Identification of Residues in the 558-Loop of Factor VIIIa A2 Subunit That Interact with Factor IXa*

Indu Jagannathan, H. Travis Ichikawa, Tricia Kruger, and Philip J. Fay1
From the Department of Biochemistry and Biophysics, University of Rochester School of Medicine, Rochester, New York 14642

Factor VIIIa is comprised of A1, A2, and A3C1C2 subunits. Several lines of evidence have identified the A2 558-loop as interacting with factor IXa. The contributions of individual residues within this region to inter-protein affinity and cofactor activity were assessed following alanine scanning mutagenesis of residues 555–571 that border or are contained within the loop. Variants were expressed as isolated A2 domains in Sf9 cells using a baculovirus construct and purified to >90%. Two reconstitution assays were employed to determine affinity and activity parameters. The first assay reconstituted factor Xase using varying concentrations of A2 mutant and fixed levels of A1/A3C1C2 dimer purified from wild type (WT), baby hamster kidney cell-expressed factor VIIIa, factor IXa, and phospholipid vesicles to determine the inter-molecular $K_D$ for A2. The second assay determined the $K_D$ for A2 in factor VIIIa by reconstituting various A2 and fixed levels of A1/A3C1C2. Parameter values were determined by factor Xa generation assays. WT A2 expressed in insect cells yielded similar $K_D$ and $k_{cat}$ values following reconstitution as WT A2 purified from baby hamster kidney cell-expressed factor VIIIa. All A2 variants exhibited modest if any increases in $K_D$ values for factor VIIIa assembly. However, variants S558A, V559A, D560A, G563A, and I566A showed >9-fold increases in $K_D$ for factor Xase assembly, implicating these residues in stabilizing A2 association with factor IXa. Furthermore, variants Y555A, V559A, D560A, G563A, I566A, and D569A showed >80% reduction in $k_{cat}$ for factor Xa generation. These results identify residues in the 558-loop critical to interaction with factor IXa in Xase.

Factor VIIIa is an essential blood coagulation protein that acts as a cofactor for the serine protease factor IXa in the conversion of factor X to Xa during the propagation phase of coagulation. Defects or deficiencies in factors VIII and IX result in hemophilia A and B, respectively. Factor VIII is synthesized as a multidomain, single chain precursor with a molecular mass of ~300 kDa and domain structure A1-A2-B-A3-C1-C2. It circulates primarily as a heterodimer resulting from proteolysis at the B-A3 junction wherein the heavy chain (A1A2B) and light chain (A3C1C2) are associated by metal ion-dependent and independent linkages (see Ref. 1 for review). Factor VIII is activated by limited proteolysis catalyzed by thrombin or factor Xa to the active cofactor, factor VIIIa. These cleavages convert the heterodimer or single chain precursor to the heterotrimer (2, 3) with the heavy chain-derived A1 subunit and light chain-derived A3C1C2 subunit maintaining stable association, whereas the A2 subunit is weakly associated with the A1/A3C1C2 dimer through electrostatic interactions (4, 5). Factor VIIIa can be reconstituted from the isolated A2 subunit and A1/A3C1C2 dimer to regenerate high levels of cofactor activity (3, 5).

Association of factors VIIIa and IXa on a phospholipid surface forms the intrinsic factor Xase complex, which increases the catalytic efficiency of factor IXa-catalyzed activation of factor X by >104 (6, 7). Fluorescence studies examining the active site of factor IXa have attributed this increase in $k_{cat}$ to the modulation of its active site by factor VIIIa (8), and in particular, the A2 subunit of the cofactor (9). Factor IXa in turn has been shown to stabilize factor VIIIa in the presence of Ca2+ and phospholipid (10) by promoting association of A2 subunit with A1/A3C1C2 (11, 12). This stabilization is derived from multiple interactions within the proteins. Studies involving monoclonal antibody targeted to the A3 domain and synthetic peptides suggest that residues 1811–1818 in that domain bind to the epidermal growth factor 1/2 regions of factor IXa (13). This interaction is of high affinity ($K_D$ ~ 15–50 nM) as determined by solid phase, non-equilibrium binding assay (14) or steady state fluorescence (15). Interaction between the A2 domain of factor VIIIa and the protease domain of factor IXa has been shown to be of low affinity ($K_D$ ~ 300 nM) (9). A number of studies involving fluorescence, peptide, or antibody inhibition and analyses of point mutations in the hemophilia A data base have identified several sequences involved in this interaction (see Ref. 16 for a review). One region in A2, the 558-loop was identified as a factor IXa-interactive site by both peptide inhibition studies (17) and by the capacity of factor IXa to selectively protect factor VIIIa from activated protein C-catalyzed cleavage at Arg-562 (18). More recently, Bajaj et al. (19) suggested that residues within the A2 558-loop bind to the 330-helix in factor IXa and modeled this interaction. According to this model, electrostatic, hydrophobic interactions and hydrogen bonds contribute to forming this interface. In an attempt to determine the role of the 558-loop toward cofactor activity of factor VIIIa, Jenkins et al. (20) expressed and purified selected B-domainless factor VIII variants with point mutations in this loop as defined from the hemophilia A data base. Functional assays showed that the mutations affected cofactor potential by altering the catalytic rate constant of factor IXa, however, affinity values of the factor VIII variants for factor IXa remained largely

---

* This work was supported, in whole or in part, by National Institutes of Health Grants HL76213 and HL38199. This work was presented at the 22nd Congress of the International Society on Thrombosis and Haemostasis in Boston, MA, July 15, 2009.

