Factor VIII A3 Domain Residues 1954−1961 Represent an A1 Domain-Interactive Site†

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ABSTRACT: Factor VIIIa consists of subunits designated A1, A2, and A3C1C2. Reassociation of the A1 and A3C1C2 subunits monitored by the factor Xa generation assay and fluorescence resonance energy transfer yielded intersubunit affinity values (K_d) of 58.0 ± 12.5 and 58.8 ± 16.8 nM, respectively. These affinity values were equivalent to that previously determined for factor VIII heavy and light chains [Wakabayashi, H., et al. (2001) *Biochemistry 40*, 10293−10300], suggesting that the A2 domain makes a minimal contribution to the interchain affinity in factor VIII. Ca^{2+} showed no effect on A1/A3C1C2 intersubunit affinity (K_d = 51.6 ± 16.6 nM), while Cu^{2+} enhanced the A1/A3C1C2 intersubunit affinity ~5-fold (K_d = 12.5 ± 2.3 nM). A synthetic peptide to A3 domain residues 1954−1961 inhibited association of the A1 and A3C1C2 subunits (K_i = 65.8 ± 11.9 μM). Three factor VIII point mutants, His1957Ala, Gly1960Val, and His1961Asp, were stably expressed in BHK cells and purified. All mutants exhibited reduced specific activity (39, 42, and 4%, respectively) compared with that of wild-type factor VIII, and their activity was less stable following heat denaturation analysis ($t_{1/2}$ values of 13.3 ± 0.7, 8.7 ± 0.3, and 8.1 ± 0.1 min, respectively) compared with that of the wild type (18.8 ± 0.8 min). This reduced stability appeared to result from an ~2-fold increased dissociation rate for the Gly1960Val and His1961Asp dimers as judged by solid-phase binding assays. We propose that residues 1954−1961 of the A3 domain contribute to interactions with the A1 domain, facilitating their association in factor VIII.

The intrinsic factor Xase, a complex composed of serine protease factor IXa and the activated form of factor VIII (factor VIIIa) assembled on a phospholipid surface, catalyzes the conversion of zymogen factor X to serine protease factor Xa, a reaction critical for the propagation phase of the coagulation cascade (*1*−*3*). Factor VIIIa participates as a cofactor in the intrinsic Xase complex where it increases the catalytic efficiency of factor IXa toward factor X by several orders of magnitude (see ref *4* for a review). The hereditary bleeding disorder hemophilia A, a result of a defect or deficiency in plasma protein factor VIII, is characterized by a marked reduction of factor Xase activity and subsequently diminished levels of factor Xa generated.

Factor VIII circulates in plasma as a heterodimeric structure composed of a heavy chain (HC)[1] in the A1A2B domain arrangement and a light chain (LC) represented by the A3C1C2 domains (*5*, *6*). The HC is minimally represented by the A1 and A2 domains and associates with the LC via metal ion-dependent and -independent linkages (*7*−*9*). A single copper ion has been identified in factor VIII and is suggested to contribute to this linkage (*10*, *11*). Thrombin-catalyzed cleavage of factor VIII at the A1−A2 and A2−B domainal junctions and at the N-terminus of the A3 domain generates the active cofactor form, factor VIIIa (*12*). The resulting factor VIIIa molecule is a heterotrimer, with the A1 and A3C1C2 subunits maintaining the metal ion-dependent interaction, and the A2 subunit associating with this dimer via electrostatic interactions (*13*, *14*). Factor VIII (VIIIa) is inactivated by EDTA which facilitates the dissociation of the heterodimeric (heterotrimeric) structures of the protein as well as chelates metal ions that are critical to the active conformation (*6*, *15*, *16*). However, factor VIII (VIIIa) activity can be reconstituted by combining the isolated subunits in the presence of Ca^{2+} (*9*, *17*). The reassociation of the factor VIII HC and LC to form a functional heterodimer has been studied recently (*9*). However, the mechanism of intersubunit association in factor VIIIa remains unclear.

In this study, we evaluate subunit association in factor VIIIa using fluorescence energy transfer and activity assays, and site-directed mutation analysis. Affinity parameter values that were determined suggest that the A1 and A3C1C2 domains mediate the bulk of intersubunit binding energy in factor VIII with the A2 domain making little if any contribution. Ca^{2+} appears to have no effect on A1/A3C1C2

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[1] Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; BSA, bovine serum albumin; HC, factor VIII heavy chain; LC, factor VIII light chain; Ac-A3C1C2, acrylodan-labeled A3C1C2 subunit; Fl-A1, fluorescein 5-maleimide-labeled A1 subunit; BHK, baby hamster kidney.

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intersubunit affinity, whereas Cu^{2+} enhances the A1/A3C1C2 intersubunit affinity ~5-fold. Results from peptide and site-directed mutant analysis suggest that residues 1954−1961 of the A3C1C2 subunit contribute to the interaction with the A1 subunit in factor VIII(a).

