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Review

Coagulation factor VIII: structure and stability

Wei Wang*, Y. John Wang, Drew N. Kelner[1]

Analytics & Formulation, Biotechnology Division, Bayer Healthcare, 800 Dwight Way, Berkeley, CA 94701, USA

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Abstract

Factor VIII (FVIII), a coagulation factor in the blood, is one of the most complex proteins known today. To facilitate the rapid development of a more convenient and safer FVIII product and to improve the quality of life for hemophilia patients, this short article reviews the recent investigations on the structure, activity, and more importantly, stability of FVIII.

Keywords: FVIII; Structure; Stability; Formulation; Aggregation; Oxidation

1. Introduction

Factor VIII (FVIII) is an essential coagulation factor in the blood. In the complex blood clotting cascade, it serves as a co-factor for factor IXa (FIXa) in the activation of factor X (FX) to factor Xa (FXa). A deficiency or defect in FVIII is the cause of classical hemophilia (type A), a hereditary life-threatening bleeding disorder. Currently, the only therapy for this hereditary disease is life-long administration of a FVIII product. However, the limited in vivo stability of FVIII requires frequent drug administration for both preventive and therapeutic purposes and the limited in vitro stability of FVIII requires lyophilization of FVIII for long-term storage, creating inconvenience for self-administration and compromising the quality of life for hemophilia patients.

To overcome the need for frequent drug administration, FVIII gene therapy is being widely investigated for hemophilia treatment, which, if successful, may provide the ultimate therapy for hemophilia. Recent results showed that a high level of FVIII could be expressed in FVIII-deficient dogs using an adenoviral vector, but FVIII expression lasted only 5–10 days and all treated dogs developed liver toxicity, a transient drop in platelets, and anticanine FVIII antibodies (Gallo-Penn et al., 2001). While development of an effective and safe FVIII gene product is still in progress, it will likely be many years until such a therapeutic approach can be implemented in humans.

The limited in vivo/vitro stability of FVIII and slow progress in the development of a FVIII gene product is at least partly due to the relatively large size and complexity of the FVIII molecule. To facilitate the rapid development of a more convenient and safer FVIII product, this short article summarizes recent investigations on FVIII structure and stability.

2. FVIII structure and activity

In this section, we briefly discuss the complex FVIII structure and its activity, as such topics have been extensively reviewed in the past (Fay, 1993; Lollar,

* Corresponding author. Tel.: +1-510-705-4755; fax: +1-510-705-5629.
E-mail address: wei.wang.b@bayer.com (W. Wang).
[1] Present address: Amgen Inc., One Amgen Center Dr., Thousand Oaks, CA 91320, USA.

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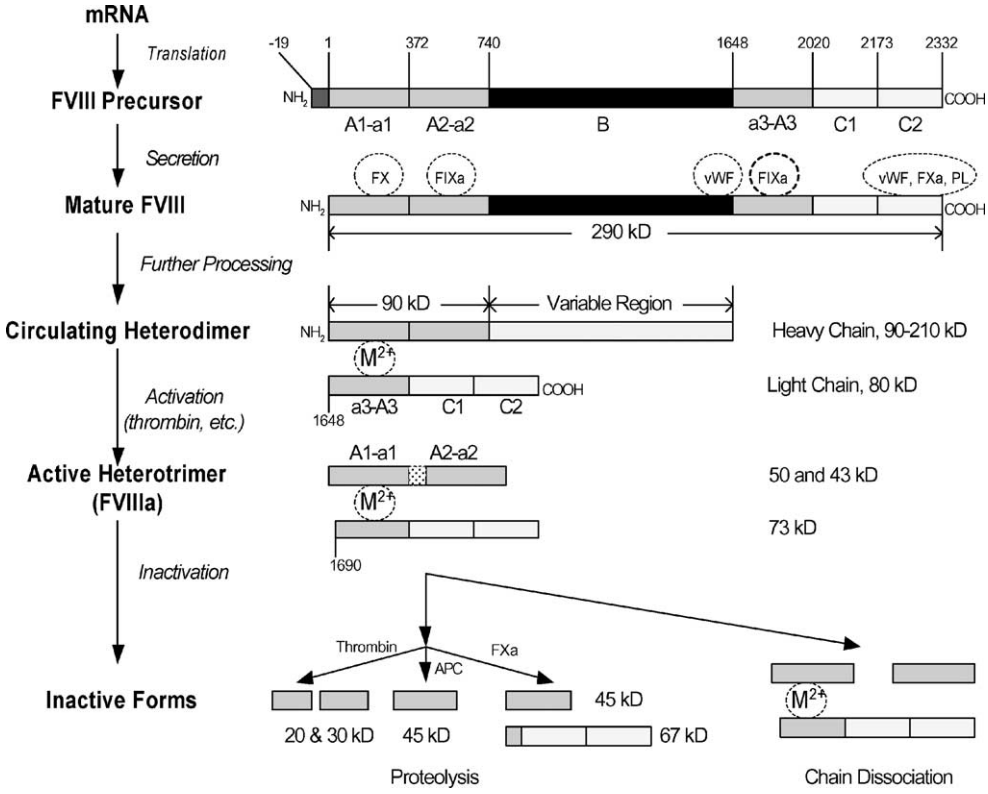