1 To whom correspondence should be addressed: P. O. Box 712, University of Rochester Medical Center, 601 Elmwood Ave., Rochester, NY 14642. Tel.: 585-275-6576; Fax: 585-275-6007; E-mail: Philip_Fay@urmc.rochester.edu.
unchanged, likely due to the primary factor IXa-binding interactions involving the A3C1C2 subunit of the cofactor.

In this study, we have investigated the contributions of residues 555–571 that border or are contained within the 558-loop region of factor VIIIa to inter-protein affinity and activity. To this end, we have reconstituted factor VIIIa and Xase complexes using baculovirus-expressed A2 domains (bA2), containing point mutations of each amino acid residue to Ala in the loop region, and A1/A3C1C2 dimer prepared from BHK cell-expressed factor VIIIa. The reconstituted complexes were evaluated for stabilization of A2 binding and Xa generating activity. With all the A2 variants we observed little or no change in $K_d$ values within reconstituted factor VIIIa compared with that of the WT A2. However, we have identified several residues including Ser-558, Val-559, Asp-560, Gly-563, and Ile-566 where Ala replacement variants resulted in marked (>9-fold) increases in $K_d$ within factor Xase complex, implicating a role for these residues in stabilizing A2 association with factor IXa. In addition, we have identified an overlapping set of residues including Tyr-555, Val-559, Asp-560, Gly-563, Ile-566, and Asp-569 whose Ala variants show >80% reduction in catalytic rates for factor Xa generation indicating a role for these residues in cofactor function.

**MATERIALS AND METHODS**

Reagents—Factor VIII was a kind gift from Lisa Regan of Bayer Corporation (Berkeley, CA). The plasmid xh-A2 (21) was kindly provided by Andrey Sarafanov and Evgueni Saenko (University of Maryland). Factors IXa, X, and Xa were obtained from Enzyme Research Laboratories, and the chromogenic substrate for factor Xa, Pefa-5523 (Pefachrome FXa) was purchased from Centerchem. Phospholipid vesicles containing 20% phosphatidylserine (PS), 40% phosphatidylcholine (PC), and 40% phosphatidylethanolamine (PE) were prepared using octyl glucoside as described previously (22). The A1/A3C1C2 dimer was purified by Mono S chromatography following activation of WT factor VIII by thrombin as described (23). The A2 domain-specific monoclonal antibody R8B12 was obtained from Green Mountain Antibodies (Burlington, VT).

**Construction and Expression of Recombinant A2 Domain and Variants**—The plasmid xh-A2 was modified using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as follows. The Gly residue at position 374 of the A2 domain in xh-A2 was replaced with Val, the authentic residue at this position (24). In addition, the factor Xa cleavage site in xh-A2 was deleted and a 7-amino acid AcTEV recognition site (Glu-Asn-Leu-Tyr-Phe-Gln-Ser) for cleavage by TEV was introduced at the N terminus of the A2 open reading frame between the His$_6$ tag and A2 sequence resulting in the plasmid designated pFastBac-wtA2. Cleavage of the TEV site occurs at the Gln-Ser bond and Ser was chosen for the seventh residue in the TEV site to be the first authentic N-terminal residue (residue 373) of the A2 domain. The His tag aided in purification of A2 and this tag was eliminated following cleavage by TEV to yield the WT A2 protein. Single alanine mutations were introduced in and around the 558-loop spanning residues 555 to 571 using the QuikChange site-directed mutagenesis kit. Baculovirus expressed A2 (bA2) was prepared from SF9 cells using the Bac-to-Bac Baculovirus Expression System (Invitrogen). Optimal transduction conditions were determined as described (21). The expression of bA2 was confirmed by Western blot analysis using the R8B12 antibody (25).

**Purification of bA2**—The secreted bA2 proteins were purified from culture medium. The supernatant from baculovirus-infected SF9 cells expressing the bA2 protein was loaded onto a 5-ml heparin column, washed with Buffer A (20 mM HEPES, pH 7.2, 150 mM NaCl, 0.01% Tween 20), bA2 was eluted with Buffer B (20 mM HEPES, pH 7.2, 800 mM NaCl, 0.01% Tween 20), and fractions (1 ml) were collected. The fractions containing bA2 were determined using SDS-PAGE, pooled, and loaded onto a 1-ml Talon column that was pre-equilibrated with Buffer C (20 mM HEPES, pH 7.2, 150 mM NaCl). The Talon column was first washed with 20 mM HEPES, pH 7.2, 1 mM NaCl, 10 mM imidazole followed by 20 mM HEPES, pH 7.2, 500 mM NaCl. bA2 was eluted with 20 mM HEPES, pH 7.2, 300 mM NaCl, 150 mM imidazole in 500–µl fractions. Fractions with bA2 were pooled based upon Bradford assay (26) and an equal volume of Buffer C was added. The TEV tag was cleaved from the protein with addition of AcTEV protease (1 µl/30 µg of protein) for 2 h at 25 °C to ensure complete cleavage of the tag. Removal of the protease and concentration of bA2 was accomplished using an SP-Sepharose column (0.6 ml) pre-equilibrated with 20 mM MES, pH 6.0, 0.01% Tween. The pooled protein was diluted 1:10 with the pre-equilibration buffer and loaded onto the column. The column was washed with Buffer A and bA2 was eluted with Buffer B in 200–µl fractions. The bA2 forms obtained were >90% pure as determined by SDS-PAGE. Fractions were pooled and concentrated using a MicroCon YM-10 (Millipore) as needed. The final concentrations were determined by Bradford assay and verified by running different amounts of the protein on SDS-PAGE gels with bovine serum albumin as the standard.