MATERIALS AND METHODS

Reagents. Recombinant factor VIII (Kogenate) was a gift from L. Regan of Bayer Corp. (Berkeley, CA). The synthetic peptides representing A3C1C2 subunit residues 1716−1724 (HVLRNRAQS), 1954−1961 (HSIHFSGH) and its histidine to alanine substituted form (ASIAFSGA), and 1966−1977 (RKKEEYKMALYN) were obtained from BioSource (Camarillo, CA). Peptides were >90% pure as judged by HPLC. Phospholipid vesicles containing 20% PS, 40% PC, and 40% PE (Sigma, St. Louis, MO) were prepared using *N*-octyl glucoside (*18*). Factor VIII-deficient plasma was prepared as previously described (*19*). Activated partial thromboplastin was purchased from General Diagnostics Organon Teknika. The anti-factor VIII monoclonal antibody R8B12 (*14*), which recognizes the COOH-terminal region of the A2 domain, was obtained from Green Mountain Antibodies. The anti-factor VIII monoclonal antibody ESH-8, which recognizes the C2 domain of the light chain, was obtained from American Diagnostica Inc. The B-domainless factor VIII expression vector (RENeo factor VIII) and Bluescript cloning vector (pBS factor VIII) were kindly provided by P. Lollar and J. Healey. All of the reagents used for the BHK cell culture were obtained from Invitrogen. The reagents α-thrombin, factor IXa, factor X, and factor Xa (Enzyme Research Laboratories, South Bend, IN), hirudin (VWR), and the chromogenic Xa substrate S-2765 (*N*-α-benzoyloxycarbonyl-D-arginyl-glycyl-L-arginyl-*p*-nitroanilide dihydrochloride; DiaPharm Group, Westchester, OH) were purchased from the indicated vendors. Fluorescein 5-maleimide and acrylodan were obtained from Molecular Probes (Eugene, OR).

Isolation of Factor VIIIa Subunits. Isolation of factor VIIIa subunits was performed as described previously (*16*) with minor modifications. Subunits were dialyzed into buffer containing 20 mM HEPES, 0.01% Tween 20, 0.1 mM EDTA (pH 7.2), and either 100 nM (A2), 300 nM (A1), or 400 nM (A3C1C2) NaCl and stored at −80 °C. The A1/A3C1C2 dimer was isolated from thrombin-treated factor VIII using Mono-S column chromatography, and the residual A2 subunit was removed using an anti-A2 subunit monoclonal antibody coupled to Affi-Gel 10 as previously described (*20*).

Assays. Factor VIII cofactor activity was determined using a factor Xa generation assay (*21*). A1 and A3C1C2 were mixed at the indicated concentrations in 20 mM HEPES (pH 7.2), 300 mM NaCl, 25 mM $CaCl_2$, and 0.01% Tween 20 at 4 °C overnight. The mixture was diluted 10-fold, and factor VIIIa was formed by the addition of indicated amounts of the A2 subunit in 20 mM HEPES (pH 7.2), 5 mM $CaCl_2$, 50 mM NaCl, 0.01% Tween 20, 100 μg/mL BSA, and 10 μM PSPCPE vesicles. Factor IXa (50 nM) was added to the resultant factor VIIIa, and time course reactions were initiated with the addition of 300 nM 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 the addition of the chromogenic substrate, S-2765 (0.46 mM final concentration). Reactions were read at 405 nm using a Vmax microtiter plate reader (Molecular Devices, Sunnyvale. CA). All reactions were carried out at room temperature. Data points represent means of three separate determinations.

Factor VIII activity measured in a one-stage clotting assay was performed using substrate plasma chemically depleted of factor VIII as previously described (*22*).

Factor VIII Mutagenesis, Expression, and Purification. B-Domainless factor VIII cDNA was excised from the RENeo factor VIII expression construct using endonucleases XhoI and NotI and cloned into the pBS factor VIII vector. Mutations were introduced into the shuttle construct using the Stratagene QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as described previously (*22*). The point mutations, His1957Ala, Gly1960Val, and His1961Asp, were separately constructed. The presence of only the desired mutation was confirmed by DNA sequence analysis. The mutated factor VIII cDNA was then excised and ligated back into the RENeo expression vector and subjected to a second round of sequence analysis to confirm the presence of only the desired mutation.

The factor VIII expression constructs were transfected into BHK cells by liposome-mediated transfection. The selection, subcloning, and cloning of stable transfectants were carried out by standard methods, and the cloned cells were cultured in roller bottles (*22*). The conditioned medium was collected daily, and the expressed factor VIII protein was purified following chromatography on a SP-Sepharose column as described previously (*22*). Active fractions were detected using a one-stage clotting assay. The level of factor VIII protein was measured by a sandwich ELISA using the LC antibody ESH-8 (10 μg/mL) as a capture antibody and the HC antibody biotinylated R8B12 (3 μg/mL) as a detection antibody as previously described (*22*). Resultant factor VIII molecules were typically >80% pure as judged by SDS−PAGE with albumin representing the major contaminant. Factor VIII samples were stored at −80 °C.

Labeling of Subunits. A1 and A3 domains of factor VIII each possess a free sulfhydryl group (*23*). The thiol-specific fluorophores fluorescein 5-malemide (excitation and emission wavelengths of 490 and 515 nm, respectively) and acrylodan (excitation and emission wavelengths of 395 and 500 nm, respectively) were coupled to the A1 and A3 domains, respectively, using the following conditions. The A1 subunit in 20 mM HEPES (pH 7.2), 100 mM NaCl, 5 mM $CaCl_2$, and 0.01% Tween 20 was reacted with a 50-fold molar excess of fluorescein overnight at 4 °C in the dark. The A3C1C2 subunit, in the buffer described above containing 400 mM NaCl, was similarly reacted with a 10-fold molar excess of acrylodan. Exhaustive dialysis was performed to remove unbound fluorophores.

