which all scissile bonds including the P1 Arg-1721 are cleaved.

DISCUSSION

Activation of factor VIII by thrombin occurs through proteolysis at three P1 residues, Arg-740, Arg-1689, and Arg-372. Two recent studies have assessed thrombin-catalyzed proteolysis at the Arg-372 (25) and Arg-740 (10) residues after site-directed mutagenesis to replace Arg with either His or Gln to slow or eliminate, respectively, cleavage at the site in question. Proteolysis at Arg-740 occurs at the A2-B junction and is rapid relative to cleavage at the other P1 sites (10, 19), liberating the B domain (or its fragments) from the contiguous A1-A2 domains of the factor VIII heavy chain. Mutations at Arg-740 showed dramatic effects at the two alternate sites, with the His replacement reducing cleavage rates at Arg-372 and Arg-1689 by 20- and 40-fold, respectively (10). Substitution of Arg-740 with the non-cleavable Gln residue further reduced these rates by ∼700- and 140-fold, respectively, relative to the value seen with wild-type factor VIII. On the other hand, these same mutations at the rate-limiting P1 residue Arg-372, although resulting in a marked reduction (for the His mutant) or absence (for the Gln mutant) of procofactor activation, showed no significant effects related to altered rates of proteolysis at the alternate P1 sites (25). Thus, these results were consistent with a largely ordered pathway for thrombin-catalyzed cleavage of factor VIII with Arg-740 > Arg-1689 > Arg-372.

In the current study mutagenesis to replace Arg-1689 with either His or Gln was used to evaluate whether this residue makes a contribution to the pathway of thrombin activation of factor VIII, as cleavage at this site proceeds at an intermediate rate compared with the other rate values. As expected, cleavage at the His-replaced P1 residue resulted in a significant rate reduction (∼350-fold) at this site. This value was ∼4-fold greater than the ∼fold reductions observed when either Arg-740 (10) or Arg-372 (25) was replaced by His, and the reason for this more substantial rate decrease is not known. Replacement with Gln yielded a non-cleavable site. Surprisingly, we observed that reducing catalysis at this site reduced cleavage at both factor VIII heavy chain P1 residues. The rate of A1 subunit generation, a direct measure of cleavage rate at Arg-372, was reduced ∼3- and ∼7-fold by substitution of His and Gln, respectively, at the P1 1689 site. A somewhat more substantial reduction in the rates of A2 subunit generation, a measure of cleavage at Arg-372 as well as Arg-740 for A2 derived from single chain factor VIII, showed ∼4- and ∼11-fold rate reductions, respectively, for the His and Gln variants.

A primary function of light chain cleavage at Arg-1689 in vivo is the liberation of the a3 segment, which constitutes a portion of the binding site for von Willebrand factor (19), allowing for dissociation of factor VIIIa from its carrier protein and subsequent association of the cofactor with both anionic phospholipid membranes and factor IXa, forming the Xase complex. Loss of this cleavage in vivo would have a significant impact on cofactor activity as illustrated by the naturally occurring mutations R1689H and R1689Q, which yield hemophilia A phenotypes (30). Studies in the current report examined cleavage rates for the purified factor VIII variants, and resultant activity profiles were performed in the absence of von Willebrand factor in the factor Xa generation assays, whereas the plasma-based one-stage clotting assays used dilutions of recombinant factor VIII well below the Ka for interaction with von Willebrand factor.

Reconstitution studies have also demonstrated that cleavage at Arg-1689 enhances the specific activity of the cofactor by several fold (17). The mechanism for this activity increase is not known but may reflect removal of detrimental interactions of the acidic a3 segment within the Xase complex and/or with substrate factor X. Consistent with this effect, we observed reduced specific activity parameters of the His- and Gln- substituted Arg-1689 of ∼50 and ∼20% the wild-type value. The specific activity for the non-cleavable R1689Q variant is predicted from the ∼5-fold reduction in this parameter when factor VIIIa was reconstituted with an a3-A3-C1-C2 light chain (17), whereas the ∼50% wild-type value for the R1689H variant suggested fractional cleavage of the light chain during activation. This latter result is also suggested in Fig. 4, which shows a peak value for factor Xa generation that is ∼55% wild type at 2 min.