**Factor Xa Generation Assays**—The rate of conversion of factor X to Xa was determined by reconstituting factor Xa from purified A1/A3C1C2 (20 nM) and WT or variant bA2 (2–400 nM) in the presence of PS/PC/PE vesicles (10 µM) and limiting factor IXa (1 nM) by incubating the above components for 10 min at 25 °C. Alternatively, factor VIIIa was reconstituted by the addition of WT or variant bA2 (2–500 nM) to limiting A1/A3C1C2 (1 nM) for 10 min at 25 °C followed by addition of factor IXa (40 nM) and PS/PC/PE vesicles (10 µM) and further incubation for 1 min. Reactions were initiated with the addition of factor X (300 nM), and 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. Affinity values ($K_d$) for A2 association in the Xase complex were obtained under limiting factor IXa conditions, whereas the $K_d$ for A2 association in the factor VIIIa complex was obtained under limiting A1/A3C1C2 conditions. Rates of factor Xa generation were determined by the addition of the chromogenic substrate, Pefa-5523 (0.46 mM final concentra-

---

2 The abbreviations used are: bA2, A2 subunit expressed in insect cells using a baculovirus construct; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; WT, wild type; BHK, baby hamster kidney; MES, 4-morpholineethanesulfonic acid; TEV, tobacco etch virus.
Residues in the 558-Loop of A2 That Interact with Factor IXa

...tion). Reactions were read at 405 nm for 5 min using a sample microtiter plate reader (GE Healthcare).

**Competition Assays**—To determine $K_d$ values, various concentrations (50–500 nM) of bA2 variants showing impaired rates of factor Xa generation and/or affinity for factor IXa were added to reactions containing WT bA2 (80 nM). Factor VIIIa and Xase complexes were reconstituted, and factor Xa generation was performed as described above.

**Data Analysis**—Inter-protein affinity ($K_d$) and activity ($V_{max}$) parameters for factor Xa generation were calculated from initial rate data by fitting the data using non-linear least-squares regression analysis to a single site ligand binding model.

$$A = B_{max}[A2]/(K_d + [A2])$$

(Eq. 1)

Where $A$ is activity of factor Xase (nm min$^{-1}$ (nm factor IXa$^{-1}$)) or factor VIIIa (nm min$^{-1}$ (nm A1/A3-C1-C2$^{-1}$)). $[A2]$ is the A2 subunit concentration, $B_{max}$ is indicated by $V_{max}$, the maximum velocity, and $K_d$ is the dissociation constant for A2 in Xase or VIIIa.

The inhibition constant ($K_i$) for bA2 variants was determined from data fitted globally using non-linear least squares regression analysis according to a competitive inhibition model.

$$A = V_{max}[A2]/(K_d(1 + [I]/K_i) + [A2])$$

(Eq. 2)

Where $[I]$ is the concentration of the variant bA2, $[A2]$ is the concentration of WT bA2, $V_{max}$ is the maximum velocity for Xa generated, and $K_i$ is the dissociation constant for WT-bA2 in factor Xase or VIIIa.

RESULTS

**Construction, Purification, and Characterization of the bA2 Variants**—The A2 subunit of factor VIIIa interacts with the protease domain of factor IXa and modulates its active site, yet contributes only fractionally to the binding energy of the proteins in complex. To study contributions of the A2 subunit to binding and functional interactions within factor Xase, we made two modifications, as described under “Materials and Methods,” to an existing baculovirus A2 domain construct for its expression in insect cells. The first involved replacing the factor Xa cleavage site that liberates the His$_6$ tag from the A2 product with a site for TEV protease. This step was performed to ensure no residual factor Xa would carry over with purification of A2 inasmuch as this is the product of reaction catalyzed by factor Xase and could cloud experimental results. The second modification involved inserting the authentic residue (Gly at 374 to Val) from the A2 sequence near the N terminus of the A2. This was performed based on observations that the A2 subunit derived from the original construct showed ~10% of the activity as WT A2 following reconstitution analyses (21). WT and variant A2 subunits were expressed and purified using a baculovirus-directed construct from Sf9 cells as described under “Materials and Methods.” SDS-PAGE confirmed complete digestion of the TEV tag upon AcTEV protease treatment and >90% homogeneity was achieved for the purified bA2 proteins (Fig. 1).

