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An engineered interdomain disulfide bond stabilizes human blood coagulation factor VIIIa

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Summary. The blood coagulation disorder, hemophilia A, is caused by deficiency of coagulation factor (F)VIII. Hemophilia A is now treated by infusions of pure FVIII, but the activity of FVIII is limited because it is unstable following activation by thrombin. This instability of activated FVIII is the result of dissociation of the A2 subunit. To obtain increased stability in FVIIIa, a disulfide bond between the A2 domain and the A3 domain, preventing A2 subunit dissociation, has been engineered. Structural analysis of the FVIII A domain homology model allowed us to identify residues 664 and 1826 as a potential disulfide bond pair. A FVIII mutant containing Cys664 and Cys1826 was produced and purified (C664-C1826 FVIII). Immunoblotting showed that a disulfide bond did form to link covalently the A2 and the A3 domains. Following activation of the recombinant C664-C1826 FVIII by thrombin, the mutant FVIIIa had increased stability and retained more than 90% of its clotting activity at a time at which wild-type FVIIIa lost more than 90% of its activity. This remarkably stable C664-C1826 FVIIIa provides a unique approach for studies of the cofactor activity of FVIIIa and also for new, improved therapy for hemophilia A.

Keywords: disulfide bond, factor VIII, molecular modeling, protein engineering.

Deficiency of the blood coagulation factor (F)VIII is responsible for the hemorrhagic bleeding disorder hemophilia A. Hemophilia A is an X-linked genetic disorder and affects approximately 1 in 5000 males [1]. FVIIIa, the activated form of FVIII, is the cofactor for FIXa in the intrinsic pathway of the

coagulation cascade. Deficiency of FIX also causes hemophilia (hemophilia B). Though the severity of hemophilia varies depending on the mutation causing the defect of FVIII or FIX, commonly there is risk of life-threatening bleeding episodes, particularly as a result of trauma or surgery. It can also result in permanent disability as a result of chronic bleeding into muscles and joints [2]. Hemophilia can now be mostly controlled by infusions of purified plasma-derived or recombinant FVIII or FIX. However, this treatment is expensive and plasma-derived clotting factors carry some risk of viral transmission. Recombinant clotting factors are even more expensive [2].

FVIII is a large, 2332 amino acid protein with a sequential domain structure from NH₂ to COOH termini designated A1-a1-A2-a2-B-a3-A3-C1-C2. The A domains are homologous to one another and additionally are homologous to the A domains of FV and ceruloplasmin [3]. A molecular model of the FVIII A domains was made based on the structure of ceruloplasmin [4].

Thrombin activates FVIII by catalyzing proteolytic cleavages at Arg372, Arg740 and Arg1689. This produces an active heterotrimer, FVIIIa, which consists of an A1 subunit (residues 1–372), an A2 subunit (residues 373–740) and the thrombin-cleaved light chain (residues 1690–2332) [5,6]. FVIIIa is quite unstable due to spontaneous dissociation of the A2 subunit and has a half-life of approximately 2 min [7–9]. Additionally, the anticoagulant protein, activated protein C, cleaves FVIIIa at Arg336 and Arg562 resulting in inactive FVIIIa [5,10,11].

Because of the high cost of FVIII replacement therapy, improvements in the stability of FVIIIa could provide significant therapeutic benefit. Previously, we introduced a disulfide bond between the A2 and A3 domains of FV by site-directed mutagenesis, using a molecular model of FVa to identify a potential site for disulfide bond introduction [12]. This disulfide bond variant produced a fully active FVa molecule that was significantly more resistant to inactivation by activated protein C [13]. In FVIIIa a similar disulfide bond would prevent spontaneous dissociation of the A2 subunit, the primary mechanism for the instability of FVIIIa. Studies have been done with chemical crosslinks of FVIIIa, in which apparent inter-

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subunit crosslinks stabilized the resulting FVIIIa [14,15]. In the present study we identified several potential disulfide bond pairs that could potentially link the A2 subunit to the rest of the molecule. One of these, between residues 664 and 1826, aligns in primary sequence with the successful disulfide bond pair created in FV at residues 609 and 1691 [13]. Therefore, we made the double mutant, Tyr664-Cys, Thr1826-Cys (C664-C1826 FVIII) and then analyzed the mutation's effects on FVIIIa stability.

