Increasing Hydrophobicity or Disulfide Bridging at the Factor VIII A1 and C2 Domain Interface Enhances Procofactor Stability*

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Factor VIII (FVIII) consists of a heavy (A1A2B domains) and light chain (A3C1C2 domains), whereas the contiguous A1A2 domains are separate subunits in the cofactor, FVIIIa. FVIII x-ray structures show close contacts between A1 and C2 domains. To explore the role of this region in FVIII(a) stability, we generated a variant containing a disulfide bond between A1 and C2 domains by mutating Arg-121 and Leu-2302 to Cys (R121C/L2302C) and a second variant with a bulkier hydrophobic group (A108I) to better occupy a cavity between A1 and C2 domains. Disulfide bonding in the R121C/L2302C variant was >90% efficient as judged by Western blots. Binding affinity between the A108I A1 and A3C1C2 subunits was increased ~3.7-fold in the variant as compared with WT as judged by changes in fluorescence of acrylodan-labeled A1 subunits. FVIII thermal and chemical stability were monitored following rates of loss of FVIII activity at 57 °C or in guanidinium by factor Xa generation assays. The rate of decay of FVIIIa activity was monitored at 23 °C following activation by thrombin. Both R121C/L2302C and A108I variants showed up to ~4-fold increases in thermal stability but minimal improvements in chemical stability. The purified A1 subunit of A108I reconstituted with the A3C1C2 subunit showed an ~4.6-fold increase in thermal stability, whereas reconstitution of the variant A1 with a truncated A3C1 subunit showed similar stability values as compared with WT A1. Together, these results suggest that altering contacts at this A1-C2 junction by covalent modification or increasing hydrophobicity increases inter-chain affinity and functionally enhances FVIII stability.

Factor F (FVIII)² is a plasma protein that is decreased or defective in individuals with hemophilia A. FVIII is expressed as both single chain and heterodimer forms in heterologous cells. The latter consists of a heavy chain composed of A1(a1)A2(a2)B domains and a light chain composed of (a3)A3C1C2 domains, with the lowercase “a” representing short segments rich in acidic residues (see Ref. 1 for review). FVIII is activated following limited proteolysis catalyzed by thrombin or FXas at Arg-372 (a1A2 junction), Arg-740 (a2B junction), and Arg-1689 (a3A3 junction). The resulting product, FVIIIa, is a heterotrimer composed of subunits designated A1, A2, and A3C1C2 that functions as a cofactor for FIXa in the membrane-dependent activation of FX to FXa (see Ref 1 for review).

Earlier structural studies identified the C2 domain as making a primary contribution to anionic phospholipid membrane binding through a combination of electrostatic and hydrophobic interactions (2). More recently, the intermediate resolution x-ray structures of FVIII (3, 4) showed that the C1 and C2 domains are aligned such that both domains may interact with the phospholipid membrane surface. In addition, these structures show close contact between A1 (heavy chain) and C2 (light chain) domains. We recently reported that an FVIII variant lacking the C2 domain retained the capacity to bind phospholipid membranes, albeit with a marked reduction in affinity (5), supporting a direct role for the C1 domain in this interaction. Furthermore, deletion of the C2 domain did not grossly alter a number of functional properties including the rate of procofactor activation by thrombin, affinity of FVIIIa for FIXa, $k_m$ of Factor Xase (FXase) for substrate FX, or $k_{cat}$ for FXa generation. However, this deletion did significantly destabilize the cofactor, as judged by increased rates of activity decay following exposure to elevated temperature or chemical denaturants. These observations suggested that increased protein lability combined with modest defects in various inter-molecular interactions resulted in significant reductions in the thrombin-generating capacity of this FVIII variant.

Although contacts between the A1 and C2 domains of FVIII appear to contribute to protein, and in particular heterodimer stability, little information is available on specific interactions and their functional significance. In this study, we examine the A1-C2 interface and explore approaches to potentially enhance the stability of this region through either covalent modification by nascent disulfide bridging or increasing its hydrophobic character. Results from this study show that an inter-domain disulfide can be constructed by replacement of selected residues with Cys and that the inter-domain affinity may also be enhanced by maximizing hydrophobic interactions. In both cases, the resultant FVIII variants demonstrate increased stability parameters, indicating that interactions at this interface are important for FVIII structural integrity.
EXPERIMENTAL PROCEDURES

