Cofactor Activity in Factor VIIIa of the Blood Clotting Pathway Is Stabilized by an Interdomain Bond between His\textsuperscript{281} and Ser\textsuperscript{524} Formed in Factor VIII*

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The factor VIII (FVIII) crystal structure suggests a possible bonding interaction of His\textsuperscript{281} (A1 domain) with Ser\textsuperscript{524} (A2 domain), although the resolution of the structure (~4 Å) does not firmly establish this bonding. To establish that side chains of these residues participate in an interdomain bond, we prepared and examined the functional properties of a residue swap variant (H281S/S524H) where His\textsuperscript{281} and Ser\textsuperscript{524} residues were exchanged with one another and a disulfide-bridged variant (H281C/S524C) where the two residues were replaced with Cys. The latter variant showed efficient disulfide bonding of the A1 and A2 domains. The swap variant showed WT-like FVIII and FVIIIa stability, which were markedly reduced for H281A and S524A variants in an earlier study. The disulfide-bridged variant showed ~20% increased FVIII stability, and FVIIIa did not decay during the time course measured. This variant also yielded 35% increased thrombin peak values compared with WT in a plasma-based thrombin generation assay. Binding analyses of H281S-A1/A3C1C2 dimer with S524H-A2 subunit yielded a near WT-like affinity value, whereas combining the variant dimer or A2 subunit with the WT complement yielded ~5- and ~10-fold reductions, respectively, in affinity. Other functional properties including thrombin generation potential, FIXa binding affinity, $K_m$ for FX, thrombin activation efficiency, and down-regulation by activated protein C showed similar results for the two variants compared with WT FVIII. These results indicate that side chains of His\textsuperscript{281} and Ser\textsuperscript{524} are in close proximity and contribute to a bonding interaction in FVIII that is retained in FVIIIa.

Factor VIII (FVIII), a plasma protein that is decreased or defective in individuals with hemophilia A, circulates as a heterodimer of a heavy chain comprised of A1(a1)A2(a2)B domains and a light chain comprised of (a3)A3C1C2 domains (see Ref. 1 for review). The A and C domains contain >30% internal sequence homology, whereas sequences designated by the lowercase “a” are short (~30–40 residue) segments rich in acidic residues. FVIII is activated by thrombin- or FXa-catalyzed cleavages at the a1A2, a2B, and a3A3 junctions. The resulting product, FVIIIa, is a heterotrimer comprised of subunits designated A1, A2, and A3C1C2.

FVIIIa functions as a cofactor for the serine protease FIXa in the phospholipid membrane-dependent conversion of zymogen FX to the serine protease, FXa (see Ref. 1 for review). FVIIIa is intrinsically unstable because of weak electrostatic interactions between the A2 subunit and the A1/A3C1C2 dimer (2, 3). Dissociation of the A2 subunit readily occurs at physiologic FVIII concentration and results in a loss of cofactor activity and consequent dampening of FXa activity (2, 3).

The earlier ceruloplasmin-based homology model for the A domains of factor VIII (4), as well as the more recent intermediate resolution (~4 Å) x-ray crystal structures of FVIII (8, 9), indicate an extended interface between the A2 domain and the A1 and A3 domains, with multiple potential contacts contributing to binding interactions. In an earlier study prior to availability of the FVIII x-ray structures, we examined hydrogen-bonding interactions at this interface following mutation of selected charged/polar residues spatially separated by <2.8 Å (5). Approximately half of the residues examined showed loss of function as judged by increased rates of FVIII decay at 55 °C and/or rates for FVIIIa decay caused by A2 subunit dissociation relative to WT. These results suggested that multiple residues at the A1A2 and A2A3 domain interfaces contributed to the stabilization of FVIII. Among those residues identified, His\textsuperscript{281} (amino acid residue numbering is based on a mature form of FVIII protein) in A1 domain and Ser\textsuperscript{524} in A2 domain were particularly interesting because Ala mutation at either residue showed loss of both FVIII and FVIIIa stability, with FVIIIa stability affected to a somewhat greater extent (5). Furthermore, both residues are located in close proximity at A1-A2 interdomain interface in the FVIII crystal structure (6, 7).
In this study we prepared two FVIII mutants, one with a residue swap where residues at His\textsuperscript{281} and Ser\textsuperscript{524} were exchanged with one another (H281S/S524H) and the other where Cys residues were substituted for His\textsuperscript{281} and Ser\textsuperscript{524} (H281C/S524C). The rationale for the former mutation was to determine whether a bonding interaction between side chains of the residues in the native protein would be maintained in the swap variant. The rationale for constructing the latter mutation was to determine whether side chains were positioned to allow for nascent disulfide bridge formation, and if so, whether covalent bonding at this site would enhance functional properties. The consequences of these mutations were assessed using a variety of function assays, and results indicate that the side chains of His\textsuperscript{281} and Ser\textsuperscript{524} are in close proximity and contribute to a bonding interaction in FVIII that is retained in FVIIa.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant FVIII (Kogenate\textsuperscript{TM}) was a generous gift from Dr. Lisa Regan of Bayer Corporation (Berkeley, CA). Dioleoyl phospholipids (phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine) were purchased from Avanti Polar Lipids (Alabaster, AL). FVIII antibodies ES8 (American Diagnostica) and R8B12 (GMA8011; Green Mountain Antibody, Burlington, VT) were purchased from the indicated vendors. The reagents \(\alpha\)-thrombin, FVIIa, FIXa, and FXa (Enzyme Research Laboratories, South Bend, IN); hirudin (DiaPharma, West Chester, OH); the chromogenic Fxa substrate Pefachrome Xa (Pefa-5523, CH\textsubscript{3}OCO-D-CHA-Gly-Arg-pNA-AcOH; Centerchem Inc. Norwalk CT); Enhanced Chemiluminescence reagent (GE Healthcare); recombinant human tissue factor (rTF); Innovin (Dade Behring, Deerfield, IL); fluorescent substrate Z-Gly-Gly-Arg-AMC (Calbiochem, San Diego, CA); thrombin calibrator (Diagnostica Stago, Parissipany, NJ); rabbit thrombomodulin (TM) (Hematologic Technologies, Inc., Essex Junction, VT); and human activated protein C (APC) (Enzyme Research Laboratories, South Bend, IN) were purchased from the indicated vendors.