Fluorescence Resonance Energy Transfer. Fifty nanomolar A3C1C2 (or Ac-A3C1C2) and 0−300 nM A1 (or Fl-A1) were reconstituted in the presence or absence of metal ions overnight at 4 °C in 20 mM HEPES (pH 7.2), 300 mM NaCl, 0.01% Tween 20, and 200 μg/mL BSA. Fluorescence measurements were performed using an Aminco-Bowman Series 2 spectrometer at room temperature at an excitation wavelength of 395 nm and a bandwidth of 4 nm. Emission fluorescence was measured at 420−550 nm, and all spectra were corrected for background. The percentage of donor

fluorescence quenching as a result of subunit reassociation was calculated from integrated fluorescence intensities at λ values of 460−490 nm. The relative fluorescence was calculated as follows:

$$F(\%) = [(F_{DA} - F_A)/F_D] \times 100 \quad (1)$$

where F_{DA} is the fluorescence intensity of the labeled donor and the labeled acceptor (Ac-A3C1C2−Fl-A1), F_A is the fluorescence intensity of the unlabeled donor and the labeled acceptor (A3C1C2−Fl-A1), and F_D is the fluorescence intensity of the labeled donor and the unlabeled acceptor (Ac-A3C1C2−A1). Triplicate values were obtained for each sample and two independent analyses performed.

Functional Stability Assay. Wild-type or mutant factor VIII (50 nM) was incubated at 55 °C, and at the indicated times, an aliquot was diluted 10-fold into a factor Xa generation reaction mixture. Factor VIII was activated in the presence of 5 nM factor IXa and 10 μM PSPCPE vesicles by the addition of 10 nM thrombin, and after 1 min, thrombin was inhibited by the addition of hirudin (2.5 units/mL). The conversion of factor X to factor Xa was initiated by addition of 300 nM factor X, and rates of factor Xa generation were determined as described above. Data points represent means of three separate determinations.

ELISA-Based Stability Assay. Wild-type or mutant factor VIII (50 nM) was incubated at 50 °C, and at the indicated times, an aliquot was diluted 20-fold into phosphate-buffered saline (pH 7.4) and applied to a sandwich ELISA assay to quantitate the amount of factor VIII heterodimer. The amount of factor VIII heterodimer was measured by an ELISA using the LC antibody, ESH-8 (10 μg/mL), as a capture antibody and the HC antibody, biotinylated R8B12 (3 μg/mL), or the LC antibody, biotinylated 10104 (0.25 μg/mL), as a detection antibody as previously described (*22*).

Data Analysis. Analysis of the interaction between A1 and A3C1C2 subunits using the factor Xa generation assay was performed using the following equation by nonlinear least-squares regression:

$$A = k[L_0 + H_0 + K_d - \sqrt{(L_0 + H_0 + K_d)^2 - 4L_0H_0}] \quad (2)$$

where A represents activity (% of maximal activity), L_0 is the A3C1C2 concentration, H_0 is the A1 concentration, K_d represents the dissociation constant, and k is a constant.

Analysis of the interaction between A1 and A3C1C2 subunits using fluorescence resonance energy transfer was performed using the following equation by nonlinear least-squares regression:

$$F = 100 - k[L_0 + H_0 + K_d - \sqrt{(L_0 + H_0 + K_d)^2 - 4L_0H_0}] \quad (3)$$

where F represents the relative fluorescence (%), L_0 is the A3C1C2 concentration, H_0 is the A1 concentration, K_d represents the dissociation constant, and k is a constant.

Analysis of the inhibition of the interaction between A1 and A3C1C2 subunits by synthetic peptides using the factor Xa generation assay was performed using the following equation by nonlinear least-squares regression:

$$Y = CL_0/[(1 + I_0/K_i)K_d + L_0] \quad (4)$$

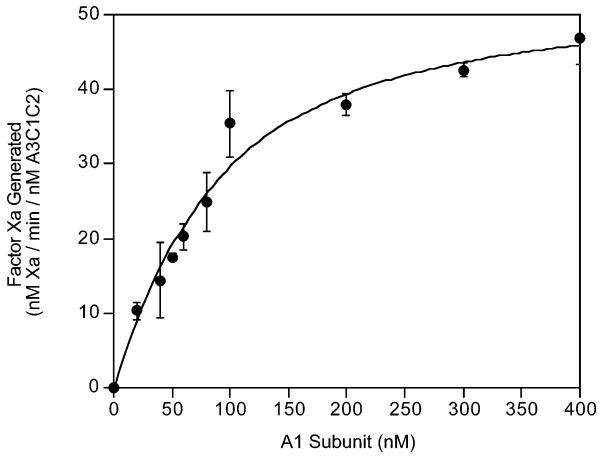

FIGURE 1: Affinity of A1/A3C1C2 interaction determined by the activity assay. Factor VIIIa was reconstituted from 50 nM A3C1C2 and the indicated amounts of A1 followed by addition of a saturating level of A2 (500 nM) and assayed for cofactor activity as described in Materials and Methods. Data were fitted to the equilibrium binding model described in Materials and Methods.