Materials—Recombinant FVIII (Kogenate™) and the monoclonal antibodies 58.12 and 2D2 were generous gifts from Dr. Lisa Regan of Bayer Corp. (Berkeley, CA). Phospholipid vesicles containing 20% phosphatidylycholine (PC), 40% phosphatidylethanolamine (PE), and 40% phosphatidylserine (PS) were prepared using octylglucoside as described previously (6). The reagents α-thrombin, FIXα, FX, and Fxa (Enzyme Research Laboratories, South Bend, IN), hirudin (Dif Pharma, West Chester, OH), phospholipids (Avanti Polar Lipids, Alabaster, AL), the chromogenic Xa substrate, Pefachrome Xa (Pef-5523, CH3OCO-D-CHA-Gly-Arg-pNA·AcOH; Centerchem, Inc., Norwalk CT) and acrylodan (Molecular Probes, Eugene, OR) were purchased from the indicated vendors.

Construction and Expression of FVIII WT and Variants—A double mutation of R121C and L2302C (designated R121C/L2302C) and A108I were prepared as B domain-deleted FVIII (lacking residues Gln-744–Ser-1637 in the B domain (7)) using methods previously described (8). Recombinant wild-type (WT) and variant FVIII forms were stably expressed in baby hamster kidney cells and purified as described previously (18). Protein yields for the variants ranged from >10 to ~100 μg from two 750-cm² culture flasks, with purity >90% as judged by SDS-PAGE. The primary contaminant in the FVIII preparations was albumin. FVIII concentrations were measured using an enzyme-linked immunosorbent assay (ELISA), and FVIII activity was determined by one-stage clotting and two-stage chromogenic FXa generation assays described below.

A1 (WT and A108I), A2, and A3C1C2 Subunit Purification—A1 subunit was purified from WT or A108I FVIII. FVIII (1–3 μM) was reacted with thrombin (50 nM) in 20 mM HEPES, pH 7.2, 0.1 M NaCl, 0.01% Tween 20 (buffer A) for 30 min and treated with 50 mM EDTA overnight at 4 °C. After a 1:4 dilution with buffer A, the samples were subjected to chromatography using a heparin-Sepharose column (1.5 × 0.7 cm in diameter, GE Healthcare). The flow-through fraction was collected and applied to a Q-Sepharose column (1.5 × 0.7 cm in diameter, GE Healthcare). After the column was washed with buffer A, bound A1 subunit was eluted with 20 mM HEPES, pH 7.2, 0.8 M NaCl, 0.01% Tween 20, and purified A1 subunit was kept frozen at ~80 °C until use. A2 and A3C1C2 subunits were completely absorbed by the heparin-Sepharose column step, and the final A1 product was >95% pure as judged by SDS-PAGE. A2 and A3C1C2 subunits were purified from recombinant FVIII (Kogenate) as described previously (9). A3C1 subunit was purified from C2 domain-deleted FVIII (5) using the same method for A3C1C2 purification.

Acrylodan Labeling of A1 Subunits and Interactions with A3C1C2 Subunit as Detected by Changes in Acrylodan Fluorescence—Purified A1 subunit from WT and A108I FVIII was labeled with acrylodan by sulfhydryl-specific protein modification as described previously (10). A1 (15 nm) from WT or A108I FVIII was reconstituted with A3C1C2 subunit (0–300 nm) at 37 °C for 2 h in buffer containing 20 mM HEPES, pH 7.2, 0.1 M NaCl, 0.01% Tween 20, 0.01% BSA, 5 mM CaCl₂ (buffer B) at pH 7.4. Fluorescence measurements were performed using an Aminco-Bowman Series 2 luminescence spectrophotometer (Thermo Spectronic) at 23 °C at an excitation wavelength of 395 nm (2 nm bandwidth), Fluorescence emission was monitored at 480 – 490 nm (8 nm bandwidth), and all spectra were corrected for background. Data were fitted to a quadratic equation by non-linear least squares regression, and parameter values were obtained.

ELISA—A sandwich ELISA was performed as described previously (11) using purified commercial recombinant FVIII (Kogenate, Bayer Corp.) as a standard. FVIII capture used the anti-C2 monoclonal antibody (GMA8003, Green Mountain Antibodies), and the anti-A2 monoclonal antibody, R8B12 (GMA8012, Green Mountain Antibodies) was employed for FVIII detection following biotinylation.