Construction, Expression, and Purification of WT and Variant FVIII—WT FVIII, the variant H281S/S524H where residues at 281 and 524 were swapped, and the variant H281C/S524C where both residues were replaced with Cys were constructed as B-domainless FVIII, lacking residues Gln744–S524C where both residues were replaced with Cys were used. The consequences of these mutations were assessed using a variety of function assays, and results indicate that the side chains of His\textsuperscript{281} and Ser\textsuperscript{524} are in close proximity and contribute to a bonding interaction in FVIII that is retained in FVIIa.

**SDS-PAGE and Western Blotting**—FVIII proteins (0.34 \(\mu\)g) in 20 mM HEPES, 0.1 M NaCl, 5 \(\mu\)M CaCl\(_2\), 0.01% Tween 20, 0.01% BSA, pH 7.2 (HEPES buffer), were incubated with 20 nM thrombin for 1 h to convert FVIII to FVIIa. Samples were subjected to electrophoresis under reducing (0.1 M dithiothreitol) or nonreducing conditions using 8% polyacrylamide gels at constant voltage (150 V). Proteins were transferred to a polyvinylidene fluoride membrane and probed with an anti-A2 monoclonal antibody (R8B12), and protein bands were visualized by chemiluminescence (525 nm) using Molecular Imager Gel Doc XR+ system (Bio-Rad).

One-stage Clotting Assay—One-stage clotting assays were performed using substrate plasma chemically depleted of FVIII as previously described (15) and assayed using a Diagnostica Stago clotting instrument.

**FXa Generation Assay**—The rate of conversion of FX to FXa was monitored in a purified system (16) according to methods previously described (17, 18). FVIII (1 nM) in HEPES buffer containing 20 \(\mu\)M PSPCPE vesicles (phosphatidylserine:phosphatidylcholine:phosphatidylethanolamine, 3:2:5) was activated with 20 nM \(\alpha\)-thrombin for 1 min. Reactions were 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 that were then terminated after 1 min by the addition of 50 mM EDTA. The FXa generated was determined following reaction with the chromogenic substrate Pefachrome Xa (final concentration, 0.46 \(\mu\)M). All reactions were run at 23°C.

**FVIII Thermal Decay**—FVIII variants (4 nM) in HEPES buffer were incubated at 55°C, aliquots were removed at the indicated time points, and activity was determined using the FXa generation assay.

**FVIIIa Activity Decay**—FVIII variants (1.5 nM) in HEPES buffer 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.

**FVIIIa Subunit Binding Analysis**—FVIIIa A1 (500 nM WT or H281S) and 1 \(\mu\)M A3C1C2 (WT) subunits were incubated overnight at 4°C in 10 mM MES, 0.3 M NaCl, 0.01% BSA, 0.01% Tween 20, 25 mM Ca\(^{2+}\), pH 6.5. Mixtures were diluted 1:10 with HEPES buffer and reacted with the indicated concentration of WT or S524H A2 subunit for 30 min at 23°C. After appropriate dilution, reconstituted FVIIIa activity was measured by FXa generation assay.

**Thrombin Generation Assay**—The amount of thrombin generated in plasma was measured by calibrated automated thrombography (19) using methods previously described (20). Briefly, FVIII-deficient plasma (<1% residual activity, platelet-poor) from a severe hemophilia A patient lacking FVIII inhibitor (George King Bio-Medical, Overland Park, KS) was mixed at 37°C with a final concentration of 0.25 nM FXIII, 0.06–0.25 pm rTF, 0–2 nM TM, 4 \(\mu\)M PSPCPE vesicles, 433 \(\mu\)M fluorogenic substrate, 13.3 mM CaCl\(_2\), and with or without 105 nM thrombin.
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calibrator (for calibration). The development of a fluorescence signal was monitored at 8-s intervals using a Microplate Spectrofluorometer (Spectramax Gemini; Molecular Devices, Sunnyvale, CA) with a 355-nm (excitation)/460-nm (emission) filter set. Fluorescence signals were corrected by the reference signal from the thrombin calibrator samples (19), and actual thrombin generation in nM was calculated as previously described (20).