Fig. 1. FVIII structure, function, and processing. The sites of FVIII interaction with other clotting factors, vWF, phospholipids (PL), and metal ions (M^{2+}) are illustrated by dotted circles.

1995; Lenting et al., 1998). A schematic representation of FVIII structure, function and processing is shown in Fig. 1.

2.1. Primary structure

The primary structure of human FVIII was first deduced based on the DNA sequence in 1984 (Gitschier et al., 1984; Vehar et al., 1984). The X-linked FVIII gene, which has a size of 186 kb including 26 exons (Peake, 1995), encodes a polypeptide of 2351 amino acids. After processing of the signal peptide (19 amino acids), a mature FVIII molecule of 2332 amino acids is secreted with a calculated molecular weight of 264,763 Da. FVIII protein species of up to 330 kDa were observed on SDS–PAGE under non-reducing conditions, probably due to extensive glycosylation and/or disulfide crosslinking (Vehar et al., 1984). Based on homology analysis, the FVIII sequence is divided into three A, two C and a large B domain(s), abbreviated as NH_2-A1-A2-B-A3-C1-C2-COOH (Vehar et al., 1984). The three A domains are connected by three short acidic sequences: a1 (337–372); a2 (711–740); and a3 (1649–1689). Thus, a more detailed FVIII sequence is NH_2-A1-a1-A2-a2-B-a3-A3-C1-C2-COOH and the respective sequences are A1-a1: 1–372; A2-a2: 373–740; B: 741–1648; a3-A3: 1649–2020; C1: 2021-2173; and C2: 2174-2332 (Fig. 1).

Commercial FVIII concentrates showed on SDS–PAGE as mixtures of FVIII heterodimers, consisting of 90–210 kDa heavy chain (HC; A1-A2-B) and 80 kDa light chain (LC; A3-C1-C2) (Eaton et al., 1986; Bihoreau et al., 1991). Less than 1% was found to be single chain molecules on SDS–PAGE either for plasma-derived FVIII (pdFVIII) (Stoylova et al., 1999) or baby hamster kidney (BHK)-derived rFVIII (Eaton et al., 1987). The heterogeneous heavy chain

polypeptides, minimally represented by A1-A2, are derived from the 210 kDa polypeptide through proteolysis of the B-A3 junction and within the B-domain.

FVIII molecules contain many glycosylation sites and the B-domain has 19 of the 25 Asn-glycosylation sites (N-linked) (Vehar et al., 1984). Although these sites have not been completely delineated, the six Asn-glycosylation sites outside the B region are located at 41, 239, 582, 1685, 1810 and 2118 (Sandberg et al., 2001). In addition, human FVIII has multiple Ser- and Thr-glycans (O-linked) (Pittman et al., 1994; Kumar et al., 1996). Due to the complexity of extensive glycosylation and heterogeneity, the 90–210 kDa proteins of either rFVIII or pdFVIII could not be focused using several gel systems under both native and denaturing conditions, but the 80 kDa protein was focused to a pI of 6.5 and a cluster of pI's from 6.9 to 7.2 (Eaton et al., 1987).