One-stage Clotting Assay—One-stage clotting assays were performed using substrate plasma chemically depleted of FVIII (12) and assayed using a Diagnostica Stago clotting instrument. Plasma was incubated with activated partial thromboplastin time reagent (General Diagnostics) for 6 min at 37 °C after which a dilution of FVIII was added to the cuvette. After 1 min, the mixture was recalculated, and clotting time was determined and compared with a pooled normal plasma standard.

Two-stage Chromogenic FXa Generation Assay—The rate of conversion of FX to FXa was monitored in a purified system (13) according to methods previously described (10, 14). FVIII (1 nm) in buffer B, containing 20 μM PS/PC/PE vesicles, was activated with 20 nM α-thrombin for 1 min. The reaction was stopped by adding hirudin (10 units/ml), and the resulting FVIIa was reacted with FIXa (40 nM) for 1 min. FX (300 nm) was added to initiate reactions, which were quenched after 1 min by the addition of 50 mM EDTA. FXa generated was determined following reaction with the chromogenic substrate Pefachrome Xa (0.46 mM final concentration). All reactions were run at 23 °C.

Western Blotting—FVIII proteins (0.34 μg) were activated by thrombin (20 nM) for 30 min at 23 °C and subjected to electrophoresis under either non-reducing or reducing (0.1 M dithiothreitol) conditions using 10% polyacrylamide gels run at constant voltage (150 V). Gels were transferred to a polyvinylidene fluoride membrane and probed with an anti-A1 domain (58.12) or anti-A3 domain (2D2) monoclonal antibody, and protein bands were visualized using chemiluminescence.

FVIII Activity Decay at Elevated Temperatures—WT FVIII or FVIII variants (4 nM) in buffer B were incubated at 57 °C (pH at this temperature = 6.94). Aliquots were removed at the indicated times and cooled to room temperature, and residual FVIII activity was determined using a FXa generation assay.

FVIIa Activity Decay—WT and mutant FVIII (1.5 nM) in buffer B containing 20 μM PS/PC/PE vesicles were activated using 20 nM thrombin for 1 min at 23 °C. Reactions were immediately quenched by hirudin (10 units/ml) to inactivate thrombin, aliquots were removed at the indicated times, and activity was determined using the FXa generation assay following the addition of FIXα (40 nM) and FX (300 nM).

FVIII Activity Inhibition by Guanidinium Chloride—WT and FVIII variants (50 nM) in buffer B plus 0–1.8 M guanidinium chloride were incubated for 2 h at 23 °C. Aliquots were diluted (1/50) in buffer A containing 20 μM PS/PC/PE vesicles and activated by 5 nM thrombin for 1 min. Reactions were immediately
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quenched with hirudin (10 units/ml), and activity was determined by FXa generation assay following the addition of FIXa (40 nM) and FX (300 nM). Residual guanidinium chloride (<36 mM) did not inhibit the proteolytic activation of FVIII or its cofactor activity.

**Thermal Denaturation of Reconstituted A1 and A3C1C2 or A3C1 Subunit as Detected by FXa Generation Assay—** A1 subunit (50 nM) from WT or A108I FVIII was reconstituted with A3C1C2 (200 nM) or A3C1 (500 nM) at 37 °C for 2 h in 10 mM MES, pH 6.5, 0.15 M NaCl, 0.01% Tween 20, 0.01% BSA, 5 mM CaCl₂. Samples were incubated at 55 °C (A3C1C2) or 52 °C (A3C1) (pH at this temperature = 6.94), and aliquots were taken at the indicated times and further incubated with 200 nM A2 subunit at 23 °C for 30 min. Samples were then diluted 1:20 with buffer B containing 20 μM PS/PC/PE vesicles, and reconstituted FVIIIa activity was measured directly by FXa generation assay in the absence of the thrombin activation step. Data were fitted to the single exponential decay equation by non-linear least squares regression, and parameter values were obtained.

**Data Analysis—** For activity decay analysis of FVIII/FVIIIa, activity values as a function of time were fitted to a single exponential decay curve by non-linear least squares regression using the equation,

\[ A = A_0 \times e^{-k \times t} \]  
(Eq. 1)

where \( A \) is residual FVIIIa activity (nm/min/nM FVIII), \( A_0 \) is the initial activity, \( k \) is the apparent rate constant, and \( t \) is the time (minutes) of reaction of FVIII (for FVIII thermal decay experiments) or of FVIIIa after thrombin was quenched (for FVIIIa decay measurements).