Materials and methods

FVIII-deficient plasma and pooled normal human plasma were purchased from George King Biomedical Inc. (Overland Park, KS, USA). Biotinylated goat antimouse IgG, and streptavidin-horseradish peroxidase conjugate were purchased from Pierce (Rockford, IL, USA). Recombinant FVIII, the plasmid p25D, the FVIII A2-domain monoclonal antibody R8B12 [7] and the FVIII monoclonal antibody C7F7 [16] were gifts from Bayer Corporation (Research Triangle Park, NC, USA). Refacto B domain-deleted FVIII was from Wyeth-Parma GmbH (Munster, Germany).

The program MODIP [17] was used to identify sites for introduction of disulfide bonds in the homology model of the FVIII A domains [4]. Proposed disulfides were constructed into the FVIIIa A domains model using Xfit [18] and refined in X-PLOR [19] using the Charmm22 all atom force field as described previously [13]. The plasmid p25D expresses B domain-deleted FVIII in which residues 744–1637 from the B domain are deleted. The double cysteine mutant was created in this plasmid using the Quikchange mutagenesis kit from Stratagene (La Jolla, CA, USA) by simultaneous addition of four mutagenic primers, which mutated Tyr664 and Thr1826 to Cys residues (codon TGT). Mutations were confirmed by DNA sequencing.

Wild-type and mutant plasmids were cotransfected into HEK293 cells with the plasmid pRSVneo [20] using the Effectene transfection reagent according to the manufacturer's instructions (Qiagen, Santa Clarita, CA, USA). Stable clones were selected with G418 and screened for FVIII expression. FVIII was collected in conditioned media containing 5% fetal bovine serum and was immediately filtered through filter paper and protease inhibitors were added (5 mM benzamidine, 0.5 mM PMSF, 2 nM PPACK). Conditioned media was stored at -80°C until purification.

For purification, a $10 \times$ buffer stock was added to conditioned media containing recombinant FVIII at final conditions of 0.01 M imidazole, 0.7 M NaCl, 0.01 M CaCl_2 , 0.01% Tween 20, pH 7.0. This media was then loaded onto a 2-mL anti-FVIII antibody C7F7-CPG resin column equilibrated in the above buffer. The column was washed in the same buffer and eluted with 0.01 M imidazole, 0.5 M NaCl, 1.0 M CaCl_2 , 0.01% Tween 20, pH 7.0. Fractions containing FVIII were pooled, concentrated and switched into HBS (50 mM HEPES, 150 mM NaCl, pH 7.4) with 0.1% bovine serum albumin (BSA), using Amicon Centricons.

FVIII antigen level was determined using the Immubind Factor VIII ELISA kit from American Diagnostica (Greenwich, CT, USA) using the standard protocol and FVIII standards provided in the kit. FVIII activity level was determined using the Chromogenix Coatest VIII:C/4 kit (Diapharma Group, Inc., West Chester, OH, USA) with pooled normal plasma used for the standard curve. The protocol was followed as described in the kit except that samples were assayed in Corning/Costar half-area 96-well plates and volumes were reduced accordingly.

FVIIIa stability following thrombin activation was measured in a standard activated partial thromboplastin time (APTT) clotting assay as follows. Different preparations of FVIII at approximately $350\text{--}450\text{ mU mL}^{-1}$ were incubated in HBS/0.5% BSA/5 mM CaCl_2 . At the zero time an aliquot was removed and assayed in the APTT assay, then thrombin was added to 200 ng mL^{-1} final concentration. After 1 min, hirudin was added to 1 U mL^{-1} final concentration to inactivate the thrombin. Then aliquots were removed at time points and assayed in the APTT assay. The APTT assay was run as follows. FVIII-deficient plasma ($50\text{ }\mu\text{L}$) was mixed with $50\text{ }\mu\text{L}$ Platelin LS (bioMérieux, Inc., Durham, NC, USA) and incubated at 37°C for 3 min. A $5\text{-}\mu\text{L}$ aliquot of FVIIIa was added, immediately followed by $50\text{ }\mu\text{L}$ of HBS/0.5% BSA/25 mM CaCl_2 . Clotting time was measured in the Diagnostica Stago ST4 coagulometer. A FVIII standard curve was made using pooled normal human plasma as the FVIII source.