There are six tyrosine sulfation sites in rFVIII. Three are located at 346 (HC), 1664, 1680 (LC) and the others are located at 718, 719, and 723 in the C-terminal of A2 subunit (Pittman et al., 1992; Severs et al., 1999). Although tyrosine sulfation seems to be required for normal FVIII activity, possibly by contributing to thrombin binding (Pittman et al., 1992; Pemberton et al., 1997), partial or full sulfation at Tyr718, Tyr719 and Tyr723 in B-domain-deleted two-chain FVIII did not affect the activation by thrombin (Kjalke et al., 1995). FVIII also contains a total of 23 cysteine residues and 19 of them (3 free and 8 disulfide bonded) are in the A and C domains (McMullen et al., 1995).

2.2. Interactions between heavy and light chains

It has long been believed that a metal ion is responsible for the association between the light and heavy chains of FVIII (Fay et al., 1986). This conclusion is based on the fact that chelation of the metal ion with EDTA leads to dissociation of the heterodimer with a concomitant loss of the procoagulant activity of a variety of FVIII forms, such as pdFVIII (Stoylova et al., 1999), rFVIII (Eaton et al., 1987) and a B-domain-deleted rFVIII (Bihoreau et al., 1991). Additional evidence for an existing metal ion in FVIII is the regeneration of 80% activity of EDTA-inactivated FVIII upon addition of excessive $MnCl_2$ (50 mM) and 30% activity for $CaCl_2$ (50 mM) (Eaton et al., 1987). A generation of 30% activity was also observed through re-association of HC and LC subunits upon addition of 25 mM Ca^{2+} (Wakabayashi et al., 2001).

Although a metal ion seems to be present in FVIII, the identity, number and role of the metal ion(s) in FVIII are still debatable. An early study with atomic absorption spectrophotometry indicated the presence of calcium in the highly-purified pdFVIII at an apparent molar ratio of 1:1 while other metals such as manganese, strontium, zinc, aluminum, magnesium, copper and iron were not present in a significant amount (<0.05 mol/subunit) (Mikaelsson et al., 1983). It was concluded that human FVIII circulates in normal plasma as a calcium-linked protein complex. In 1994, the metal was identified to be copper at a molar ratio of 1:1 in both pdFVIII heterodimers (ranging from 90/80 to 210/80 kDa dimers) and recombinant FVIII-ΔII, a B-domain-deleted FVIII (Bihoreau et al., 1994). The copper ion remained in the inactive dimer of 50/70 kDa until being released upon dissociation of this dimer, suggesting its essential role in the association of FVIII heavy and light chains. The presence of copper(I) in FVIII at a similar binding ratio was later confirmed by electron paramagnetic resonance spectroscopy (EPR) and supported by site-directed mutation studies (Tagliavacca et al., 1997). This study suggested the location of Cu(I) within the A1-domain and also demonstrated that Cu(I), not Cu(II), was able to reconstitute FVIII activity from dissociated FVIII chains. Therefore, it is possible that both copper and calcium ions are involved in HC/LC association.

In addition to metal-induced association, hydrophobic interactions between the heavy and light chains also play a significant, if not dominant, role. Two hydrophobic sites were identified by (1,1′)-bi-(4-anilino)naphthalene-5,5′-disulfonic acid (bis-ANS) binding in the heavy and light chains and the higher affinity site on each isolated subunit contributes to the divalent metal ion-dependent, intersubunit interaction (Sudhakar and Fay, 1996). A more recent study showed that the high-affinity hydrophobic association of the heavy and light chains ($K_d = 53.8$ nM) was enhanced by approximately 100-fold in the presence of 0.5 μM Cu^{2+} (Wakabayashi et al., 2001). Since Ca^{2+} at 25 mM did not affect the hydrophobic association but increased the specific activity (~60% of native FVIII activity) in this study, it appears that Ca^{2+} promotes the formation of the active conformation of

FVIII while Cu^{2+} primarily enhances the intersubunit affinity. The affinity enhancement of a metal ion may explain why low ionic strength promotes association of isolated LC and HC subunits (Donath et al., 1995).