FVIII activity inhibition by guanidinium was fitted to a linear equation by least squares regression using the equation,

\[ A = 50 - k \times (X - IC_{50}) \]  
(Eq. 2)

where \( A \) is the normalized activity (= 100 (%)), \( IC_{50} \) is the inhibitor (guanidinium chloride) concentration (m) at 50% activity, \( X \) is the guanidinium chloride concentration (m), and \( k \) is the slope.

Determination of A1-A3C1C2 binding affinity used the quadratic equation

\[ F = \frac{F_{\text{max}}}{B} \times \left( \frac{B + K_d + X)^2 - \sqrt{(B + K_d + X)^2 - 4 \times B \times X}}{2} \right) \]  
(Eq. 3)

where \( F_{\text{max}} \) is the maximum increase in fluorescence at saturation, \( B \) is the A1 concentration (= 15 nM), \( K_d \) is the dissociation constant, and \( X \) is the concentration of A3C1C2 in nM. Non-linear least squares regression analysis was performed using KaleidaGraph (Synergy, Reading, PA). A Student’s \( t \) test was performed for statistical analysis.

**RESULTS**

**Rationale for Construction of A1-C2 Domain Interface Variants—** The A1 and C2 domains show close proximity to one another in the FVIII crystal structure (Fig. 1, upper left panel). Inasmuch as an earlier study (5) suggested a role for this region in contributing to protein stability and to maintenance of the FVIII heterodimer, we investigated this region with the aim toward enhancing inter-domain interactions to positively alter affinity and stability parameters. Examination of the A1-C2 interface suggested two possible approaches for increasing the inter-domain affinity. Although the orientation of side chains cannot be discerned due to the resolution (~4 Å) of the structure (4), the putative spatial separation of several paired residues suggested that mutagenesis of these residues to Cys could result in formation of a nascent inter-domain disulfide bridge. Paired A1/C2 domain residues were identified that appeared to meet this distance requirement and included Ser-119/Pro-2300, Gln-120/Pro-2300, Arg-121/Leu-2302, Ala-108/L2302, and Trp-106/Ala-2308. Double mutations where Ala residues to Ile, Leu, and Val (A108L, A108V, A2328I, A2328L, and A2328V). Of the variants prepared, we found that the...
A108I FVIII variant showed minimal effects on FVIII specific activity (~74% of the WT value, Table 1).

Western Blotting of the R121C/L2302C FVIII Variant—Evidence for high efficiency disulfide bridging between FVIII A1 and A3C1C2 domains in this double mutant, as judged by Western blotting, is shown in Fig. 2. For this analysis, WT FVIII and the R121C/L2302C FVIII variant were cleaved with thrombin to generate the FVIIIa heterotrimer prior to SDS-PAGE, which was then run in the absence and presence of disulfide bond reduction using DTT. Blots were probed with an anti-A1 antibody (58.12, lanes 1–4) and an anti-A3 antibody (2D2, lanes 5–8). Both A1 and A3C1C2 subunits derived from the FVIII R121C/L2302C variant were detected at the ~120 kDa band, consistent with the sum of their molecular masses under non-reducing conditions (lanes 2 and 6), whereas reduction by 0.1 M DTT yielded the separated subunits (lanes 4 and 8). Based upon the band densities of bridged and free subunits in the non-reduced lanes, it appeared that ~90% of the variant molecules were disulfide-linked.

Affinity of the WT and A108I FVIII Variant A1 Subunits for A3C1C2—To assess the affinity of the A108I A1 domain for C2 domain, the purified FVIII variant and WT were treated with thrombin, and the A1 subunits were separately purified as described under “Experimental Procedures.” A1 subunits were then reacted with the environment-sensitive fluorescent probe, acrylodan, and these reagents were used to probe binding with the A3C1C2 subunit. The site for acrylodan attachment is likely the lone free thiol in A1 at Cys-310, which is in close proximity (~15 Å) to residues in the A3 domain of the light chain (4). Indeed, increases in the emission fluorescence from acrylodan-labeled A1 subunits have been previously observed when A3C1C2 was bound to the molecule (10, 15). Titration of acrylodan-labeled A1 subunits with A3C1C2 was performed as described under “Experimental Procedures,” and the results are shown in Fig. 3. Acrylodan-labeled A1 (15 nM) fluorescence from both the WT and the A108I subunits saturably increased as the A3C1C2 concentration increased. The estimated $K_d$ values of this interaction for WT and A108I A1 subunits were 88.7 ± 9.8 and 24.1 ± 4.1 nM, respectively. The $K_d$ value for WT was somewhat higher than a previously reported value (~50 nM) (15), likely due to slightly higher pH (7.4) employed for the binding conditions (16). This result indicated an ~4-fold increase in affinity of A108I A1 for A3C1C2 as compared with the WT A1 subunit for A3C1C2. The estimated maximal values in fluorescence for WT and A108I were 0.221 ± 0.009 and 0.245 ± 0.011, respectively, and were not significantly different ($p > 0.1$).