where Y represents activity (% of initial), L_0 is the A3C1C2 concentration, K_i is the dissociation constant for the A1 subunit−peptide complex, K_d is the dissociation constant for the A1/A3C1C2 dimer, I_0 represents inhibitor concentration, and C is a constant. Nonlinear least-squares regression analysis was performed using the above equation. Analysis of the intramolecular stability of the various forms of expressed factor VIII molecules using the functional and ELISA-based assays was performed using the following equations:

$$A = A_0 \times \exp(-10^{-C} \times t) \quad (5)$$

where A represents activity or HC bound (% of initial), A_0 is the activity or HC bound at initial time point, t is time, and C is $-\log k$, where k is the rate constant.

$$t_{1/2} = 10^C \times \ln(2) \quad (6)$$

where $t_{1/2}$ represents the half-life of exponential decay and C is defined as in eq 5. Parameter values (A and C) and their standard deviations were estimated by nonlinear least-squares regression analysis using eq 5. Half-life ($t_{1/2}$) values were obtained using eq 6. The Student's t-test was performed on the average value for C.

RESULTS

Association of Factor VIIIa Subunits. While residues in the A1 domain clearly participate in the association of the HC with the LC, on the basis of the generation of a stable A1/A3C1C2 dimer following thrombin activation (*14*), the contribution of the A2 domain to this intersubunit interaction-(s) is not known. A comparison of the K_d for A1/A3C1C2 interaction with that for HC−LC interaction was undertaken to provide insights into the extent of the contribution of the A2 domain to the affinity of the HC for the LC. To estimate the K_d for A1/A3C1C2 interaction, varying amounts of the A1 subunit and 50 nM A3C1C2 were reacted in the presence of 25 mM Ca^{2+} and saturating amounts of the A2 subunit (500 nM) in a factor Xa generation assay as described in Materials and Methods. The results are shown in Figure 1.

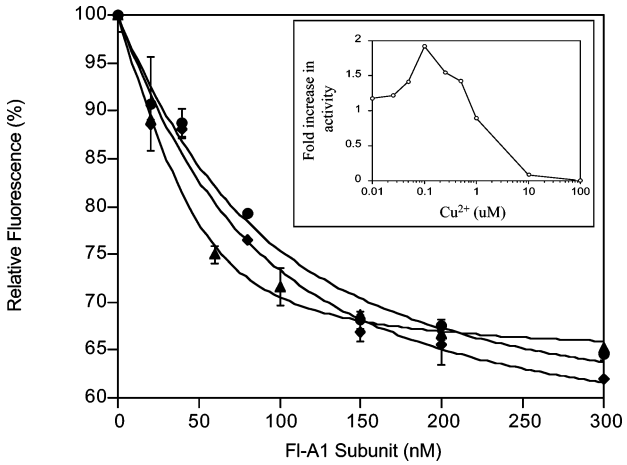

FIGURE 2: Subunit reassociation measured as a function of relative fluorescence. Ac-A3C1C2 (50 nM) and varying levels of the Fl-A1 subunit in the absence of exogenous metal ions (●) or presence of Cu^{2+} (0.1 μM) (▲) or Ca^{2+} (25 mM) (◆) were reacted overnight at 4 °C. The fluorescence of Ac-A3C1C2 was measured at λ values of 460−490 nm. The relative fluorescence (%) represents the ratio of Ac-A3C1C2 fluorescence intensity in the presence of the Fl-A1 to that of Ac-A3C1C2 alone, and was plotted as a function of Fl-A1 concentration and fitted by nonlinear least-squares regression to the formula shown in Materials and Methods. The inset shows the effect of Cu^{2+} on A1/A3C1C2 activity. Indicated amounts of Cu^{2+} were titrated into reconstitution mixtures containing A1 and A3C1C2 subunits (250 nM each). After overnight incubation, the A2 subunit (100 nM) was added to form factor VIIIa. Cofactor activity of the reconstituted factor VIIIa was measured in a factor Xa generation assay as described in Materials and Methods.

The A1/A3C1C2 interaction occurred with estimated V_{max} and K_d values of 53.4 ± 3.4 min^{-1} and 58.0 ± 12.5 nM, respectively. The estimated affinity parameter is similar to that determined for HC−LC association in the presence of 25 mM Ca^{2+} (K_d = 48.7 ± 15.4) using the fluorescence energy transfer assay (*9*). This result suggests that the A1 and A3C1C2 domains may mediate the bulk of intersubunit binding energy in factor VIII.

Association of Factor VIIIa Subunits in the Absence or Presence of Metal Ions. To further assess the mechanism of factor VIIIa subunit association, fluorescein 5-maleimide-labeled A1 and acrylodan-labeled A3C1C2 subunits were prepared as described in Materials and Methods. Activity of the labeled subunits was determined following reconstitution of factor VIIIa from Fl-A1, Ac-A3C1C2 subunits, and saturating amounts of the unlabeled A2 subunit. Factor VIIIa reconstituted from Fl-A1 and Ac-A3C1C2 possessed >60% of the activity of factor VIIIa prepared from unlabeled subunits as judged by the FXa generation assay (data not shown), indicating the subunits retained significant activity upon fluorophore incorporation.