Immunoblots were done as follows. Samples were run on an Invitrogen Bis/Tris 4–12% pre-poured gel with MOPS-SDS buffer (Invitrogen, Carlsbad, CA, USA). The gel was transferred to PVDF membrane in a BioRad Semi-dry Blot apparatus (BioRad, Hercules, CA, USA). The blots were developed with the monoclonal antibodies R8B12, specific for the FVIIIa A2 domain, and C7F7, specific for the N-terminus of the light chain. The secondary antibody was biotinylated goat antimouse IgG and the bands were visualized with streptavidin-conjugated horseradish peroxidase and Pierce Supersignal West-Pico chemiluminescent substrate.

Results and discussion

Our previous successful introduction of a disulfide bond between the A2 and A3 domains of the homologous FVa [13] provided confirmation that existing comparative models, based on ceruloplasmin, were accurate. The disulfide-crosslinked FVa protein had normal affinity for FXa and the prothrombinase complex created with the disulfide-crosslinked FVa had normal kinetic properties for cleavage of prothrombin, suggesting a native-like conformation for disulfide-crosslinked FVa. Therefore, we hypothesized that introducing a disulfide crosslink into the homologous FVIIIa will yield a stable native-like molecule that may have improved therapeutic properties for treatment of hemophilia A.

The program MODIP, which predicts sites for introduction of disulfide bonds, predicted 15 potential sites between the A2 domain and either the A1 or A3 domains. One pair that was identified, Tyr664 and Thr1826, aligned exactly with the

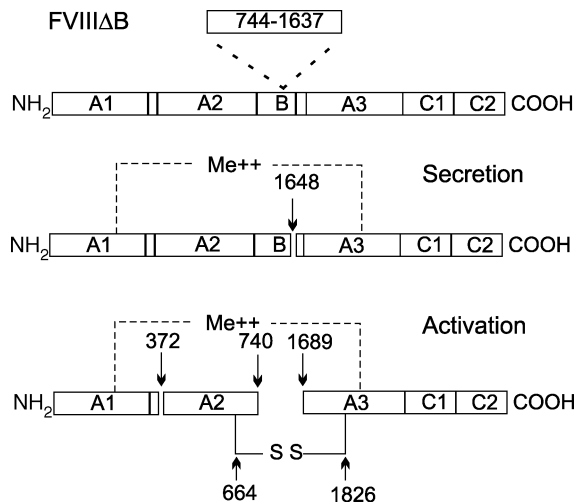


Fig. 1. FVIII activation and inactivation cleavages. FVIII Δ B has much of the B domain deleted (residues 744–1637) but all of the critical activating and inactivating cleavage sites remain. During the course of intracellular processing and secretion much of FVIII is cleaved at Arg1648 in the B domain. FVIII is activated primarily by thrombin via cleavages at Arg372, Arg740 and Arg1689 resulting in a heterotrimer that requires divalent metal ions to remain complexed.

cysteine pair successfully introduced into FVa to create a disulfide bond (609–1691 [13]). Therefore, this pair was selected for mutagenesis. Figure 1 shows a schematic of the FVIII Δ B construct we used with the location of the engineered disulfide bond. The 664–1826 disulfide bond crosslinks the A2 domain within its C-terminal half to the A3 domain in the light chain.

To evaluate potential artefacts in the engineering of the 664–1826 disulfide bond, we analyzed naturally occurring cysteine residues in the model of FVIIIa A domains. It should be noted that the FVIIIa A domains share 34% sequence identity with ceruloplasmin. Although certain details of the model are expected to contain some inaccuracies, in particular the interatomic distances, sequence alignment and interresidue distances in the model are reasonably well defined at this level of similarity. In each A domain, there are two disulfide bonds and a free cysteine residue [21]. According to the FVIIIa model, Fig. 2 shows the closest distances between the 664–1826 disulfide bond and other disulfide or free cysteine residues. The closest distance to any cysteine side chain, the disulfide bond between Cys1832 and Cys1858, is about 15 Å. Similarly, the closest distance between residues 664–1826 to any free cysteine residue (692 in the A2 domain) is about 21 Å. Assuming that interresidue distances are accurate in the FVIIIa model, these distances are incompatible with the formation of a mispaired disulfide bond, between either Cys664 or Cys1826 and any other cysteine residue. In other words, no alternate conformation of any cysteine side chains could allow the formation of an artefact disulfide bond without significant disruption of the native fold of FVIIIa. Furthermore, because of the tight packing at the interface between the A domains, rearrangements that would lead to artefact disulfide bonds are also unlikely. In fact, in experimental systems in which folding of disulfide bonded proteins has been studied, it has universally been found that