2.3. Secondary and tertiary structure

FVIII contains mainly β-sheet structure (Sudhakar and Fay, 1998a,b; Grillo et al., 2001). A small amount of α-helices are located in the A domains (Stoilova-McPhie et al., 2002). A detailed analysis shows that B-domain-deleted rFVIII SQ contains 41% β-sheet, 14% α-helix, 26% random structure, and 19% turn by circular dichroism (CD) while the respective percentages of B-domain-deleted pdFVIII are 43, 13, 27, 18% (Fatouros and Sjostrom, 2000). In comparison, the light chain of human FVIII had 36% β-sheet, 22% α-helix, and 42% unordered structure by CD (Bihoreau et al., 1992). Activation of FVIII to FVIIIa increases significantly its β-sheet content by CD (Curtis et al., 1994).

Due to the complexity and heterogeneity of the purified FVIII, a complete high-resolution three-dimensional crystal structure of FVIII has not been determined. A few studies, however, have been conducted to characterize the overall shape of the molecule, to determine the detailed structure of its individual domains (Pratt et al., 1999), and recently, to deduce the three-dimensional structure based on low-resolution electron crystallography of two-dimensional crystals of B-domain-deleted FVIII bound to phospholipids (Stoilova-McPhie et al., 2002).

Based on the structure of nitrite reductase and ceruloplasmin, the three A domains in human FVIIIa have been modeled and the proposed structure contains 6 β-barrels (D1 through D6, two per domain) of 987 amino acids in total, roughly in a sphere size of 16 nm in diameter (Pan et al., 1995). The size is very close to what was observed for FVIII heterodimers under an electron microscope, a globular structure (contributed from A and C domains) of ∼14 nm in diameter with a two-stranded tail of different lengths (contributed from B-domain) (Fowler et al., 1990), but a smaller surface coverage of 8 nm × 5 nm was observed by electron crystallography for one pdFVIII molecule when crystallized on a phospholipid membrane (Stoylova et al., 1999). The proposed model successfully explains the location of six disulfide bonds, the copper-binding site(s), the activated protein C (APC) cleavage site, and an inhibitor epitope. However, the copper-binding sites are debatable based on another modeling study (Pemberton et al., 1997).

2.4. FVIII activity

FVIII itself has minimal or no detectable procoagulant activity before proteolytic activation by thrombin or FXa (Lollar et al., 1993; Donath et al., 1995). The rate and extent of FVIII activation depend on reaction conditions, such as the relative concentrations of FVIII and thrombin or FXa. Maximal thrombin-induced activation was observed at 0.5–1 min (Sandberg et al., 2001), 2 min (Fay et al., 1986), and 10 min (Wood et al., 1984) with a corresponding potentiation of FVIII activity of approximately 20-, 13–15-, and 40-fold, respectively. As high as 40-fold (Fay et al., 1991a,b,c) or even 80-fold (Eaton et al., 1987) increase in FVIII activity was observed. Yet, the role of Ca^{2+} in the activation of FVIII by thrombin has not been clearly established (Eaton et al., 1986; Wakabayashi et al., 2001). The activated FVIII (FVIIIa) binds to FIXa at a 1:1 molar ratio (Duffy et al., 1992; Lenting et al., 1994) through multiple interactive sites in the A2 and A3 domains (Lollar et al., 1993; Fay et al., 1994; O'Brien et al., 1995; Pemberton et al., 1997; Bajaj et al., 2001). FVIIIa, FIXa (a serine protease), Ca^{2+}, and phospholipid constitute the FXase complex. FVIIIa increases the k_{cat} of FIXa-dependent conversion of FX by several orders of magnitude (Fay and Koshibu, 1998).

FVIIIa is a heterotrimer consisting of 73, 50, and 43 kDa fragments on SDS–PAGE (Vehar et al., 1984; Eaton et al., 1986, 1987). The association among the three FVIIIa subunits has not been clearly defined. Data suggest that a metal ion links the A1 and A3-C1-C2 subunits (Bihoreau et al., 1994) with a significant contribution from hydrophobic interaction (Sudhakar and Fay, 1996), while A2 is likely to interact primarily with A1 subunit ionically (Fay et al., 1991a,b,c, 1999). Because of the weak interaction between A1 and A2, the associated heterotrimer readily dissociates at physiologic pH (Fay and Koshibu, 1998). The dissociated individual subunits have less than 1% of the FVIIIa activity (Fay and Koshibu, 1998).