**Reconstitutions of Xase with bA2 WT and 558-Loop Variants**—To evaluate the role of individual residues in and around the 558-loop in association within Xase, we individually mutated residues 555–571 that border or are contained within this loop region to Ala and expressed and purified the bA2 variants as described above. Analysis of WT and mutant bA2 forms used factor Xa generation assays following reconstitution of Xase using purified components. Reactions contained purified A1/A3C1C2 dimer (20 nM) from thrombin-treated BHK-factor VIII, limiting concentrations of factor IXa (1 nM), PS/PC/PE vesicles (10 μM), and the indicated concentrations of the individual bA2 subunits. Thus the bA2 subunit was the only varying component in Xase formation thereby eliminating any variability caused by differential activation of the procofactor forms. Reactions were initiated by addition of a $V_{max}$ concentration of factor X (300 nM). Because the amount of factor Xa generated is directly proportional to the concentration of reconstituted Xase, the concentration of A2 subunit required to yield maximal levels of factor Xa generation is an indicator of the affinity of the A2 subunit for Xase. Assays performed in the absence of bA2 did not generate detectable amounts of factor Xa (data not shown) indicating that A2 subunit is essential for cofactor activity. Factor Xase reconstituted with WT bA2 yielded similar affinity ($K_d = 5.3$ nM) and maximal activity (61.1 min$^{-1}$ nm factor IXa$^{-1}$) values to that formed using A2 subunit purified from thrombin-activated factor VIII purified from BHK cells ($K_d = 4.8$ nM; $V_{max} = 60.9$ min$^{-1}$ nm factor IXa$^{-1}$) (Fig. 2 and Table 1). This result indicated that bA2 was functionally equivalent to the A2 subunit purified from factor VIII produced in mammalian cells in generating cofactor activity, and that the baculovirus-expressed reagent was an appropriate model for examining the role of the A2 subunit in Xase formation. Furthermore, reconstitution reactions run for 30 min with WT bA2 yielded equivalent activity and resulted in equivalent $K_d$ values as reactions run for 10 min (data not shown), indicating the latter time was sufficient to achieve equilbrium.

Results showing rates of factor Xa generated as a function of A2 concentration for selected variants are shown in Fig. 2 with calculated parameter values shown in Table 1. Results for the remaining variants are not shown but parameter values are presented in Table 1. Mutations of residues Ser-558, Val-559, Asp-560, Gly-563, and Ile-566 to Ala resulted in ~10–15-fold...
Residues in the 558-Loop of A2 That Interact with Factor IXa

![Graph](image_url)

**FIGURE 2. Effects of alanine mutation of 558-loop residues on factor Xase reconstitution as measured by factor Xa generation assays.** Factor Xase was reconstituted with the indicated concentrations of A2 subunit, 20 nM A1/A3C1C2, 1 nM factor IXa, and 10 μM PS/PC/PE vesicles. Reactions were initiated with the addition of 300 nM factor X and initial rates of reactions are plotted as a function of A2 subunit concentration and fitted to a single site ligand binding equation by nonlinear least squares regression. The assays were performed at least three times and mean values with the standard deviations are shown. The variants are grouped based on similar $K_d$ values. Panel a, WT bA2, solid line, closed circles; factor VIIIa-derived A2, dashed line, closed triangles; Q565A, solid line, closed circles; R562A, solid line, closed diamonds; N564A, solid line, closed squares; and S558F, dashed line, closed circles. Panel b, Y555A, solid line, open triangles; V559A, solid line, open diamonds; D560A, solid line, open squares; D560K, solid line, open circles; G563A, dashed line, closed squares; 1566A, dashed line, closed diamonds; and D569A, dashed line, open triangles.

Increases in $K_d$ compared with WT, indicating a significant contribution of these residues toward A2 association within Xase (see Fig. 2b and Table 1). Furthermore, Ala variants of residues Tyr-555, Val-559, Asp-560, Gly-563, Ile-566, and Asp-569 yielded about 5–40-fold reductions in rates of factor Xa generation suggesting that these mutations variably impaired cofactor function. Among these, Y555A and D569A showed only moderate increases (3–5-fold) in $K_d$ suggesting that these residues act predominantly by modulating the cofactor activity and play a modest role in association of the A2 subunit with the cofactor with the stabilization of A2 conferred by Xase. In this series of experiments, factor VIIIa was reconstituted with various concentrations of bA2 (2 nM–500 nM) and limiting factor IXa relative to dimer (40 nM), and reactions were initiated using a $V_{max}$ concentration of factor X (300 nM). The amount of factor Xa generated in this case is directly proportional to the level of reconstituted factor VIIIa (Fig. 3) and was measured as described under “Materials and Methods.”

The affinity for WT bA2 within reconstituted factor VIIIa ($K_d = 30.1 \text{ nM}$) was found to be similar to that observed for A2 purified from BHK-expressed factor VIII (22.4 nM) (see Table 1) confirming that S9 purified WT bA2 functions similarly to the BHK cell-derived A2 subunit with respect to its intersubunit

function but not in binding interactions. Finally, similar affinity and activity parameters to WT bA2 observed for Ala variants for residues Arg-562, Asn-564, and Gln-565 indicated that these residues do not appear to play a significant role in the interactions of the 558-loop residues regarding cofactor binding or function.