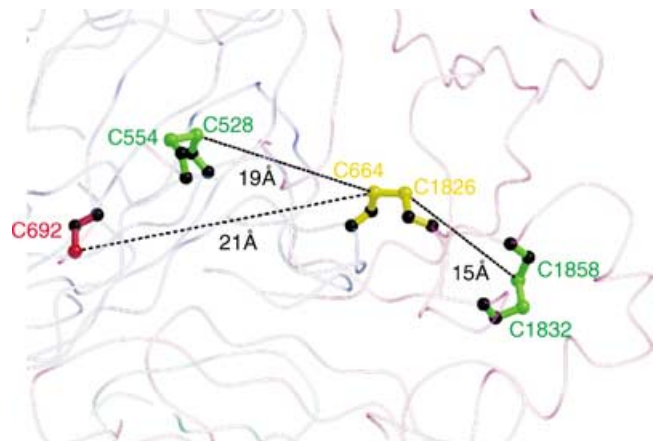


Fig. 2. Location of cysteine residues in FVIIIa A domains near the engineered 664–1826 disulfide bond. A trace linking each C α atom of the FVIIIa A domain backbone is drawn (transparent colors) with domain A2 in blue (on the left), and domain A3 in magenta (on the right). Cysteine residues are displayed as balls and sticks where black balls represent carbon atoms and colored balls sulfur atoms. Green sticks represent native FVIIIa disulfide bonds whereas red sticks represent native FVIIIa free cysteine residues. The engineered disulfide bond 664–1826 is drawn in yellow sticks in the center. The closest distances between residues 664–1826 and other cysteine residues are shown as dotted black lines where the distances are between sulfur atoms.

only the correct disulfide bonds appear to allow formation of stable tertiary structure and stable misfolded proteins have not been observed [22]. Judging by the excellent clotting activity of C664–C1826 FVIII, disruption of the native fold is improbable. Therefore, although further biochemical characterization of the C664–C1826 disulfide bond is required, we feel that it is unlikely that an incorrect disulfide has formed in this molecule.

Both B domain-deleted wild-type FVIII and C664–C1826 FVIII were purified from conditioned media in parallel, as described in Materials and methods. Expression levels were low, with 10–20 mU mL⁻¹ activity in the conditioned media (1 unit equals the amount of FVIII in 1 mL of plasma). However, these levels are similar to levels seen in unamplified transient transfections of other FVIII variants [23] and recombinant human FVIII does typically express poorly in mammalian tissue culture [24]. Certainly our low levels of expression are not specifically due to the introduced disulfide bond, as the wild-type B domain-deleted FVIII also had the same low levels of expression. Following purification, wild-type B domain-deleted FVIII and C664–C1826 FVIII were assayed for antigen with the Immubind Factor VIII ELISA kit using the FVIII standards provided with the kit. Purification yielded 1 mU per mL of conditioned media of pure wild-type B domain-deleted FVIII and 3 mU per mL of conditioned media of pure C664–C1826 FVIII (from 2 to 3 L of conditioned media). Given the low expression and recovery of recombinant FVIII it was not possible to analyze samples on protein-stained gels for purity or for quantification of specific peptide species. In addition, it was necessary to add BSA to improve recovery. Therefore, we assume that these FVIII preparations are only partially pure. These purified FVIII preparations were also assayed for FVIII