Stability of FVIII R121C/L2302C and A108I Variants—The above results demonstrate that introduction of the disulfide bridge or increasing the hydrophobic character at the A1-C2 interface stabilizes this inter-domain interaction. To test the functional consequences of these mutations, stability parameters of FVIII (FVIIIa) were evaluated by several methods. Thermal denaturation experiments were performed at 57 °C as described under “Experimental Procedures.” Data shown in Fig. 4A were fitted to a single exponential decay curve using
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FIGURE 4. FVIII R121C/L2302C and A108I variant stability. A, FVIII activity decay at elevated temperature. FVIII (4 nM) was incubated at 57 °C, and at the indicated times, aliquots were removed, and activity was measured by FXa generation assays as described under “Experimental Procedures.” Data were fitted to a single exponential decay curve by non-linear least squares regression. WT FVIII (circles) decayed to ~40% of the initial activity level in 6–7 min at 57 °C. On the other hand, the R121C/L2302C variant (triangles) retained >40% activity up to 20 min, whereas the A108I variant (squares) retained this level for >30 min. Overall, decay rates for the R121C/L2302C and A108I variants obtained by curve fitting were reduced by 3.1- and 4.2-fold, respectively, as compared with the WT FVIII value (Table 1).

In a complementary series of experiments, FVIII stability was examined following a 2-h exposure to 0.6–1.2 M guanidinium (Fig. 4B). As the concentration of guanidinium increased, FVIII activity was reduced to near zero as an indication of denaturation. Using the range of linear response (~0.6–1 M), data points were fitted by a linear equation, and the IC50 values were obtained (Table 1). FVIII activity of the A108I variant was significantly more stable than WT, showing an ~10% higher IC50 value as compared with WT (p < 0.001), whereas the IC50 determined for the R121C/L2302C variant was only slightly increased (~2% greater than WT, Table 1). Overall, the stability data for the disulfide-bridged variant suggested that the covalent bond between A1 and C2 subunits significantly increased FVIII thermal stability while showing little stabilizing effect in the presence of guanidinium. This result suggested that dissociation of FVIII heavy and light chains may be a prominent cause for activity loss at elevated temperature but that chain dissociation may not represent a primary mode for activity loss due to chemical denaturation. Alternatively, the A108I mutation demonstrated a more global protective effect in increasing FVIII stability toward either thermal or chemical denaturation. Control experiments were performed to determine whether there was any time-dependent change in activity following the thermal or chemical denaturation step and return of FVIII to either ambient temperature or dilution of denaturant, respectively. FVIII was assayed from 30 s to 1 h, and no significant change in activity was observed (data not shown).

FVIIIa activity is labile due to A2 subunit dissociation following proteolytic activation (17–19). To determine whether these mutations affected FVIIIa decay, we performed experiments to assess rates of loss of FVIIIa activity over time. As shown in Fig. 4C, reaction conditions employed resulted in the loss of ~50% of WT FVIIIa activity at ~6 min after thrombin activation, whereas ~10% activity remained after 16 min. The observed FVIIIa activity decay was slightly reduced for both R121C/L2302C and A108I variants, which showed ~40% activity in 7–8 min and demonstrated decay rates that were 1.2- and 1.3-fold greater than WT FVIII, respectively (Table 1). These results suggested at best minor effects on the inter-subunit interactions involving A2 subunit following modification of the A1 and C2 domain interface.