**FIXa Binding Affinity**—FVIII (0.5 nM) in HEPES buffer containing 20 μM PSPCPE was activated by 20 nM thrombin for 1 min, immediately reacted with hirudin (10 units/ml). Samples were then reacted with the indicated concentration of FIXa, and activity was measured by FXa generation assay.

**Michaelis-Menten Kinetics**—Thrombin-activated FVIIIa as described above (0.5 nM) in HEPES buffer containing 20 μM PSPCPE was incubated with 40 nM FIXa, and FXa generation was initiated by adding the indicated concentrations of FXa. The data were fitted to the Michaelis-Menten equation by nonlinear least squares regression, and parameter values were obtained.

**Rate of FVIII Activation by Thrombin**—FVIII (20 nM) in HEPES buffer containing 20 μM PSPCPE was incubated with 1 nM thrombin at 23 °C. At the indicated times, aliquots were taken, appropriately diluted, and mixed with hirudin (10 units/ml)/FIXa (40 nM) solution, and activity was measured by FXa generation assay.

**Inactivation of FVIIIa by APC**—FVIII (150 nM) was activated by thrombin (30 nM) in HEPES buffer for 10 min at 37 °C. After thrombin was inhibited by addition of hirudin (20 units/ml), the FVIIIa product was reacted with APC (5 nM) in the presence of phospholipid vesicles (100 μg/ml). Reactions were run at 37 °C. Aliquots were removed at the indicated time and assayed by one-stage clotting assay.

**Data Analysis**—Values for FVIII/FVIIIa activity decay as a function of time were fitted to a single exponential decay curve by nonlinear least squares regression using the following equation,

\[
A = A_0 \cdot e^{-k \cdot t}
\]  
(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 after FVIII activation when thrombin was quenched.

FVIIIa subunit binding affinity used the following equation,

\[
A = \frac{V_{\text{max}} \cdot (B + K_d + X)^2 - \sqrt{(B + K_d + X)^2 - 4 \cdot B \cdot X}}{2}
\]  
(Eq. 2)

where \( A \) is initial velocity (nM/min/nM A1), \( X \) is the concentration of A2 in nM, \( K_d \) is the dissociation constant, \( B \) is the A1 subunit concentration (50 nM), and \( V_{\text{max}} \) is the maximum activity at saturation.

The estimation of FIXa-FVIIIa binding affinity also used the above equation where \( A \) is initial velocity (nM/min/nM FVIII), \( X \) is the concentration of FIXa in nM, \( K_d \) is the dissociation constant, \( B \) is the FVIIIa concentration, and \( V_{\text{max}} \) is the maximum activity at saturation.

Rates for thrombin activation of FVIII or APC inactivation of FVIII utilized a third order polynomial equation as previously employed (21) for an unbiased estimation of the initial reaction rate,

\[
[FVIIIa] = A + Bt + Ct^2 + Dt^3
\]  
(Eq. 3)

where \([FVIIIa]\) is the FVIIIa concentration in nM, \( t \) is the time in min, \( A \) is the FVIIIa concentration at time 0 (\( A = 0 \) for thrombin activation, and \( A = 150 \) for APC inactivation), and the absolute value of \( B \) is the slope at time 0, which corresponds to rates of thrombin activation or APC inactivation. Computation for nonlinear least squares regression analysis was performed using a standard curve-fitting algorithm (Gauss-Newton algorithm using the method of Levenberg-Marquardt).

**RESULTS**

FVIII Variants Possessing a Residue Swap (H281S/S524H) and a Nascent Disulfide Bridge (H281C/S524C)—Fig. 1A shows the FVIII molecular structure model based on its intermediate resolution x-ray crystal structure (7) in a van der Waals surface
display format. Although this resolution (\(-4 \text{ Å}\)) does not allow for accurate side chain location, the model shows side chains of His\(^{281}\) (A1 domain) and Ser\(^{524}\) (A2 domain) as facing each other at the domain interface and in close proximity to the A3 domain. In an earlier study (5), both residues showed contribution to FVIII and FVIIIa stability as judged by individually replacing these residues with Ala. Because of the close proximity of these residues, we predicted and presented suggestive evidence that the side chains of His\(^{281}\) and Ser\(^{524}\) contributed to interdomain bonding interactions that would result in stabilizing the protein. To further assess the role of this interaction, the variant H281S/S524H was constructed to test the reciprocity of side chain interaction. In addition, a double Cys variant was prepared to assess whether a nascent disulfide bridge could be formed, and if so, how this could further contribute to FVIII stability and function.

Disulfide bond formation in the H281C/S524C variant was examined by SDS-PAGE run in the presence and absence of reducing agent after the FVIII was converted to FVIIIa by thrombin (Fig. 1B). Western blotting using an anti-A2 domain antibody (R8B12) showed that the A2 subunit of both the WT (lane 1) and variant (lane 2) possessed equivalent mobility migrating at \(-40 \text{ kDa}\) when samples were reduced. However, in nonreducing conditions WT A2 subunit (lane 3) migrated slightly faster than the reduced A2 sample, a result consistent with the perseverance of two intradomain disulfide bonds. On the other hand, the A2 domain of the H281C/S524C variant migrated at \(-90 \text{ kDa}\) position (lane 4), which corresponded to an A1-A2 dimer, indicating that H281C/S524C possessed an additional interdomain covalent bond resulting from disulfide bridge formation. Furthermore, the lack of detectable A2 subunit migrating at its authentic position of \(-40 \text{ kDa}\) in this sample indicated that the interdomain disulfide bond was formed with high efficiency (\(>90\%\)).