Asp-560, a charged residue contained within the 558-loop, demonstrated a key role in both A2 association and cofactor activity of factor VIIIa within the Xase complex, as indicated by significant reductions in affinity and activity parameters upon mutation to Ala. To gain additional insights into the role of this residue, we tested the effect of charge reversal (Asp to Lys) on affinity and rate parameters. Reconstitution of Xase using the D560K A2 variant did not significantly alter the affinity of A2 with FIXa ($K_d = 40.1 \text{ nM}$) relative to the D560A mutation ($K_d = 70.3 \text{ nM}$) where the negative charge was neutralized (Fig. 2b). However, the charge reversal at this site decreased the rate of Xa generation by a further ~10-fold compared with the observed rate for Xase formed with D560A A2 (~40-fold relative to the WT bA2), suggesting that the Asp residue at this position modulates cofactor activity through a negative electrostatic interaction.

Reconstitution of Factor VIIIa

Reconstitution of Factor VIIIa was performed with factor VIIIa-derived A2 (2 nM–500 nM) and limiting factor IXa relative to dimer (40 nM), and reactions were initiated using a $V_{max}$ concentration of factor X (300 nM). The amount of factor Xa generated in this case is directly proportional to the level of reconstituted factor VIIIa (Fig. 3) and was measured as described under “Materials and Methods.”
interactions within the cofactor. Reconstitution reactions run for 30 min with WT bA2 yielded equivalent activity and resulted in equivalent $K_d$ values as reactions run for 10 min (data not shown). Furthermore, the $K_d$ for A2 in factor VIIIa was about 6-fold higher relative to that within the factor Xase complex. This observation is consistent with results from previous studies showing that interaction with factor IXa partially stabilizes association of the A2 subunit (11, 12). This effect was further verified by modifying the factor VIIIa reconstitution assay using WT bA2 to decrease or increase the duration of reaction of the reconstituted factor VIIIa with factor IXa prior to reaction with factor X. Reduction of the standard reaction time from 1 min to 30 s yielded a modest increase (~25%) in $K_d$, whereas increasing this reaction time to 20 min yielded a >3-fold reduction in this value (results not shown). These observations were consistent with the extended contact time with IXa allowing for a re-equilibration of reactants to approach the affinity value obtained with A2 for Xase.

Mutation of individual residues to Ala within and around the 558-loop resulted in no or a modest change on $K_d$ values as reactions run for 10 min (data not shown). Furthermore, the $K_d$ for A2 in factor VIIIa as compared with WT (Fig. 3a). These observations suggested that these residues may affect affinity by impacting the orientation and or structure of the loop. The Ala variants for residues Ser-568, Asp-569, Lys-570, and Arg-571 that border the C-terminal end of the loop region also showed ~3-fold decreases in $K_d$ values. These residues, therefore, might extend a modest contribution toward interaction of A2 within the cofactor. However, the remaining residues that border or are contained within the 558-loop showed little (~2-fold or less) effect compared with WT bA2 on inter-subunit affinity within factor VIIIa suggesting that these residues do not play a significant role in the association of A2 to A1/A3C1C2 in the absence of factor IXa.

Binding Competition between WT bA2 and Selected bA2 Variants—A series of experiments were performed in which selected high $K_d$, low activity Ala variants were used as competitors to WT bA2 in forming Xase to verify that the low activity observed for the variants was indeed due to impaired association of the variant in the presence of factor IXa. A2 variants employed for this analysis included Y555A, V559A, D560A, G563A, I566A, and D569A, which showed both marked increases in $K_d$ and decreases in activity relative to WT A2 in Xase. For these experiments factor Xase was reconstituted following the addition of purified A1/A3C1C2 dimer (20 nM), limiting factor IXa (1 nM), PS/PC/PE vesicles (10 μM), various amounts of variant A2 (50–500 nM), and WT bA2 (80 nM). Factor Xa was generated with the addition of factor X (300 nM) and the initial rates of the reactions were measured as described under “Materials and Methods.” In all cases, the rate of Xa generation decreased in a dose-dependent manner with the addition of the variant suggesting that the variant competed with WT bA2 for association within the factor Xase complex. Addition of a 6-fold excess of the variant led to inhibition of reaction rates that ranged from 40% for G563A to 60% for Y555A (Table 2 and Fig. 4a) after correcting for the contribution of the Ala variant to Xa generation rate. The apparent $K_i$ values estimated for the variants (Table 2) were found to be similar (within ~2-fold) to their $K_d$ values determined for formation of the Xase complex (Table 1). $K_i$ values for the Ala variants ranged from 5-fold higher than the $K_d$ for WT bA2 in the case of Y555A to 13-fold higher for G563A. These observations suggest that although the variants exhibiting low activity effectively compete with WT bA2 for binding within Xase, their capacity to functionally interact within this complex is significantly impaired compared with the WT A2.