The large B-domain in FVIII (741–1648) does not contribute to the activity of FVIII and has no

known functions (reviewed by Fay, 1993). Several B-domain-deleted or partially deleted FVIII forms have been engineered, including $FVIII_{des\text{-}797–1652}$ (Eaton et al., 1986), $FVIII_{des\text{-}771–1666}$ (FVIII-ΔII, 165 kDa) (Bihoreau et al., 1991), and $FVIII_{des\text{-}760–1639}$ (LA-VIII) (Pittman et al., 1993). FVIII SQ, another B-domain-deleted FVIII variant in which Ser743 is linked directly to Gln1638, is now an approved commercial product (Sandberg et al., 1991, 2001). These variants do have similar clotting activity as the intact FVIII, such as $FVIII_{des\text{-}760–1639}$ (Pittman et al., 1993), and FVIII SQ (Sandberg et al., 2001). Due to their smaller molecular masses, their specific activities are generally higher than that of the full-length molecule (Eaton et al., 1987; Bihoreau et al., 1991; Sandberg et al., 2001).

3. In vivo FVIII stability

FVIII has limited in vivo stability, which is influenced by many factors, including binding to plasma proteins and antibodies, proteolytic inactivation, and non-proteolytic degradation. The half-life of high-purity FVIII in man was determined between 15 and 19 h (Ludlam et al., 1995). A single-phase in vivo decay was observed after infusion of FVIII concentrate with added calcium chloride into a hemophiliac (Foster et al., 1988).

3.1. Stabilization by von Willebrand Factor (vWF)

FVIII interacts non-covalently with vWF in plasma via at least two binding sites. A high-affinity vWF-binding site ($K_d = 2.1 \times 10^{-10}$ M) is located in A3-domain (Lys1673-Arg1689), interacting with only 1–2% of the vWF subunits (Leyte et al., 1989). The acidic region (residues 1649–1689) maintains the optimal conformation (for maximal binding) of the high-affinity vWF-binding site (Saenko and Scandella, 1997). Another vWF-binding site is located in residues 2173–2332 of the C2-domain, which also binds to phospholipids (Saenko et al., 1994; Saenko and Scandella, 1997; Jacquemin and Saint-Remy, 1998). The stoichiometry between FVIII and vWF varies depending on the study conditions (Vlot et al., 1995).

FVIII interaction with vWF results in significant stabilization and survival of FVIII in the circulation (see review by Sadler, 1998). Possible stabilization mechanisms include (1) protection of the proteolytic sites, such as Arg1689; (2) promotion of association of the heavy and light chains (Wise et al., 1991); and (3) inhibition of FVIII neutralization by FVIII antibodies (Jacquemin and Saint-Remy, 1998). These stabilization mechanisms may explain why the stability of FVIII SQ in homogenates of human subcutaneous tissue was greater in the presence of vWF (Fatouros et al., 2000) and the activity of rFVIII in immuno-depleted plasma was higher after addition of vWF (Barrowcliffe, 1994).

3.2. Effect of thrombin

Thrombin not only activates but also inactivates FVIII. Thrombin activation of either pdFVIII or rFVIII generates three major polypeptides of 73, 50, and 43 kDa on SDS–PAGE (Vehar et al., 1984; Eaton et al., 1986, 1987). Typical thrombin cleavage sites in FVIII are at Arg followed by either Ser or Ala (Vehar et al., 1984). The 73 kDa fragment comes from cleavage at Arg1689 (light chain) while the other two come from cleavages of Arg372 (between A1 and A2), and Arg740 (between A2- and B-domain) (Vehar et al., 1984; Eaton et al., 1987; Donath et al., 1995; Lenting et al., 1998). Therefore, FVIIIa is a heterotrimer of A1-a1/A2-a2/A3-C1-C2. It was noted, however, that maximum activity was actually observed some time after generation of these fragments (Eaton et al., 1986).

Thrombin-induced inactivation of FVIIIa depends on the actual study conditions, relative amount of thrombin, and the length of thrombin treatment, but not on the size of the heavy chain (Fay et al., 1986). It was demonstrated that thrombin-activated rFVIII could be stable for at least 1 h at 37 °C in one study (Eaton et al., 1987), but in a different study, treatment of human FVIII with an adequate amount of thrombin inactivated FVIII in 30 s in a buffer at pH 7.4 (Lollar et al., 1992). Even at −80 °C, loss of activity of thrombin-activated FVIII could be observed with a $t_{1/2}$ of approximately 1 week (Curtis et al., 1994).