Specific activity values for the FVIII variants were examined by both one-stage and two-stage assays (Table 1). The activity of H281S/S524H was similar to WT values (86–95% of WT). H281C/S524C showed WT-like activity in one-stage assay (94%), whereas slightly reduced activity (60% WT) was observed in the two-stage assay. The reason for this disparity is not clear.

**Stability of FVIII Variants—**FVIII thermal decay at 55 °C, as measured by FXa generation assay, was used to monitor protein stability (6) and the contributions of the mutations to this parameter. Results from these experiments are shown in Fig. 2A. Results obtained for WT FVIII showed \(~60\%\) activity remaining after 10 and 18 min, respectively. The rate of FVIII activity decay was determined as described under "Experimental Procedures" and is shown in Table 2. Both variants showed similar rates of decay (Fig. 2A and Table 2) with essentially no difference observed for the H281S/S524H swap variant, whereas the rate of decay for the nascent disulfide-bonded variant was reduced by \(~20\%\) compared with WT. This latter result suggests that some enhancement in protein stability was obtained by covalently stabilizing the inter-A1-A2 domain interaction.

FVIIIa activity decay is governed by the dissociation of A2 subunit (22). Fig. 2B shows results for FVIIIa activity decay for the WT variants. FVIIIa activity of both WT FVIII and H281S/S524H FVIII showed identical rates of decay (Fig. 2B and Table 2) with \(~50\%\) activity remaining at 4 min. This result, coupled with the result above for the swap variant showing essentially identical results with the WT, provides evidence that the bond formed by residues His\(^{281}\) and Ser\(^{524}\) in the WT protein is maintained in the Ser\(^{281}/\text{His}^{524}\) variant. Furthermore, as predicted, the activity of H281C/S524C did not show any appreciable

### Table 1: Specific activity of FVIII variants

|                   | One-stage clotting | FXa generation |
|-------------------|--------------------|----------------|
|                   | units/μg           | units FXa/min/μM FVIII |
| WT                | 4.5 ± 0.5 (1)      | 44.1 ± 2.5 (1) |
| H281S/S524H       | 4.3 ± 0.4 (0.95)   | 37.8 ± 1.4 (0.86) |
| H281C/S524C       | 4.3 ± 0.3 (0.94)   | 26.2 ± 0.1 (0.59) |

**FIGURE 2.** FVIII thermal stability at 55 °C (A) and FVIIIa spontaneous decay (B). A, FVIII proteins (4 nM) were incubated at 55 °C, aliquots were taken at the indicated time points, and the activity was measured by FXa generation assay as described under "Experimental Procedures." B, thrombin-activated FVIIIa (1.5 nM) was incubated at 23 °C, aliquots were taken at indicated time points, and activity was measured by FXa generation assay as described under "Experimental Procedures." Each point represents a value averaged from three separate determinations. The data were fitted to a single exponential decay curve by nonlinear least squares regression, and solid (FVIII mutants) and dashed (WT) lines were drawn. Symbols denote WT (circles), H281S/S524H (triangles), and H281C/S524C (squares).
TABLE 2

FVIII and FVIIa stability of FVIII variants
FVIII thermal decay at 55 °C and FVIIa spontaneous decay as described under "Experimental Procedures" and plotted in Fig. 2. The data were fitted to a single exponential decay curve, and rate constant values ± S.D. were obtained. The values in parentheses are relative to the WT value. ND, not determined.

| FVIII thermal decay | FVIIa spontaneous decay |
|---------------------|-------------------------|
| rate constant       | rate constant           |
| WT                  | 0.047 ± 0.001 (1)       | 0.162 ± 0.004 (1)       |
| H281S/S524H         | 0.051 ± 0.003 (1.09)    | 0.161 ± 0.003 (0.99)    |
| H281C/S524C         | 0.033 ± 0.003 (0.79)    | ND                      |