The competition assay experiments were also performed under factor VIIIa reconstitution conditions as described above. The Ala mutants with low activity were added in various amounts (50–500 nM) to a fixed level of WT bA2 (80 nM). Factor VIIIa was reconstituted by adding limiting A1/A3C1C2 (1 nm) and factor Xa was generated following the addition of PS/PC/PE vesicles (10 μM), factor IXa (40 nM), and factor X (300 nM). The reaction rates in this case are proportional to the amounts of factor VIIIa generated and were observed to decrease with increasing amounts of the Ala variant added to the reconstitution mixture. With the addition of 6-fold excess of the mutant, inhibition was about 60% for the mutants except for D555A and D569A where the inhibition was about 75 and 80%, respectively (Fig. 4b). In general, the apparent $K_i$ values for these low activity mutants were similar to the inter-subunit $K_d$ values for WT bA2 suggesting that these residues did not appreciably contribute to inter-subunit affinity within factor VIIIa. The lone exception was the D569A variant, which showed an apparent $K_i$ about 4-fold lower than the $K_d$ of WT bA2. The reason for this disparity is not clear but may suggest that this residue contributes directly or indirectly to the inter-

### TABLE 1

| Residues in the 558-Loop of A2 That Interact with Factor IXa |

| A2 variant | Xase reconstitution$^a$ | VIIIa reconstitution$^b$ |
|------------|-------------------------|-------------------------|
|            | $K_d$ (nM) | $V_{\text{max}}$ (nM IXa/min) | $K_d$ (nM) | $V_{\text{max}}$ (nM IXa/min) |
| WT         | 5.3 ± 0.3 | 61.1 ± 0.7 | 30.1 ± 2.2 | 57.9 ± 1.3 |
| FVIII-A2    | 4.8 ± 0.4 | 60.9 ± 0.8 | 22.4 ± 1.6 | 50.4 ± 2.3 |
| Y555A       | 26.9 ± 1.5 | 29.9 ± 0.1 | 20.1 ± 1.5 | 21.1 ± 0.1 |
| K565A       | 18.2 ± 1.4 | 29.9 ± 0.6 | 39.8 ± 4.1 | 40.1 ± 1.1 |
| E557A       | 4.8 ± 0.6 | 26.1 ± 0.6 | 20.9 ± 2.3 | 35.0 ± 1.1 |
| S558A       | 73.4 ± 5.4 | 44.0 ± 1.4 | 85.4 ± 4.2 | 35.9 ± 0.6 |
| V559A       | 89.4 ± 9.9 | 11.9 ± 0.6 | 56.2 ± 1.3 | 3.5 ± 0.2 |
| D560A       | 70.1 ± 10.7 | 10.7 ± 0.7 | 6.3 ± 2.2 | 10.1 ± 0.4 |
| D560K       | 40.2 ± 5.1 | 1.5 ± 0.1 | 22.6 ± 5.0 | 35.0 ± 1.1 |
| Q561A       | 21.4 ± 1.8 | 41.3 ± 1.2 | 5.8 ± 7.7 | 34.2 ± 1.4 |
| R562A       | 9.9 ± 1.3 | 66.3 ± 2.3 | 31.1 ± 2.2 | 45.8 ± 1.3 |
| G563A       | 75.8 ± 1.4 | 3.1 ± 0.2 | 26.4 ± 1.2 | 6.2 ± 0.1 |
| N564A       | 10.6 ± 2.4 | 57.5 ± 0.6 | 17.6 ± 1.5 | 46.9 ± 1.0 |
| Q565A       | 8.5 ± 1.0 | 82.1 ± 0.5 | 8.8 ± 0.8 | 65.1 ± 1.3 |
| S566A       | 46.2 ± 2.5 | 3.4 ± 0.1 | 292.2 ± 2.8 | 125.3 ± 0.5 |
| M567A       | 30.0 ± 2.1 | 39.2 ± 0.9 | 67.2 ± 3.9 | 22.3 ± 0.5 |
| S568A       | 5.1 ± 0.8 | 31.9 ± 1.2 | 14.1 ± 1.6 | 25.9 ± 1.6 |
| D569A       | 14.9 ± 3.0 | 0.6 ± 0.1 | 11.7 ± 1.7 | 2.5 ± 0.1 |
| K570A       | 4.6 ± 0.4 | 23.1 ± 0.4 | 12.4 ± 2.2 | 40.5 ± 1.5 |
| R571A       | 4.6 ± 0.3 | 34.9 ± 0.4 | 11.9 ± 1.5 | 23.9 ± 0.7 |

$^a$ Reconstitution of factor Xase was performed using limiting factor IXa (1 nM).
$^b$ Reconstitution of factor VIIIa was performed using limiting A1/A3C1C2 dimer (1 nM).
Residues in the 558-Loop of A2 That Interact with Factor IXa

In this report we employed Ala-scanning mutagenesis using a baculovirus construct of the isolated A2 domain to identify critical residues within and bordering the 558-loop of factor VIIIa that function in factor Xase formation and activity. Reconstitution of factor Xase from purified components indicated that residues Ser-558, Val-559, Asp-560, Gly-563, and Ile-566, when replaced with Ala, resulted in >9-fold increases in the $K_d$ for A2 subunit in Xase. Because these mutations showed marginal if any effect on the inter-subunit affinity in factor VIIIa, these results indicate a role for these residues in stabilizing the interaction of factor VIIIa with factor IXa, likely through direct contact with the protease. In addition, an overlapping set of residues that includes Tyr-555, Val-559, Asp-560, Gly-563, Ile-566, and Asp-569 when mutated to Ala resulted in >80% reductions in $K_{cat}$ for factor Xa generation. This observation suggested that some contributions of 558-loop residues to cofactor function may be independent of direct binding to factor IXa.