The affinity of isolated Fl-A1 for isolated Ac-A3C1C2 was measured using fluorescence resonance energy transfer. The extent of donor (acrylodan) fluorescence quenching was used as an indicator of Ac-A3C1C2 and Fl-A1 binding, with appropriate unlabeled controls (Figure 2 and Table 1). The affinity value estimated for A1/A3C1C2 interaction (K_d = 58.8 ± 16.8 nM) in the absence of exogenous metal ions was equivalent to the value for HC−LC interaction (53.8 ± 14.2 nM) determined under similar conditions (*9*). Evaluation of the thermodynamic stability values (*24*) indicated similar values for both HC−LC and A1/A3C1C2 interactions, suggesting little if any contribution of the HC A2 domain to the binding affinity for the LC, assuming no gross changes in the A1/A3C1C2 interactions following activation of factor VIII by thrombin.

Table 1: Affinity and Fluorescence Energy Transfer Parameters for A1/A3C1C2 Interactions[a]

conditions	K_d[b] (nM)	RF_{max}[c] (%)
−	58.8 ± 16.8	65 ± 0.5
25 mM Ca^{2+}	51.6 ± 16.6[d]	62 ± 1
0.1 μM Cu^{2+}	12.5 ± 2.3[e]	65 ± 1

[a] Reactions were performed as described in Materials and Methods. [b] K_d values ± standard deviations were estimated by nonlinear least-squares regression of the data shown in Figure 2. [c] Relative fluorescence values of Ac-A3C1C2 were determined at a saturating Fl-A1 concentration. [d] $p > 0.5$ compared with that of the A1/A3C1C2 interaction in the absence of metal ions. [e] $p < 0.02$ compared with that of the A1/A3C1C2 interaction in the absence of metal ions.

Association of Fl-A1 and Ac-A3C1C2 subunits in the presence of 25 mM Ca^{2+} yielded a K_d of 51.6 ± 16.6 nM (Figure 2 and Table 1). This value was similar to that obtained for reactions carried out in the absence of exogenous metal ions (58.8 ± 16.8 nM), suggesting that Ca^{2+} does not influence the intersubunit affinity in factor VIIIa.

Earlier experiments identified the presence of a single copper ion [Cu^+ (*11*)] in factor VIII (*10*). Cu^{2+} has been shown to effectively substitute for Cu^+ in enhancing factor VIII reconstitution (*25*) given the tendency of Cu^+ to oxidize. The concentration of Cu^{2+} (0.1 μM) yielding maximal reconstituted factor VIIIa activity (Figure 2 inset) was assessed for its effect on the K_d for the A1/A3C1C2 interaction. Compared with the absence of exogenous metal ions, the presence of Cu^{2+} yielded an ~5-fold increase in A1 and A3C1C2 affinity (K_d = 12.5 ± 2.3 nM) (Figure 2 and Table 1).

Effect of A3 Domain Peptides on A1/A3C1C2 Interaction. The ceruloplasmin-based factor VIII homology model of Pemberton and colleagues (*26*) provides a useful tool for exploring various predictions about the gross features of factor VIII, such as inter- and intrasubunit interactions. Examination of the A1−A3 domainal interface suggests a number of prospective intersubunit interactive sites. With a goal of identifying residues within the A1 and A3 domains contributing to intersubunit association, we examined the following A3 domain segments, residues 1716−1724 (HVLRNRAQS), 1954−1961 (HSIHFSGH), and 1966−1977 (RKKEEYKMALYN), that appeared to be closest to the A1 domain based on the model. Synthetic peptides corresponding to the above A3 domain residues were prepared, and their ability to inhibit reconstitution of factor VIIIa activity was assessed as follows. Each peptide was titrated into a solution containing A1 (250 nM) and A3C1C2 (250 nM) subunits, and the mixtures were incubated overnight at 4 °C. Factor VIIIa was subsequently reconstituted by the addition of 100 nM A2 subunit and cofactor activity determined as described in Materials and Methods. The results are shown in Figure 3. A3 domain peptides 1716−1724 and 1966−1977 exhibited no inhibitory effect on the reconstitution of factor VIIIa activity, while A3 domain peptide 1954−1961 inhibited regeneration of cofactor activity with an estimated K_i value of 65.8 ± 11.9 μM.

To ensure that the inhibition observed was specific to the A1/A3C1C2 interaction and not a result of perturbing other

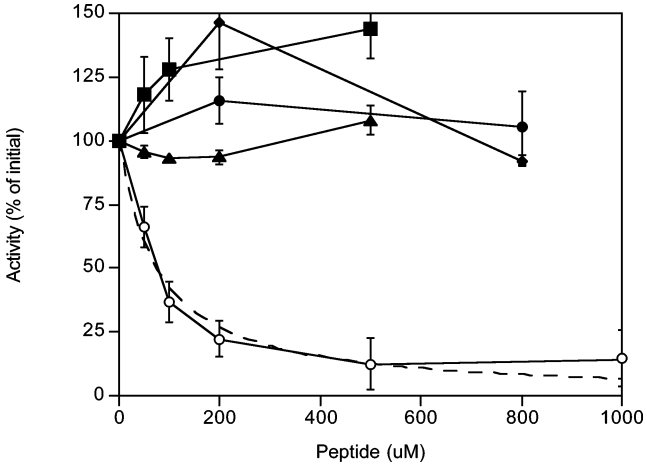

FIGURE 3: Effect of synthetic peptides on factor VIIIa activity reconstitution. Indicated amounts of synthetic peptides to A3 domain residues 1716−1724 (◆), 1954−1961 (○), 1966−1977 (●), or 1954−1961 His to Ala (■) were titrated into reconstitution mixtures containing A1 and A3C1C2 subunits (250 nM each). The 1954−1961 peptide was also reacted with the preformed A1/A3C1C2 dimer [25 nM (▲)]. The A2 subunit (100 nM) was added, and the cofactor activity of reconstituted factor VIIIa was measured in a factor Xa generation assay as described in Materials and Methods. The dashed line represents the curve fit for titration with peptide 1954−1961. Each point represents the mean and standard deviation of three individual measurements.