Stability of Reconstituted A108I or WT A1 Subunit with A3C1C2 or A3C1—The thermal stability for the A1/A3C1C2 dimer was assessed following its reconstitution from isolated subunits. Purified WT A1 or A108I A1 subunits were reconstituted with A3C1C2 subunit at 37 °C for 2 h, and the stability of the A1/A3C1C2 dimer at elevated temperature (55 °C) was measured by FXa generation assay following the addition of A2 subunit as described under “Experimental Procedures.” As shown in Fig. 5A, FVIIIa activity reconstituted from WT A1
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Similar reconstitution experiments were performed using the A3C1 subunit derived from the C2 domain-deleted FVIII variant. The rationale for this experiment was that if the enhanced stability of the A108I A1 was due to interaction with the C2 domain following reassociation with A3C1C2, then use of the truncated A3C1 for reconstitution would abrogate the enhanced stability of the variant. Experiments performed with a C2 domain-deleted FVIII, described in an earlier study (5), showed that this variant was markedly less stable than WT FVIII at elevated temperatures and that rates of decay needed to be monitored at a relatively lower temperature (52 °C). Under these conditions, the C2 domain-deleted FVIII variant decayed ~40-fold faster than WT FVIII. For this reason, stability studies following FVIII reconstitutions with the A3C1 light chain were performed at 52 °C. Furthermore, in an earlier study (20), we showed that FVIII stability measured over a range of temperatures from 52 to 60 °C yielded similar relative rates of decay when comparing a given FVIII variant with WT. Consistent with observations using the C2 domain-deleted FVIII, we observed that reconstitutions using either A1 form with A3C1 yielded an overall faster decay (Fig. 5B), with ~50% activity reduction at 4 min at 52 °C, than results observed following reconstitutions with the intact A3C1C2. The estimated decay rates for WT and the variant FVIIIa forms were similar (0.166 ± 0.001 and 0.154 ± 0.013 min⁻¹, respectively). That the observed increase in thermal stability of the A108I variant was also observed following reconstitutions using purified components supports the conclusion that the enhanced stability of the variant as compared with WT derived from improved interaction(s) between A1 and A3C1C2 subunits and required the presence of C2 subunit.

FIGURE 5. Thermal stability of A1 subunit reconstituted with A3C1C2 (A) or A3C1 (B) subunit. A and B, thermal decay of reconstituted heterodimer of A1 subunit from WT (circles) or A108I (triangles) with WT A3C1C2 (A) or A3C1 (B) subunit at 55 °C (A) or 52 °C (B) was detected by residual FVIIIa activity following the addition of A2 subunit as described under “Experimental Procedures.” Data were fitted to a single exponential decay curve by non-linear least squares regression. Data points averaged from three separate determinations were fitted to a single exponential decay curve to obtain rates. Decayed to ~25% of its original value at 20 min (circles), whereas ~80% of the original activity level remained for the A1 subunit containing the A108I mutation (triangles). The estimated decay rates for WT and the mutant FVIIIa forms were 0.066 ± 0.005 and 0.014 ± 0.001 min⁻¹, respectively, showing a 4.6-fold rate reduction for the variant as compared with WT FVIII.

DISCUSSION

In this study, we investigate interactions at the interface between FVIII A1 and C2 domains following preparation of two novel variants, R121C/L2302C FVIII, possessing a nascent disulfide bond spanning the domains, and A108I FVIII, which has a larger hydrophobic side chain to better fill the inter-domain space. Several other mutations were prepared at this region in an attempt to create a disulfide bond or to increase hydrophobicity. These variants yielded low specific activity values, possibly resulting from unfavorable changes in conformation, and their characterization was not pursued further. However, both variants studied exhibited enhanced inter-A1-C2 domain affinity resulting in increases in the observed stability of the FVIII variants, especially related to thermal denaturation.

The intermediate resolution (~4 Å) x-ray structure of FVIII (4) predicts the close proximity of Arg-121 and Leu-2302 with 7.7 Å separating Ca atoms (Protein Data Bank (PDB) number 3CDZ). This spatial separation suggested the potential to bridge this distance by a disulfide bond (~4–6 Å) following replacement of these residues with Cys and provided that the side chains were in an acceptable orientation. Results evaluating the R121C/L2302C FVIII protein by Western blotting in the absence and presence of disulfide bond reduction showed high efficiency bridging (>90%) constituting experimental proof for the opposing orientation of side chains of these two residues in FVIII. In addition, the x-ray structure also showed that the A1-C2 junction adjacent to Ala-108 is rich in hydrophobic groups represented by the Cβ carbon of Ala-108 from the C6 of Leu-2302, the Cβ of Ala-2328, or the Cγ of Gln-2329 (Fig. 1, lower panels). Thus, it is possible that extended alkyl groups of side chains larger than the methyl group of Ala might contribute to enhanced binding energy. Of several variants prepared to this region, replacement of Ala-108 with Ile yielded a variant possessing near WT-like specific activity.