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Thrombin Generation Potential of FVIII Variants—FVIII variant activity was assessed by thrombin generation assays performed at low rTF concentration (0.25 pm) using FVIII-deficient plasma. Fig. 4 shows a thrombogram for FVIII variants, with parameter values listed in Table 4. Control experiments without FVIII yielded essentially no thrombin generation. Thrombin generation using WT FVIII initiated at ~9 min, and peak thrombin generation (114 nM) was reached at ~22 min. A similar profile was seen for the H281S/S524H variant with slightly reduced peak and endogenous thrombin potential (ETP) values compared with WT (~0.8- and 0.9-fold, respectively). Interestingly, although initiation of thrombin generation using the H281C/S524C variant was somewhat delayed (1.7-fold compared with WT time), the peak time was similar (~1.1-fold difference). In addition, the peak thrombin and ETP values for this variant were also modestly improved (~1.4- and ~1.2-fold, respectively). In the presence of reduced concentration of rTF (0.13 and 0.06 pm), thrombin generation potential decreases in all FVIII variants (Table 4), and this effect has been previously observed (23). Although we did observe a slight increase in the ETP value for the H281C/S524C variant compared with WT FVIII at 0.06 pm rTF presence (~1.6-fold), we also examined thrombin generation in the presence of TM.
TABLE 4
Thrombin generation assay parameter values
Thrombin generation assays in the presence of 0.25 nM FVIII proteins, 4 μM PSPCPE vesicles, and either 0.06 – 0.25 pm rTF or 0.25 pm rTF plus 0 – 2 nM TM were performed, and parameter values were calculated as described under “Experimental Procedures.” The data represent the average values ± S.D. of triplicate samples. The values in parentheses are relative to the WT value obtained in the presence of 0.25 pm rTF.

|                | Latent time | Peak time | Peak value | ETP    |
|----------------|-------------|-----------|------------|--------|
|                | min         | min       | ns         | n/min  |
| WT             |             |           |            |        |
| 0.25 pm rTF    | 9.1 ± 0.3 (1)| 22.2 ± 0.9 (1)| 114.1 ± 20.3 (1)| 1284 ± 89.8 (1) |
| 0.13 pm rTF    | 13.5 ± 1.3 (1.48)| 25.0 ± 0.9 (1.13)| 72.4 ± 0.7 (0.63)| 742 ± 31.6 (0.58) |
| 0.06 pm rTF    | 17.3 ± 0.3 (1.90)| 29.4 ± 1.6 (1.33)| 58.9 ± 0.8 (0.52)| 628 ± 24.6 (0.49) |
| H281S/S524H    |             |           |            |        |
| 0.25 pm rTF    | 9.7 ± 1.3 (1.06)| 22.3 ± 0.8 (1)| 86.3 ± 3.7 (0.76)| 1162 ± 153.7 (0.9) |
| 0.13 pm rTF    | 13.1 ± 0.9 (1.44)| 24.6 ± 0.6 (1.11)| 68.0 ± 5.9 (0.60)| 755 ± 22.4 (0.59) |
| 0.06 pm rTF    | 18.1 ± 1.8 (1.99)| 31.0 ± 2.8 (1.40)| 33.1 ± 1.9 (0.29)| 438 ± 14.6 (0.34) |
| H281C/S524C    |             |           |            |        |
| 0.25 pm rTF    | 15.5 ± 1.1 (1.70)| 23.9 ± 0.1 (1.08)| 153.9 ± 7.7 (1.35)| 1595 ± 127.7 (1.24) |
| 0.13 pm rTF    | 15.6 ± 0.8 (1.49)| 24.3 ± 0.6 (1.09)| 117.5 ± 0.2 (1.03)| 1252 ± 12.3 (0.98) |
| 0.06 pm rTF    | 16.1 ± 0.6 (1.77)| 28.0 ± 0.3 (1.26)| 75.7 ± 6.3 (0.66)| 1058 ± 43.7 (0.82) |
| WT             |             |           |            |        |
| 0 nm TM        | 9.1 ± 0.3 (1)| 22.2 ± 0.9 (1)| 114.1 ± 20.3 (1)| 1284 ± 89.8 (1) |
| 1 nm TM        | 13.4 ± 0.4 (1.48)| 22.9 ± 0.1 (1.04)| 49.9 ± 6.5 (0.44)| 429 ± 32.3 (0.33) |
| 2 nm TM        | 16.4 ± 0.8 (1.81)| 25.4 ± 0.3 (1.15)| 29.4 ± 1.8 (0.26)| 329 ± 15.6 (0.26) |
| H281S/S524H    |             |           |            |        |
| 0 nm TM        | 9.7 ± 1.3 (1.06)| 22.3 ± 0.8 (1.00)| 86.3 ± 3.7 (0.76)| 1161 ± 153.7 (0.90) |
| 1 nm TM        | 13.9 ± 0.9 (1.53)| 22.7 ± 0.5 (1.02)| 35.0 ± 0.6 (0.31)| 319 ± 16.6 (0.25) |
| 2 nm TM        | 16.2 ± 1.1 (1.78)| 25.2 ± 1.0 (1.14)| 17.6 ± 2.3 (0.15)| 167 ± 12.1 (0.13) |
| H281C/S524C    |             |           |            |        |
| 0 nm TM        | 15.5 ± 1.1 (1.70)| 23.9 ± 0.1 (1.08)| 153.9 ± 7.7 (1.35)| 1595 ± 127.7 (1.24) |
| 1 nm TM        | 15.1 ± 0.3 (1.44)| 23.3 ± 0.5 (1.05)| 64.0 ± 5.7 (0.56)| 676 ± 46.1 (0.53) |
| 2 nm TM        | 17.9 ± 1.3 (1.96)| 26.9 ± 0.8 (1.21)| 27.9 ± 5.9 (0.24)| 325 ± 68.6 (0.25) |

which serves as a cofactor for thrombin-catalyzed activation of protein C to yield APC (24–26). As expected, in the presence of TM, thrombin generation potential decreases in all FVIII variants because of formation of APC and its catalyzed inactivation of FVIIa (Table 4). Interestingly, in the presence of TM (2 nm), the reduction of the ETP values for the H281S/S524H and H281C/S524C variants were somewhat greater (80 and 86%, respectively) than that for the WT (74%) when compared with results in the absence of TM. The reason for this difference is not clear. Taken together, these results show that the disulfide-bridged variant yields greater thrombin generation potential than either the WT or swap variant, consistent with enhanced retention of A2 subunit in this FVIIa form. Furthermore, WT and both variants show reduced thrombin generation parameter values in response to the presence of TM, consistent with down-regulation of these FVIIa forms by APC.