The 558-loop is a highly conserved structure with a high degree of sequence identity in all known species of factor VIII (27). Early evidence based upon factor IXa-dependent protection from cleavage by activated protein C at Arg-562 (18), and activity inhibition by peptides and fluorescence analysis using isolated factor VIIIa subunits (17) indicated that this region of the A2 domain likely interacts with the protease domain of factor IXa. Other regions of A2, as well as the A3 and C2 domains of factor VIII light chain have also been implicated in interactions with factor IXa (see Ref. 1 for review).

Studies employing isolated factor VIIIa subunits show that the isolated A2 subunit possesses relatively weak affinity for factor IXa ($K_d \sim 300 \text{ nM}$) (9), whereas the light chain-derived A3C1C2 subunit shows a significantly higher affinity for the proteinase ($K_d \sim 15–50 \text{ nM}$) (14, 15).

Interactions of factor VIIIa within the Xase complex have been long known to stabilize the labile activity of the cofactor (10) by promoting the association of A2 subunit in this complex (11, 12, 28). However, little experimental evidence is available regarding specific factor VIIIa residues that contribute to this effect. In an earlier report from our laboratory, Jenkins et al. (20) examined several point mutations in the 558-loop follow-
Residues in the 558-Loop of A2 That Interact with Factor IXa

The approach taken in the current study clarifies and extends the role of the 558-loop residues in binding factor IXa inasmuch as isolated A2 domain variants are used for reconstitution studies rather than use of intact factor VIII variants. Thus inter-protein interactions can be discriminated from inter-subunit ones. Factor Xase reconstituted using bA2 and A2 derived from thrombin-activated, BHK-expressed factor VIII exhibited similar $K_d$ values thus validating our experimental design. Furthermore, the values for $K_d$ for A2 binding and $k_{cat}$ for Xa generation using these reagents were similar to the parameter values obtained in the above study (20) using factor Xase formed with thrombin-activated WT factor VIIIa in the presence of phospholipid vesicles.

Examination of residues significantly affecting both inter-protein affinity and cofactor function revealed critical roles for Val-559, Asp-560, Gly-563, and Ile-566 in the 558-loop. On the other hand, mutation at Ser-558 and Met-567 markedly affected inter-protein affinity with modest effects on cofactor function, whereas the converse was true for mutation at Tyr-555 and Asp-569. The importance of these residues in binding and/or activity was supported by examination of the hemophilia A data base. For instance, natural mutations V559A and D560A result in mild hemophilia phenotypes. Valine at position 559 might be involved in a crucial hydrophobic contact with factor IXa. Although mutation to alanine does not cause any major biochemical alteration, it might reduce the hydrophobicity necessary to maintain this interaction due to fewer methyl groups. Neutralization of charge at position 560 resulted in 14-fold higher $K_d$ and 6-fold lower activity relative to the WT Asp. Furthermore, reversal of charge at Asp-560 to Lys, a positively charged residue, reduced the cofactor activity another 10-fold compared with D560A indicating that this Asp residue might be part of a key electrostatic interaction in Xase function. The decreased activities of mutations at Ile-566 and Asp-569 are suggested by the severe hemophilia A phenotypes. Valine at position 559 might be involved in a crucial hydrophobic contact with factor IXa.
contribution of Glu-557 toward cofactor activity as evident by ~2-fold reduced \( k_{\text{cat}} \) for the E557A variant compared with WT A2 is consistent with the mild hemophilia A phenotype resulting from reversal of charge at Glu-557 to Lys. This observation suggests that the Glu residue at 558 might be involved in electrostatic interaction with factor IXa, although this interaction might be of less significance to the formation of factor Xase complex. Absence of any mutation at residue Arg-562 associated with hemophilia A is consistent with our observation that Ala substitution at this residue does not affect activity and affinity parameters and therefore, the Arg-562 (side chain) does not seem to contribute to interaction with factor IXa. Although Q565A showed similar \( K_d \) and \( k_{\text{cat}} \) values to WT A2, mutations of this residue to positively charged amino acids such as His, Lys, and Arg have been reported to cause mild to severe hemophilia A. It can be inferred that whereas this Gln is not an essential residue, introduction of a positively charged residue at this position might be deleterious for interaction with the protease. A mutation at Asn-564 to Ser results in mild hemophilia A. It can be inferred that whereas this Gln is not an essential residue, introduction of a positively charged residue at this position might be deleterious for interaction with the protease. Based upon experimental data supporting a direct interaction of the 558-loop of A2 with the 330-helix of factor IXa, Bajaj et al. (19) proposed a model for this interface between the protease domain and the A2 subunit following docking of these structures. In the model, Asp-560 is suggested to electrostatically interact with Arg-338 (Arg-170 using the chymotrypsin number system) of factor IXa. Furthermore, A2 residues Ile-566 along with Met-567 form part of the significant hydrophobic patch with factor IXa residues Ile-298, Tyr-295, Phe-299, Phe-302, Phe-378, and Phe-98 at the interface. The model further proposes interaction of Lys-570 of A2 with Asn-346 of factor IXa correlating with the modest contribution extended by Lys-570 toward cofactor activity as evident from the lower \( k_{\text{cat}} \) value for K570A. Finally, the model predicts residues Arg-562 and Gln-561 of A2 interact with Asp-332 of IXa. Although the Q561A variant yielded a severalfold increase in \( K_d \) for A2 in Xase, the R562A variant showed a WT-like \( K_d \), suggesting any role for Arg-562 may be related to backbone interactions rather than its side chain. Bonding interactions for several residues, in particular Ser-558, Val-559, and Gly-563 that when mutated show significant increases in the inter-protein \( K_d \) are not identified in this model, suggesting additional refinement of the model may be in order. Bonding interactions between the 558-loop and 330-helix are also supported by a recent in silico model docking factors VIIIa and IXa on a membrane surface (30). However, interactions of specific residues within these structures are not identified. This information will require results obtained following co-crystallization of the cofactor and proteinase.