interactions involved in factor Xa generation, we evaluated the effects of the peptide using an intact A1/A3C1C2 dimer purified from factor VIIIa, which shows high stability over an extended time course (*20*). Increasing concentrations of A3 domain peptide 1954−1961 added to reaction mixtures containing 25 nM A1/A3C1C2 dimer and 100 nM A2 subunit failed to inhibit cofactor activity regeneration as judged by factor Xa generation (Figure 3). The results presented above indicate that the peptide specifically blocks A1/A3C1C2 interaction and does not block either interaction of the A2 subunit with the dimer or the subsequent factor VIIIa−factor IXa or factor Xase−factor X interactions. These results suggest that residues 1954−1961 may represent a portion of an A1-interactive site in the A3 domain.

Factor VIII A3 Domain Site-Directed Mutant Analysis. To further investigate the role of residues 1954−1961 in contributing to an A1-interactive site, the factor VIII point mutations His1957Ala, Gly1960Val, and His1961Asp were constructed, and protein was expressed as B-domainless factor VIII in BHK cells. The selection of residues to mutate and the amino acid substitutions to employ were based on the hemophilia A mutation database, and the proximity to the A1 domain as judged by the factor VIII homology model. His1957, Gly1960, and His1961 all appear to be close to the A1 domain relative to other residues in the A3 domain segment of residues 1954−1961. Furthermore, His1957 is suggested to be involved in a type 2 copper coordination site that may contribute to bridging the A1 and A3C1C2 subunits (*26*). Gly1960Val and His1961Asp are point mutations listed in the hemophilia A mutation database (*27*). His1957 is not listed in the database and was thus converted to Ala. The specific activities of the stably expressed factor VIII forms were determined by measuring factor VIII activity and protein levels of the expressed proteins by the one-stage clotting assay and the ELISA, respectively. His1957Ala and

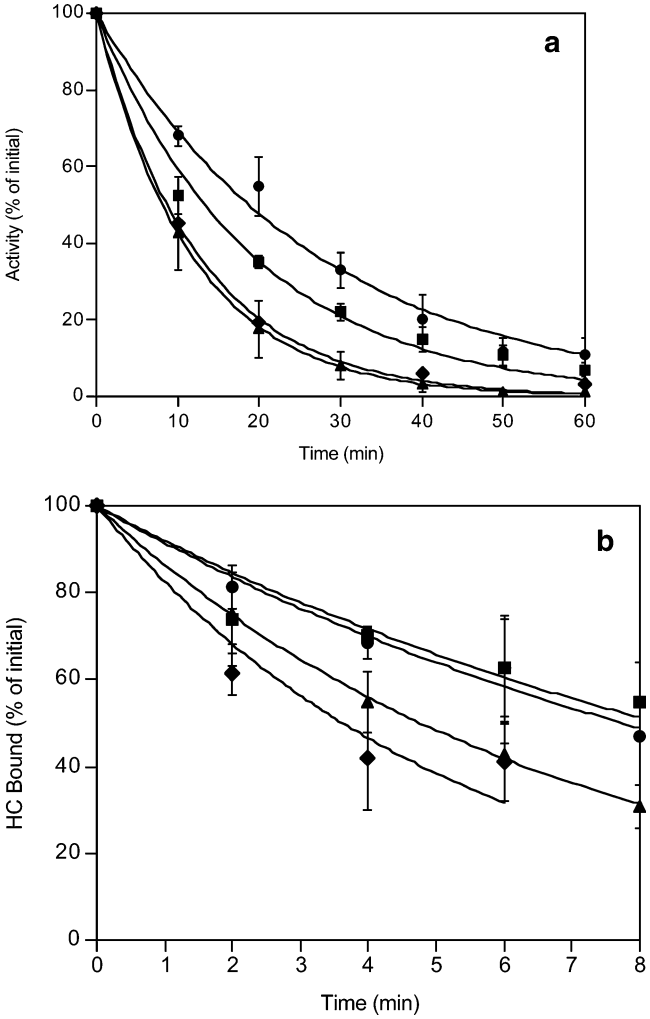

FIGURE 4: (a) Effects of A3 domain mutations on FVIII protein stability. Factor VIII wild type (●), His1957Ala (■), Gly1960Val (▲), and His1961Asp (◆) were incubated at 55 °C, and aliquots were removed at the indicated times and assayed for activity as described in Materials and Methods. Each point represents the mean and standard deviation of at least three individual measurements. (b) Effects of A3 domain mutations on FVIII intersubunit association stability. Factor VIII wild type (●), His1957Ala (■), Gly1960Val (▲), and His1961Asp (◆) were incubated at 50 °C, and aliquots were removed at the indicated times and subjected to an ELISA to measure the amount of heterodimer as described in Materials and Methods. The LC antibody ESH-8 (10 μg/mL) was used as the capture antibody and the HC antibody biotinylated R8B12 (3 μg/mL) as the detection antibody. Each point represents the mean and standard deviation of at least three individual measurements.