An earlier study from our laboratory (28) demonstrated a relatively high affinity site (Kd ∼5 nM) for thrombin with the A1 domain of factor VIII that likely involves an interaction with anion binding exosite I (ABE I) and a weaker affinity interaction (Kd ∼100 nM) in A2 domain for thrombin that appeared ABE II-dependent. Based upon these observations, we proposed a model for heavy chain cleavage where initial binding of thrombin to factor VIII would occur through the A1/ABE I-interactive site with subsequent tethering occurring through A2 domain interaction with ABE II. The latter step was suggested to result in alignment and active site docking of Arg-740 with consequent cleavage liberating the B domain. Expulsion of the P1 Arg followed by a possible ratcheting mechanism (14) would allow for sequential presentation and docking of Arg-372, with
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this rate-limiting cleavage at the A1-A2 junction liberating the separated A1 and A2 subunits.

Results from the current study add to this mechanism, showing that rates of proteolysis at light chain impact cleavage in heavy chain. We observed that either slowing or eliminating cleavage at Arg-1689 after site-directed mutagenesis resulted in reduced cleavage rates at both heavy chain sites as judged by rates of A1 and A2 subunit generation. Similarly, we previously showed that slowing or eliminating cleavage at Arg-740 in the heavy chain reduced the rate of cleavage of light chain (10). One mechanism consistent with these observations is a competition between the two factor VIII chains for exosite binding. Thus, if light chain binds first, this would engage Arg-1689 at the active site, and cleavage would result in the release of A3 and A3-C1-C2 products. Conversely, initial binding of heavy chain would allow for the ordered cleavage at Arg-740 followed by Arg-372 as described above. Ultimately, the presence of a mutation at Arg-1689 (or Arg-740) would not block exosite binding but would stall cleavage at the mutated P1 site, and in turn, reduce the rate of cleavage at the remaining unmodified P1 sites.

As shown in the current study, when cleavage at 1689 is impaired by mutation to Gln, we observed 7–11-fold rate reductions in cleavages at the heavy chain sites relative to the wild-type rates. However, mutation of Arg-740 to Gln yielded a >100-fold rate reduction in the cleavage of light chain at Arg-1689 relative to wild type. The disparity in the magnitude of these effects suggests that exosite binding of heavy chain and subsequent cleavage at Arg-740 represents the preferred pathway in the activation scheme. Thus, we would observe faster rates of cleavage at Arg-740 than Arg-1689. We further speculate that the Arg-372 site is slow relative to the other two sites as a result of the time required for active site docking after initial cleavage at Arg-740.

Further support for this competition model derives from the results of Myles et al. (12) who examined factor VIII activation using a panel of thrombin point mutants. Their results showed that mutations in exosite I resulted in reduced cleavage rates of both the factor VIII heavy chain (as monitored by A1 and A2 subunit generation after cleavage at Arg-372) and light chain (monitored by cleavage of the a3 segment). Thus, both factor VIII chains may contain an ABE I-interactive site for thrombin. Indeed the high density of acidic residues plus the presence of sulfated Tyr-1680 contained in the a3 segment suggests the possibility of an ABE I-interactive site. Furthermore, the observation that association of von Willebrand factor with factor VIII appears to accelerate thrombin cleavage at Arg-1689 (19) would be consistent with the carrier protein potentially stabilizing an exosite-interactive region in the factor VIII substrate facilitating active site docking.

Factor Xa cleavage of factor VIII revealed that mutations at Arg-1689 modestly slowed the two heavy chain cleavages as judged by A1 and A2 subunit generation. Furthermore, although factor Xa cleavage of the wild type yielded A3-C1-C2 subunits derived from cleavage at Arg-1689 and Arg-1721, cleavage of the variants light chain was reduced ~4-fold compared with wild type and yielded a single product derived from cleavage at Arg-1721. These results suggest the Arg-1721-derived product is generated more efficiently from the Arg-1689-cleaved light chain. Overall, the data suggest a cleavage mechanism of factor VIII by factor Xa that is less selective in P1 site ordering than thrombin. In comparison to thrombin, factor Xa cleavage of wild-type factor VIII light chain is reduced up to ~10-fold; however, factor Xa appears to cleave the variant substrates up to 11-fold faster than thrombin. Moreover, previous results evaluating cleavage at Arg-740 (10) showed mutations at that site had more modest rate reductions in cleavage at Arg-1689 and Arg-372 in factor Xa compared with thrombin. The smaller effects of mutation at Arg-1689 on heavy chain cleavage could be because of lack of dependence on exosite interactions, and further work is required to establish this mechanism.

In summary, our data show that mutations at Arg-1689 that slow or eliminate thrombin catalysis at this site also reduce reaction rates for activating cleavage in the heavy chain. These observations suggest a mechanism likely representing an asymmetric competition between heavy chain and light chain for exosite tethering, thereby facilitating docking of Arg-740 in the heavy chain or Arg-1682 in the light chain to the active site. Overall, this competition gives the appearance of an ordered cleavage pathway of P1 residues Arg-740 > Arg-1689 > Arg-372.

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