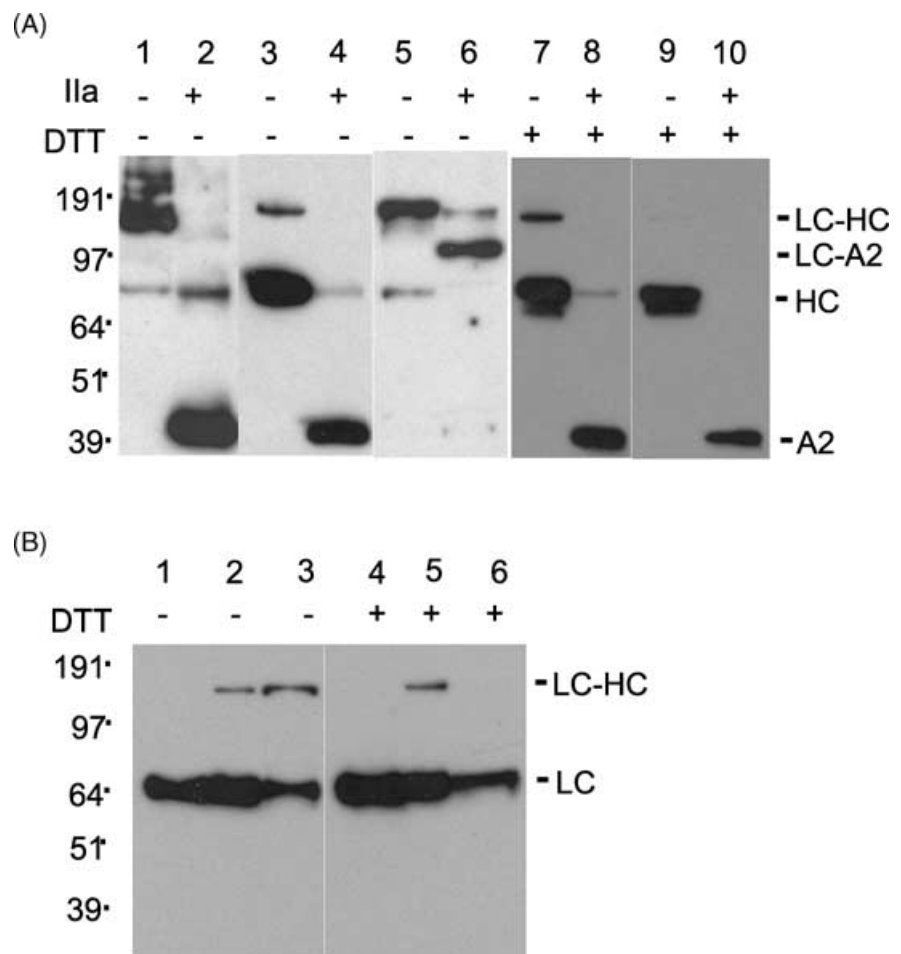
activity with the chromogenic Coatest VIII:C/4. Based on these antigen assays and activity assays, wild-type B-domain-deleted FVIII had 0.95 units of activity per unit of antigen and C664-C1826 FVIII had 1.06 units of activity per unit of antigen. Therefore, introduction of Cys664 and Cys1826 did not affect the activity of the B-domain-deleted FVIII.

To demonstrate that the desired interdomain disulfide bond did form between Cys664 and Cys1826, we immunoblotted FVIII and thrombin-activated FVIIIa. We used recombinant full-length FVIII (from Bayer), wild-type B domain-deleted FVIII (Refacto FVIII from Wyeth) and C664-C1826 FVIII. Figure 3A shows an immunoblot using the anti-A2 domain monoclonal antibody R8B12. Before activation, full-length FVIII showed a cluster of bands from >200 to 130 kDa representing heterogeneous heavy-chain species containing various parts of the B domain (lane 1). Following activation with thrombin, two bands appeared, at 86 kDa representing the A1 and A2 subunits together (heavy chain) and at 40 kDa, representing the A2 subunit alone (lane 2). Wild-type B domain-deleted FVIII (lane 3) primarily showed a heavy-chain band at 86 kDa but also showed a band corresponding to light chain and heavy chain together at an apparent molecular weight of 140 kDa. This was probably single-chain B domain-deleted FVIII. Thrombin activation of wild-type B domain-deleted

FVIII resulted in a single band at 40 kDa, representing the A2 domain (lane 4). Disulfide-crosslinked C664-C1826 FVIII in lane 5 contained an intense band at 140 kDa representing the heavy chain (A1-A2) crosslinked to the light chain and a faint band representing the heavy chain at 86 kDa. Following activation by thrombin, the 140-kDa band shifted to 108 kDa, representing the A2 subunit crosslinked to the light chain (lane 6, see schematic in Fig. 1). This suggests that the predicted disulfide bond is present in C664-C1826 FVIII. However, some amount of C664-C1826 FVIII appeared not to have a disulfide bond formed, since some heavy chain alone was visible in lane 5 and some A2 domain alone was visible in lane 6. The presence of a small percentage of FVIIIa without a disulfide bond should not taint the therapeutic value of the disulfide-crosslinked FVIIIa. Non-disulfide-crosslinked C664-C1826 FVIIIa retains the same activity as normal FVIIIa and should be inactivated via the same dissociation mechanism by which normal FVIIIa is inactivated.