Gly1960Val (a mild hemophilia phenotype) demonstrated specific activity values that were ∼39 and ∼42% of that of the wild type, respectively. His1961Asp exhibited a specific activity that was ∼4% of that of the wild type, consistent with its listing as a severe hemophilia phenotype. All factor VIII forms were similarly cleaved by thrombin as judged by SDS−PAGE analysis (data not shown), indicating the mutations did not affect interactions with this proteinase.

To determine whether the reduction in the specific activity of the mutants derived from a decrease in intramolecular stability, the stability of wild-type and mutant factor VIII forms was assessed. Factor VIII (50 nM) was incubated at 55 °C, and at the indicated times, an aliquot was assayed for factor Xa generation activity as described in Materials and Methods. Results are shown in Figure 4a and Table 2.

Table 2: Intramolecular Stability Parameters for Factor VIII Wild Type and Mutants[a]

	functional assay[b]		solid-phase binding assay[c]	
FVIII	$t_{1/2}$ (min)	p value[d]	$t_{1/2}$ (min)	p value[d]
wild type	18.8 ± 0.8		7.7 ± 0.4	
His1957Ala	13.3 ± 0.7	<0.001	8.3 ± 0.9	>0.5
Gly1960Val	8.7 ± 0.3	<0.001	4.8 ± 0.1	<0.001
His1961Asp	8.1 ± 0.1	<0.001	3.6 ± 0.5	<0.001

[a] Reactions were performed as described in Materials and Methods. [b] Parameter values and standard deviations were estimated by nonlinear least-squares regression from data shown in Figure 4a using the formula described in Materials and Methods. [c] Parameter values and standard deviations were estimated by nonlinear least-squares regression from data shown in Figure 4b using the formula described in Materials and Methods. [d] Data are compared with those of wild-type FVIII.

Factor VIII mutants His1957Ala, Gly1960Val, and His1961-Asp exhibited significantly increased rates of loss of activity compared to the wild type, suggesting a reduction in the stability of these factor VIII forms. The above results suggest that the reduced specific activity of these mutant factor VIII forms may result from reduced protein stability.

On the basis of the observed contributions of the two His residues in segment 1954−1961 to intersubunit stability, we prepared a version of peptide 1954−1961 in which all His residues (1954, 1957, and 1961) were replaced with Ala. This peptide failed to inhibit factor VIIIa activity regeneration following reconstitution of the A1 and A3C1C2 subunits (see Figure 3), suggesting the importance of these His residues for functional intersubunit interaction.

To show that the temperature-dependent decrease in factor VIII activity observed in the mutants derived from defective intersubunit interactions at the A1−A3 domainal junction, the stability of this interaction was assessed. Factor VIII (50 nM) was incubated at 50 °C, and at the indicated times, an aliquot was assayed using a sandwich ELISA as described in Materials and Methods to measure the amount of intact heterodimer remaining. Results are shown in Figure 4b and Table 2. In all cases, we observed a time-dependent loss of the HC as a result of chain dissociation. Compared to the wild type, the Gly1960Val mutant and the His1961Asp mutant exhibited a decrease in intersubunit stability, while the His1957Ala mutant showed no significant difference. Control experiments using a LC antibody that recognizes an epitope distinct from the LC capture antibody showed no significant loss of the capture antibody-bound LC over the time course of this experiment (data not shown). Taken together, these data suggest that mutations yielding the hemophilia phenotype (G1960V and H1961D) show a defective interchain interaction. On the other hand, the H1957A mutation was relatively benign with respect to this parameter, suggesting the putative type 2 copper site spanning A1 and A3 domains may not make a significant contribution to interchain affinity.

DISCUSSION

Evidence derived from observations that the A1 and A3C1C2 subunits can be isolated as a stable dimer while the A2 subunit is weakly associated with the dimer suggests interchain interaction in the factor VIII heterodimer is mediated by A1 and A3C1C2 domains (*14*). Furthermore, the recently determined crystal structure of activated protein C-inactivated bovine activated factor V (Vai) (*28*), a protein which shares strong functional and sequence homology with factor VIII, suggests that the association between the heavy and light chains is largely mediated by interaction of the A1 domain with the A3 domain. However, quantitative analysis of these interactions is lacking. A model for the triplicated A domains of factor VIII based on homology to ceruloplasmin predicts a pseudo-3-fold axis at the tightly packed hydrophobic core. Recently, Pipe et al. (*29*) have suggested that the entire core, which includes the A2 domain, contributes to the stabilization of factor VIIIa. Their conclusion is based, in part, on the observation that mutations within the predicted A2−A3 interface cause a one-stage to two-stage assay discrepancy which is reflected as an increased rate of A2 subunit dissociation. This observation, however, does not necessarily suggest any binding contribution by the A2 subunit, but rather lends itself to the notion that these mutations are disruptive to A2 retention in factor VIIIa. A comparison of the K_d value for HC−LC interaction with that for A1/A3C1C2 interaction provides an appropriate means of evaluating the contribution of the A2 domain to the interchain interaction. The affinities of the HC and LC in the absence of exogenous metal ions and in the presence of Ca^{2+} were recently assessed (K_d = 53.8 ± 14.2 and 48.7 ± 15.4 nM, respectively) using fluorescence resonance energy transfer (*9*). These affinity values are equivalent to our results for A1/A3C1C2 association in the absence of exogenous metal ions and in the presence of Ca^{2+} (K_d = 58.8 ± 16.8 and 51.6 ± 16.6 nM, respectively), indicating A1 and A3C1C2 subunits primarily mediate chain association. On the basis of this result, we speculate that the A2 domain makes little if any contribution to the intersubunit binding energy in factor VIII. Furthermore, this contention is supported by a previous study showing that the affinity of the A2 subunit for the A1 subunit was equivalent to that for the A1/A3C1C2 dimer (*8*). Thus, a mechanism whereby the A2 domain initially makes a primary contribution in binding the heavy chain with the light chain that subsequently undergoes a conformation rearrangement such that the A1 domain now becomes the primary contributor of binding energy, and that this phenomenon occurs with no overall change in thermodynamic stability, appears to be unlikely.