Both R121C/L2302C and A108I variants exhibited superior stability parameters as compared with the WT protein. For

3 H. Wakabayashi, A. E. Griffiths, and P. J. Fay, unpublished observations.
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example, the thermal decay rates for the R121C/L2302C and A108I FVIII variants were reduced by 3.1- and 4.2-fold, respectively, as compared with WT. Further dissection of the interaction with the A108I variant was obtained following reconstitution of the A1/A3C1C2 dimer using WT and variant A1 subunits as well as the truncated A3C1 subunit derived from the C2 domain-deleted FVIII. We observed enhanced stability of the variant as compared with WT using native A3C1C2 but similar stability parameter values for variant and WT in the absence of the C2 domain. Taken together, these observations support the hypotheses that modulating the A1-C2 interface, through either covalent bridging or increased hydrophobic interaction, appeared to make an important contribution to overall protein stability.

The primary cause for thermal decay of FVIII is attributed to dissociation of the heavy and light chains (15). This result is supported by the present study showing that bridging the FVIII heavy chain and light chain via a disulfide bond between A1 and C2 domains preferentially reduced thermal decay as compared with chemical denaturation. Thus, chemical denaturation appears to represent a more global effect on FVIII structure and a less specific effect for chain dissociation. We reported earlier that several residues at the A2-A3 interface (Tyr-1792, Tyr-1786, and Asp-666) possibly contributed to the binding energy only in the active FVIIIa form (21). Thus, interactions between the A2 domain of the heavy chain and A3C1C2 domains of the light chain may be minimal in the procofactor. Based on that earlier study and the present study, we speculate that in the FVIII heterodimer, the predominant sources for binding energy likely derive from A1 interactions with both A3 and C2 domains.

On the other hand, the instability of FVIIIa results from weak electrostatic interactions between the A2 subunit and the A1/A3C1C2 dimer (17, 18), and its dissociation leads to dampening of Factor Xase (FXase) activity (19, 22). Several FVIII point mutations have been shown to facilitate the rate of dissociation of A2 relative to WT, and these residues localize to either the A1-A2 domain interface (23, 24) or the A2-A3 domain interface (25). Previously, we reported that among several charged residues located at the interface between A1 and A2 domains or between A2 and A3 domains Asp-519, Glu-665, and Glu-1984 were found to be detrimental to FVIII/FVIIIa stability (20, 26). Replacing the charged residues with Ala or Val yielded increased FVIII stability and in particular enhanced retention of the A2 subunit in FVIIIa. Interestingly, neither the single mutants nor combinations of these mutations yielded FVIII variants that showed reductions in the rate of thermal decay of greater than 2-3-fold, whereas the variants examined in the present study showed thermal decay rate reductions of 3–4-fold. Thus, the magnitude of stability enhancement we observed for the A1-C2 interface variants appeared somewhat larger than for the A2 domain-mediated interactions. However, although these variants clearly showed superior FVIII stability, results from this study indicated essentially little if any effect of the interactions involving the A1 and C2 domains in stabilizing the FVIIIa cofactor, suggesting no linkage of these sites with sites involved in A2 subunit retention.

A1 domain residues 110–126 are in close contact to the C2 domain. These residues contain a Ca$^{2+}$ binding site predicted by Ala-scanning mutagenesis (8) and subsequently identified in the x-ray crystal structure (3, 4). Interestingly, preliminary experiments assessing chelation of Ca$^{2+}$ (and/or Cu$^{2+}$) in FVIII by EGTA yielded dramatic losses in activity of WT FVIII while showing more minimal effects on the activity of R121C/L2302C and A108I variants. One possible explanation for these observations may be that the functional effects of Ca$^{2+}$ occupancy at 110–126 in FVIII were replaced by enhanced stabilizing interactions between the A1 and C2 domains in the variants.

In conclusion, results from this study demonstrate that interactions between the A1 and C2 domains of FVIII contribute to the integrity of the protein, providing significant energy for stabilizing the multidomain structure of FVIII. Furthermore, observations for enhancing FVIII stability, in particular by increasing non-covalent, hydrophobic interactions at the A1-C2 domain interface, suggest that these variants could potentially represent superior therapeutics in the treatment of hemophilia.

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