In lanes 7 through 10 the samples in lanes 3 through 6 were repeated except that they were reduced with DTT. Wild-type B domain-deleted FVIII/FVIIIa (lanes 7 and 8) were unchanged following reduction. For wild-type B domain-deleted FVIII in lane 7 the light chain-heavy chain band that was present in lane 3 was still present after DTT reduction. This ruled out the possibility that the band was due to a disulfide crosslink as was

Fig. 3. Immunoblot of various FVIII and FVIIIa mutants. FVIII and FVIIIa activated by thrombin were immunoblotted according to Materials and methods. (A) Immunoblot with a monoclonal antibody against the A2 domain (R8B12). Lanes 1 through 6 are non-reduced and lanes 7 through 10 are reduced with DTT. Lane 1, Full length FVIII; lane 2, FVIIIa; lanes 3 and 7, Refacto B domain-deleted FVIII; lanes 4 and 8, Refacto B domain-deleted FVIIIa; lanes 5 and 9, C664-C1826 FVIII; lanes 6 and 10, C664-C1826 FVIIIa. (B) Immunoblot with a monoclonal antibody against the FVIII light chain (C7F7). Lanes 1 through 3 are non-reduced and lanes 4 through 6 are reduced with DTT. Lanes 1 and 4, Full-length FVIII; lanes 2 and 5, Refacto B domain-deleted FVIII; lanes 3 and 6, C664-C1826 FVIII. Molecular weight standards are indicated on the left. LC-HC, Light chain crosslinked to heavy chain (A1-A2); LC-A2, light chain crosslinked to the A2 subunit; HC, heavy chain; LC, light chain; A2, A2 subunit.



seen with C664-C1826 FVIII. This band was also visible in Fig. 3B, lanes 2 and 5, again in the absence and presence of DTT. This supports the assumption that this is probably a small amount of single-chain B domain-deleted FVIII, but in lane 9 the 140-kDa band was replaced by an 86-kDa band (heavy chain alone) and in lane 10 the 108-kDa band was replaced by a 40-kDa band (A2 subunit alone) for reduced C664-C1826 FVIII/FVIIIa. This confirmed that the predicted crosslink in C664-C1826 FVIII was due to a disulfide bond.

Figure 3B shows an immunoblot developed with the antilight chain antibody, C7F7. This antibody recognizes the N-terminus of the FVIII light chain that is cleaved off by thrombin during FVIII activation. Therefore, it only recognizes the FVIII light chain, not the FVIIIa light chain. Lanes 1 and 2 show the light chain of full-length FVIII and wild-type B domain-deleted FVIII at 70 kDa. As mentioned, lane 2 also shows a small amount of single-chain B domain-deleted FVIII. Lane 3 is C664-C1826 FVIII and shows two bands, the light chain at 70 kDa and a band at 140 kDa that was the same molecular weight as the light chain-heavy chain band visualized by the anti-A2 domain antibody. Therefore, the crosslinked band seen in Fig. 3A, lane 5 did contain light chain, as predicted. Lanes 4 through 6 repeated the samples in lanes 1 through 3 except that they were reduced with DTT. In lane 6 the light chain-heavy chain crosslink band disappeared and only the light chain alone was visible at 70 kDa. As in Fig. 3A, this confirmed that the crosslink was due to a disulfide bond. In Fig. 3A, lane 5 appeared to show that a majority of the C664-C1826 heavy chain was crosslinked, whereas in Fig. 3B, lane 3 appeared to show that a majority of the C664-C1826 light chain was not crosslinked. Clearly, one or both of these monoclonal antibodies has altered sensitivity for the disulfide-crosslinked FVIII relative to non-crosslinked FVIII. Therefore, these Western blots cannot be used to quantify the proportion of FVIII that is crosslinked. Although the amount of crosslinked C664-C1826 FVIII is unknown, the high stability suggests that most of it is crosslinked.