The factor VIII homology model predicts the presence of a single consensus type 2 copper site formed at the A1−A3 domainal junction involving His99 in A1 and His1957 in A3C1C2 (*26*). The sequences around these His residues are PVSLH^{99}AVGV and HSIH^{1957}FSGH, respectively. Analysis of these sequences reveals a high concentration of apolar residues. This observation is consistent with earlier results suggesting hydrophobic residues contribute to intersubunit interactions in factor VIII (*30*). In this study, we identify A3C1C2 subunit residues 1954−1961 (HSIHFSGH) as likely contributing to an A1 subunit-interactive site in factor VIIIa. A synthetic peptide (H^{1954}SIHFSGH^{1961}) prepared to this region inhibited A1/A3C1C2 dimer formation as judged by the factor Xa generation assay. A number of residues in this segment are listed in the hemophilia A database as point mutations that manifest mild to severe hemophilia A phenotypes (*27*). Results from site-directed mutational analysis in our study suggest the hemophilia phenotypes derive from defective intersubunit interactions at this domainal junction, with factor VIII mutants His1957Ala,

Gly1960Val, and His1961Asp exhibiting reduced stability in intersubunit interactions compared to that of the wild type.

It has recently been reported that low levels of Cu^{2+} yielded a few-fold increase in cofactor specific activity and enhanced the affinity of HC−LC interaction by as much as 100-fold (*9*). However, limitations in HC−LC interaction data analysis at low subunit concentrations, which define the upper limit to affinity, yielded large standard errors, suggesting this reported upper limit affinity value may be somewhat equivocal. We observed Cu^{2+} mediating an approximately 5-fold increase in A1/A3C1C2 affinity, a value significantly lower than the proposed upper limit value observed in the HC−LC interaction. One possible reason that may contribute to this disparity in response to copper may be due to the conformational differences between the subunits of the procofactor and activated factor VIII forms. Several studies have shown conformational changes in factor VIII following activation. For example, treatment of factor VIII with a zero-length cross-linker prior to activation with thrombin resulted in no cross-linking between A1 and A2 domains. However, treatment of factor VIIIa with the zero-length cross-linker resulted in cross-linking between the A1 and A2 subunits, suggesting the formation of a new salt bridge(s) that is absent in the contiguous A1−A2 domains of factor VIII (*20*). Examination of binding of the apolar probe bis-ANS to factor VIII and VIIIa subunits revealed alterations in exposed hydrophobic sites, also suggesting a change in conformation following factor VIII activation (*30*). Furthermore, physical analyses of factor VIII and VIIIa secondary structure using CD suggested an increase in β-sheet structure content in activated factor VIII compared to the unactivated form (*31*).

Earlier experiments have identified the presence of a single copper ion [Cu^{+} (*11*)] in factor VIII (*10*). The location of copper ion(s) in factor VIII, however, remains controversial. Consensus type 1 sites have been identified in A1 (His265, Cys310, His315, and Met320) and A3 (His1954, Cys2000, His2005, and His2010) domains (*7*). Molecular modeling studies have proposed that a single consensus type 2 copper site is formed at the A1−A3 domainal junction involving His99 in A1 and His1957 in A3C1C2 (*26*). Tagliavacca et al. (*11*) suggested the type 1 site in A1 and not the type 2 site was involved in coordinating the bound copper in factor VIII based on site-directed mutagenesis studies. Their conclusions were based on the fact that conversion of Cys2000 to Ser and His1957 to Ala did not affect factor VIII activity, while a Cys310 to Ser factor VIII showed a marked reduction in activity as measured from factor VIII in conditioned medium from transiently expressing cell lines. These observations are in agreement with our results, which show that conversion of His1957 to Ala does not affect interchain interaction.

In summary, we have demonstrated that the A1 and A3C1C2 subunits likely mediate the bulk of interchain binding energy in the factor VIII heterodimer. We also suggest that residues within segment 1954−1961 of the A3C1C2 subunit contribute to an A1-interactive site involved in A1/A3C1C2 intersubunit association. Results from site-directed mutational analyses suggest that point mutations within this region yield a hemophilia A phenotype due to the reduced stability of the interdomain interface and, on the basis of mutagenesis of H1957, suggest little potential contribution of the putative type 2 copper site to this interchain affinity.

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