**FIXa Binding Affinity**—To further confirm our hypothesis that residues His$^{281}$ and Ser$^{524}$ are interactive, we performed several FVIII functional tests. Functional affinity of FVIII variants for FIXa was measured by titrating thrombin-activated FVIIa (0.5 nM) with the indicated concentrations of FIXa followed by activity measurement by FIXa generation assay. The reconstituted FXase activity values were plotted as a function of FIXa concentration, and the results are shown in Fig. 5A. FXase activity of FVIII WT and variants increased to a saturable level as FIXa concentration was increased. From the fitted curves, $K_d$ values (Table 5) for both the H281S/S524H swap variant and the H281C/S524C disulfide bridged variant showed only modest reduced affinity values for FIXa (~2- and ~1.8-fold) as compared with WT FVIII.

**Michaelis-Menten Kinetics**—Michaelis-Menten kinetics of the FXase complex reflecting catalysis of substrate FX was measured as another assessment for the structural integrity of the FVIII variants (Fig. 5B). In all FVIII variants, the activity titrated with FX showed hyperbolic curves that were saturable. Estimated $K_m$ values for the H281S/S524H and H281C/S524C variants (Table 5) were slightly lower than WT values (~0.7- and ~0.5-fold, respectively), indicating that the mutations did not impair interactions of the cofactor with FX substrate.

**Thrombin Activation Efficiency**—FVIII contains thrombin cleavage sites at Arg$^{372}$, Arg$^{40}$, and Arg$^{1648}$, with the former site being rate-limiting (1). Cleavage at these sites converts the inactive FVIII procofactor to the active FVIIIa cofactor. Thrombin activation rates were monitored by FXa generation assays for the WT and FVIII variants, and results are shown in Fig. 5C. The cofactor activity values, obtained as rates of generation of FXa, were converted to FVIIIa concentration using individual specific activity values. FVIII activation rates occurred over a rapid time course with reactions complete within 1 min. Estimated rates of activation were calculated based upon the slope values at time 0 (Table 5) and suggested essentially no difference among the FVIII variants and WT. Collectively, the above FVIII functional characterization did not yield any evidence suggestive of structural alteration(s) caused by mutation.

**FVIIIa WT and Variant Inactivation by APC**—APC inactivation experiments were conducted by adding APC to a high concentration FVIII (150 nM) that had been completely activated to FVIIa by thrombin (30 nM for 10 min at 37°C). This high FVIIa level was used to minimize the loss of activity caused by spontaneous dissociation of A2 subunit. Fig. 5D shows the rate of loss of FVIIIa activity following the addition of 5 nM APC and incubation at 37°C. Control experiments in the absence of APC yielded essentially no activity reduction (<5%) after 30 min of
incubation, and these values were used to correct for the non-proteolytic decay in the APC-containing reactions. Using these reaction conditions, WT FVIIIa activity was rapidly inactivated by FXa showing activity attributed to ~75 nM FVIIIa remaining in 4 min. Both H281S/S524H and H281C/S524C variants showed slightly increased sensitivity to APC inactivation, with

![FIGURE 5. Functional analyses of FVIII variants. A, FVIIIa and FIXa association kinetics were analyzed by incubating thrombin-activated FVIII variants (0.5 nM) with the indicated concentrations of FIXa followed by activity measurements using FXa generation assays as described under "Experimental Procedures." Each point represents the value averaged from three separate determinations. The data were fitted to the quadratic equation by nonlinear least squares regression, and dashed (WT) and solid (mutants) lines were drawn. B, Michaelis-Menten kinetics of FXase complex using indicated concentrations of substrate FX were analyzed as described under "Experimental Procedures." Each point represents the value averaged from three separate determinations. The data were fitted to Michaelis-Menten equation by nonlinear least squares regression, and dashed (WT) and solid (mutants) lines were drawn. C, thrombin activation efficiency of FVIII measured as a rate of thrombin activation (slope value at time 0) was quantified by FXa generation assay as described under "Experimental Procedures." FVIII variants (20 nM) were incubated with 1 nM thrombin at 23 °C. The data were fitted to third order polynomial equation by nonlinear least squares regression, and dashed (WT) and solid (mutants) lines were drawn. D, APC (5 nM) inactivation of FVIIIa (150 nM) in the presence of phospholipid (100 μg/ml) was determined by a one-stage clotting assay as described under "Experimental Procedures." Active FVIIIa concentration is plotted as a function of time. Activity values were corrected for the spontaneous decay of FVIIIa in the absence of added APC, which accounted for less than 5% loss over the 30-min time course. Each point represents a value averaged from three separate determinations. The data were fitted to a second order polynomial equation by nonlinear least squares regression, and dashed (WT) and solid (FVIII mutants) lines and were drawn. Symbols denote WT (circles), H281S/S524H (triangles), and H281C/S524C (squares).