Acknowledgments—We thank Evgueni Saenko and Andrey Sarafanov for the generous gift of the xh-A2 vector and helpful discussions related to the expression of hA2. We also thank Lisa Regan for the gift of recombinant factor VIII (Kogenate), and Sandeep Chadha and Amy Griffiths for excellent technical assistance.

REFERENCES

1. Fay, P. J. (2004) Blood Rev. 18, 1–15
2. Lollar, P., and Parker, C. G. (1989) Biochemistry 28, 666–674
3. Fay, P. J., Haidaris, P. J., and Smudzin, T. M. (1991) J. Biol. Chem. 266, 8957–8962
4. Lollar, P., and Parker, C. G. (1990) J. Biol. Chem. 265, 1688–1692
5. Fay, P. J., and Smudzin, T. M. (1992) J. Biol. Chem. 267, 13246–13250
6. van Dieijen, G., Tans, G., Rosing, J., and Hemker, H. C. (1981) J. Biol. Chem. 256, 3433–3442
7. Duffy, E. J., and Lollar, P. (1992) J. Biol. Chem. 267, 7821–7827
8. Mutucumaran, V. P., Duffy, E. J., Lollar, P., and Johnson, A. E. (1992) J. Biol. Chem. 267, 17012–17021
9. Fay, P. J., and Koshibu, K. (1998) J. Biol. Chem. 273, 19049–19054
10. Lollar, P., Knutson, G. J., and Fass, D. N. (1984) Blood 63, 1303–1308
11. Lamphire, B. J., and Fay, P. J. (1992) J. Biol. Chem. 267, 3725–3730
12. Fay, P. J., Beattie, T. L., Regan, L. M., O’Brien, L. M., and Kaufman, R. J. (1996) J. Biol. Chem. 271, 6027–6032
13. Lenting, P. J., van de Loo, J. W., Donath, M. J., van Mourik, J. A., and Mertens, K. (1996) J. Biol. Chem. 271, 1935–1940
14. Lenting, P. J., Donath, M. J., van Mourik, J. A., and Mertens, K. (1994) J. Biol. Chem. 269, 7150–7155
15. Jenkins, P. V., Dill, J. L., Zhou, Q., and Fay, P. J. (2004) Biochemistry 43, 5094–5101
16. Fay, P. J., and Jenkins, P. V. (2005) Blood Rev. 19, 15–27
17. Fay, P. J., Beattie, T., Huggins, C. F., and Regan, L. M. (1994) J. Biol. Chem. 269, 20522–20527
18. Regan, L. M., Lamphire, B. J., Huggins, C. F., Walker, F. J., and Fay, P. J. (1994) J. Biol. Chem. 269, 9445–9452
19. Bajaj, S. P., Schmidt, A. E., Mathur, A., Padmanabhan, K., Zhong, D., Matri, M., and Fay, P. J. (2001) J. Biol. Chem. 276, 16302–16309
20. Jenkins, P. V., Freas, J., Schmidt, K. M., Zhou, Q., and Fay, P. J. (2002) Blood 100, 501–508
21. Sarafanov, A. G., Makogonenko, E. M., Pechik, I. V., Radtke, K. P., Khrenov, A. V., Ananyeva, N. M., Strickland, D. K., and Saenko, E. L. (2006) Biochemistry 45, 1829–1840
22. Mimms, L. T., Zampighi, G., Nozaki, Y., Tanford, C., and Reynolds, J. A. (1981) Biochemistry 20, 833–840
23. Lapan, K. A., and Fay, P. J. (1997) J. Biol. Chem. 272, 2082–2088
24. Veber, G. A., Keyt, B., Eaton, D., Rodriguez, H., O’Brien, D. P., Rotblat, F., Oppermann, H., Beck, K., Wood, W. L., Harkins, R. N., Tuddenham, E. G. D., Lawn, R. M., and Capon, D. J. (1984) Nature 312, 337–342
25. Newell, J. L., and Fay, P. J. (2009) J. Biol. Chem. 284, 11080–11089
26. Bradford, M. M. (1976) Biochemistry 72, 248–254
27. Kembal-Cook, G., Tuddenham, E. G., and Wacey, A. I. (1998) Blood Rev. 12, 205–219
28. Lollar, P., and Parker, C. G. (1989) Biochemistry 28, 666–674
29. Fay, P. J., Smudzin, T. M., and Walker, F. J. (1991) J. Biol. Chem. 266, 12481–12486
30. Ngo, J. C., Huang, M., Roth, D. A., Furie, B. C., and Furie, B. (2008) Structure 16, 597–606