To monitor the stability of recombinant FVIII variants, we followed the activity of FVIII preparations following activation by thrombin. Figure 4 shows a time course of FVIII activation by thrombin and subsequent spontaneous inactivation. Under the conditions used recombinant wild-type FVIII increased 28-fold in activity following thrombin activation. Refacto wild-type B domain-deleted FVIII increased in activity 32-fold, our wild-type B domain-deleted FVIII increased in activity 27-fold and C664-C1826 FVIII increased in activity 37-fold (Fig. 4). These variations in fold-activation are not significantly different from each other. Over the course of 40 min full-length wild-type FVIIIa and Refacto B domain-deleted FVIIIa were spontaneously reduced to about 5% of their highest activity. Our wild-type B domain-deleted FVIIIa was reduced to 10% of its highest activity, while C664-C1826 FVIIIa retained 90% of its highest activity (Fig. 4). C664-C1826 FVIIIa was also monitored for as long as 3 h without additional loss of activity (not shown). Therefore, the addition of the double cysteine mutation, which apparently leads to formation of a disulfide bond between

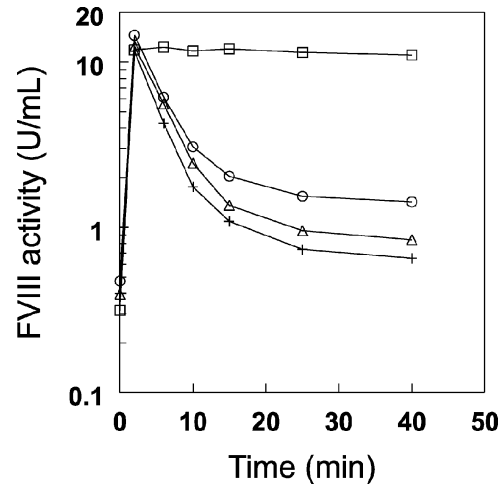


Fig. 4. Activation and inactivation of recombinant FVIII. FVIII activity of the wild-type and mutant FVIII was followed over time with an activated partial thromboplastin time (APTT) clotting assay as described in Materials and methods. (+) Full-length FVIII; (Δ) Refacto B domain-deleted FVIII; (○) our B domain-deleted FVIII; (□) C664-C1826 FVIII. APTT times were converted to units of FVIII activity based on a standard curve of pooled normal human plasma. These data are averages of two experiments.

the A2 domain and the A3 domain, resulted in a variant of FVIIIa with increased stability. It is possible that the loss observed down to 90% was due to the fraction that was not disulfide crosslinked. However, due to the low quantity of recombinant FVIIIa, it was not yet possible to quantify the fraction of disulfide-crosslinked and non-disulfide-crosslinked molecules. Detailed kinetic analyses need to be performed to determine if the C664-C1826 FVIIIa has normal kinetic properties, as was observed for the similar disulfide-crosslinked FVa. But the similarity in specific activity and activation levels by thrombin (see below) for wild-type B domain-deleted FVIII and C664-C1826 FVIII suggests that the kinetic properties are at least similar if not unchanged.

Pipe and Kaufman created an inactivation-resistant FVIIIa variant, termed IR8, in which the A2 domain was fused to the A3 domain by a 53 amino-acid polypeptide linker that replaced the B domain and in which the two APC cleavage sites at Arg336 and Arg562 were also mutated [23]. In contrast to IR8, our protein did not have any mutations of the APC inactivating cleavage sites. However, the disulfide-crosslinked FVIIIa from this work is still more resistant to spontaneous inactivation than the IR8 variant (90%, compared with about 50% for IR8 in an APTT assay). This suggests that there are structural differences between C664-C1826 FVIIIa and IR8 FVIIIa that affect the FVIIIa function. One possible explanation for the functional difference is that the long polypeptide linker in IR8 has considerably more degrees of freedom than a disulfide bond. Therefore, it is possible that the A2 domain in the IR8 construct is still able to dissociate from its native conformation, thus reducing FVIIIa activity.

In summary, we have tested the hypothesis that formation of a disulfide bond between the A2 domain and the A3 domain

would result in stabilization of the resulting FVIIIa variant. Our data suggest that this is the case. Presumably this stabilization results from prevention of the spontaneous dissociation of the A2 subunit that normally takes place. However, future studies with this FVIII variant are needed to characterize its cofactor properties in detail, as well as the chemical details of the engineered disulfide bond. C664-C1826 FVIII should be a valuable tool for detailed functional analyses of the interaction of FVIIIa with FIXa, FX and APC, because these interactions can be analyzed without the complications caused by the spontaneous inactivation of the wild-type FVIIIa. This molecule may also provide the basis for an improved therapy for hemophilia A patients by virtue of reducing the amount of FVIII that needs to be transfused and also possibly reducing the number of times that FVIII needs to be transfused to achieve normal hemostasis. Furthermore, this variant of FVIII with increased stability may be a valuable tool for correction of hemophilia A through genetic therapy, since presumably less FVIII would need to be expressed to attain functional correction of the FVIII deficiency.

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