| TABLE 5 | Functional characterization of FVIII variants |
|---|---|
| **Initial velocity of FXa generation as a function of either FIXa concentration or FX concentration** was measured as described under “Experimental Procedures” and plotted in Fig. 5 (A and B, respectively). The data were fitted to either quadratic equation or Michaelis-Menten equation, respectively, by nonlinear least squares regression, and parameter values were obtained. The amount of FVIIIa generation from each FVIII variant (20 nM) by a limiting amount of thrombin (1 nM) was measured by FXa generation as described under “Experimental Procedures,” and the data are plotted in Fig. 5C. Factor VIIIa inactivation by 5 nM APC was performed as described under “Experimental Procedures,” and the data are plotted in Fig. 5D. The data in Fig. 5 (C and D) were fitted to a third order polynomial equation by nonlinear least squares regression and initial velocity (rate at time 0) was obtained. The values in parentheses are relative to the WT value. |
| **FIXa affinity** | **Michaelis-Menten analysis** | **Thrombin activation efficiency** | **APC inactivation rate** |
| $K_a$ | $V_{max}$ | $K_a$ | $V_{max}$ | $K_{D}$/nM | $V_{max}$/nM FXa/min/nM FXa | $V_{max}$/nM FVIIIa/min/nM thrombin | $V_{max}$/nM APC/min $^{-1}$ |
| WT | 0.39 ± 0.07 (1) | 43.5 ± 1.79 (1) | 0.42 ± 0.10 (1) | 42.7 ± 2.8 (1) | 4.65 ± 0.28 (1) |
| H281S/S524H | 0.78 ± 0.10 (1.98) | 38.1 ± 1.56 (0.88) | 27.6 ± 2.4 (0.66) | 35.8 ± 1.0 (0.78) | 53.9 ± 6.9 (1.15) | 8.42 ± 0.35 (1.81) |
| H281C/S524C | 0.70 ± 0.15 (1.77) | 27.4 ± 1.68 (0.63) | 22.3 ± 2.6 (0.53) | 28.1 ± 0.9 (0.61) | 50.1 ± 6.0 (1.07) | 8.05 ± 0.43 (1.73) |
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~50 nm FVIIIa remaining in 4 min. The estimated inactivation rates of the H281S/S524H and H281C/S524C variants were slightly increased (~1.8- and ~1.7-fold relative to the WT value, respectively; Table 5). This slight increase in sensitivity to APC inactivation was consistent with the increase in inhibitory effect on thrombin generation potential in the presence of TM observed for the swap variant (Table 4) and indicated that both variants were efficiently inactivated by APC in a purified system.

DISCUSSION

The results presented in this study provide strong experimental evidence that the side chains of His^{281} and Ser^{524} participate in a bonding interaction in FVIII that is maintained following activation of FVIII to FVIIa. Although the x-ray structures of FVIII (8, 9) shows a close proximity of the residues and suggests a potential interaction, the ~4 Å resolution of these structures does not provide adequate resolution to unequivocally localize side chain positions. Furthermore, our earlier results (7) showing reductions in FVIII and FVIIa stability with the individual H281A and S524A point mutants did not prove a bonding interaction of the side chains. In the current study, the FVIII swap variant H281S/S524H showed essentially equivalent FVIII and FVIIa decay rates as the WT protein, whereas results from an earlier study (7) showed that these parameter values were increased ~2.5- and 4-fold, respectively, in the H281A variant and ~9-fold, and ~7- to ~40-fold, respectively, in the S524A variant. Furthermore, FVIIIa reconstitution studies described in the current report and used to estimate apparent Kd values for the intersubunit interactions show an ~2-fold reduced affinity of the swap mutant compared with WT, whereas ~5- and ~10-fold reduced affinity values were determined following reconstitution assays using the H281S and S524H point mutants, respectively. Thus, these data indicate a compensatory interaction in the double swap mutation compared with the single mutations. Taken together, these results provide direct evidence for the side chains of these residues contributing to an interdomain/subunit bond in FVIII/ FVIIa. We speculate that this bond is a hydrogen bond involving an imidazole nitrogen of His and the hydroxyl group of Ser.

The bonding interaction involving His^{281} and Ser^{524} was made covalent in the disulfide-bridging variant, H281C/S524C. Interdomain disulfide bond formation in this variant showed high efficiency as judged by Western blotting of the thrombin-activated variant. This result supported the close spatial location of the side chains of the two residues. In addition, the results from several functional analyses indicated that this covalent linkage was not detrimental to FVIII activity. First, this variant exhibited a WT-like specific activity by one-stage clotting assay. However, there was a reduction in the specific activity as measured by the two-stage assay (~60% of WT value). This modest reduction may be explained by the altered Cα distance between the native His-Ser interaction relative to the Cys-Cys interaction because they are not exactly equal and may yield a subtle change in structure. However, the reason for the effect observed only with the two-stage assay is not clear. Second, the thermal stability of FVIII was actually slightly (~10%) improved in the H281C/S524C variant as compared with WT, suggesting that FVIII heavy chain and light chain interactions were not globally altered. In addition, analyses of procofactor activation and formation and kinetics of FXase showed similar parameter values for the variant compared with WT FVIII. Finally, WT and variant FVIII were similarly down-regulated in a hemophilia A-plasma-based thrombin generation assay supplemented with TM, as well as the down-regulation of FVIIIa forms showing similar rates of inactivation by APC in a purified system. These results further suggest that the interactive interfaces of this mutant FVIII relative to binding FXα, FX, and thrombin, and APC were not globally altered.

Activation of the H281C/S524C FVIII variant yielded a FVIIIa form that demonstrated high stability relative to the WT FVIII. This is clearly illustrated by results presented in Fig. 2B that show essentially no cofactor activity loss over the time course measured. This result was expected inasmuch as FVIIIa decay is governed by dissociation of the A2 subunit, and this subunit is covalently linked to the A1 subunit in the H281C/ S524C variant. Our earlier results have shown that stabilizing FVIIIa by enhancing noncovalent interactions yields improved thrombin generation parameter values including peak thrombin levels and ETP (27). The basis for this improvement is the persistence of FXase activity resulting from the increased affinity of A2 subunit in FVIIa. In the present study, we observed 35 and 24% increases in peak thrombin and ETP, respectively, in the disulfide-bridged variant compared with WT FVIII, consistent with the improved stability of FVIIa. These increases were observed even though the specific activity of this variant appeared somewhat reduced compared with WT. This enhancement in thrombin generation parameters was consistent with an earlier study where the A2 subunit was stabilized following disulfide bridging with the A3 subunit (28).

The Cα distance separating His^{281} and Ser^{524} is 5.9 Å according FVIII x-ray crystal structure (7). This value falls within the range of possible Cα distances at disulfide bond forming Cys residues (4–7 Å) (29). There are several reports describing successful recombinant FVIII variant expression after introducing nascent interdomain disulfide bridges. In an earlier study, disulfide bridges in FVIII linking A2 and A3 domains at Met^{662} and Asp^{1826} and at Tyr^{664} and Thr^{1826} were formed given Cα distances for the pairings of 9.1 and 8.8 Å, respectively (30). We previously produced FVIII variants with an additional disulfide bond separating Arg^{211} and Leu^{2300} (Cα distance = 7.7 Å) at A1-C2 domain interface (10), as well as between Ala^{1866} and Ser^{2119} (Cα distance = 6.5 Å) and between Gly^{1730} and Arg^{2116} (Cα distance = 5.4 Å) at the A3-C1 domain interface and between Ser^{2029} and Pro^{2029} (Cα distance = 7.3 Å) at the C1-C2 domain interface (31). Interestingly, in the above report, we also prepared seven additional FVIII variants with double Cys mutations that did not express and/or showed no disulfide bond formation, although Cα distance values for five of the seven variants ranged from 4 to 7 Å. Thus, formation of disulfide-bridged mutants may not be successful even given the optimal Cα distance separation. This may reflect uncertainties in the predicted structures because of low resolution as well as other variables. Together, these observations indicate that preparation of these FVIII reagents may be somewhat empirical.
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The activation of FVIII is thought to result in modest changes in conformation that include full exposure of FIXa interactive site(s) in the A2 domain and changes in A2 orientation to allow for binding to FIXa (see Ref. 1 for review). Results from the present study indicate that the bond between His\textsuperscript{281} and Ser\textsuperscript{524} linking A1 and A2 domains is retained in FVIIa. In contrast, in our earlier study (5) examining hydrogen bonding interactions within the A domains, we noted several residues including Asp\textsuperscript{666}, Tyr\textsuperscript{1786}, and Tyr\textsuperscript{1792} that potentially participate in bonding interactions involving A2 with A3 domain affected FVIIa stability with little if any effect on FVIII stability. Thus, these residues may modulate the structural changes that occur with procofactor activation. Based on these observations, we speculate that bonding interactions between A1 and A2 domains exist for the most part in the procofactor and are primarily retained following activation, whereas the interactions of residues in the A2 and A3 domains are more likely to be formed following activation in response to a change in A2 subunit orientation. This speculation is consistent with residues in the A2 and A3 domains forming an extended surface for the interaction of FVIIa with FIXa in the FXase complex (32).

Individual residue interactions at the FVIII domain interfaces are crucial determinants for FVIII stability and FVIIIa function. The currently available FVIII structure information obtained by x-ray crystallography is of intermediate resolution (6, 7). Although these structural data and derived models have been extremely useful for many aspects of FVIII research, coordinates for individual residues need to be interpreted with caution. The combined mutagenesis and biochemical approach presented here is useful to experimentally confirm the coordinates for the bonding residues, as well as provide important information for functional roles of these bonding interactions.

Acknowledgments—We thank Lisa M. Regan of Bayer Corporation for the gifts of recombinant human FVIII, Pete Lollar and John Healey for the FVIII cloning and expression vector, and Jennifer Wintermute for excellent technical assistance.

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