The exact mechanism of thrombin inactivation is still not clear. At least one inactivation pathway is the conversion of 50 kDa polypeptide in FVIIIa to 20 and 30 kDa fragments but the inactivation does not corre-

late well with the formation of these two fragments by SDS–PAGE (Vehar et al., 1984). In fact, FVIIIa inactivation by thrombin was not always associated with additional cleavage (Fay et al., 1986). For example, the activity of both activated wild-type FVIII and $FVIII_{des\text{-}760–1639}$ dropped significantly 1 min after thrombin treatment but the intensity of the three major bands did not seem to change significantly in 20 min (Pittman et al., 1993). Similarly, no apparent cleavage was observed by SDS–PAGE between 5 and 22 min after thrombin treatment, but FVIIIa activity dropped in this period (Bihoreau et al., 1991). It was suggested that the non-proteolytic dissociation of the A2 subunit may be responsible for the apparent inactivation (Lollar et al., 1992).

3.3. Effect of protein C and FXa

As a feedback control for clotting, APC inactivates both FVIII and FVIIIa, while FXa both activates and inactivates FVIII like thrombin (Vehar et al., 1984; Eaton et al., 1986, 1987). APC only converts the heavy chain (90–210 kDa proteins) to a 45 kDa fragment while FXa converts the active 73 and 50 kDa fragments to 67 and 45 kDa fragments (Eaton et al., 1987). The APC-induced inactivation disrupts the interaction between A1 and A2/A3-C1-C2 subunits (Persson et al., 1995) and correlates well with the generation of the 45 kDa fragment (Eaton et al., 1986). APC-induced cleavages occur both at Arg336-Met337 (A1) and Arg562-Gly563 (A2) but it is the preferable cleavage at Arg562 that closely correlates with the loss of co-factor activity (Fay et al., 1991a,b,c; Regan et al., 1994, 1996). The binding site for APC has been localized to a region on the A3-domain of the light chain (Walker et al., 1990).

3.4. Chain dissociation—the non-proteolytic degradation

FVIII also degrades in vivo by chain (A2 subunit) dissociation—a non-proteolytic degradation mechanism, which is due to the reversible and weak interaction between A2 and the metal ion-linked A1/A3-C1-C2 dimer (Lollar et al., 1992). The chain dissociation is independent of FVIII light chain cleavage (Donath et al., 1995), but dependent on salt concentration (Fay et al., 2001) and inhibited by the presence of FIXa and phospholipid (Curtis et al., 1994). Indeed, at low salt concentrations, the FVIIIa-enhanced k_{cat} ($5.5\,min^{-1}$) for conversion of FX to FXa is approximately eight-fold greater than at a near physiological ionic strength ($0.7\,min^{-1}$) because salt interferes with the association of the A2 and A1 subunits (Fay et al., 2001). Although the relative contribution of chain dissociation to the overall inactivation of FVIII has not been determined, the higher affinity of the porcine A2 subunit for human or porcine A1/A3-C1-C2 heterodimer partly explains the higher activity of porcine FVIII relative to the human counterpart (Lollar et al., 1992; Curtis et al., 1994). Increasing the resistance to chain dissociation through mutagenesis can make FVIII more stable (Pipe and Kaufman, 1997).

4. In vitro FVIII stability

All current FVIII products are lyophilized because of its limited in vitro stability in the liquid state. This section will discuss (1) stability of FVIII in lyophilized and liquid states, during infusion, and in isolated plasma; (2) factors affecting FVIII stability; and (3) mechanisms of FVIII instability.

4.1. Stability of FVIII in lyophilized state

The stability of lyophilized FVIII products depends largely on the presence of protein stabilizers. Kogenate® and Recombinate are two representative full-length rFVIII products, that contain human serum albumin (HSA). Because of the excellent stabilizing effect of HSA, these lyophilized products are fairly stable. As much as 95% of FVIII activity can be recovered in Recombinate after storage at 30 °C for 36 months (Parti et al., 2000). To prevent potential HSA-associated pathogen exposure, the latest FVIII products are devoid of this excipient, including Kogenate® FS and ReFacto. Although a 2-year storage stability at 5–8 °C has been achieved, the overall stability of these HSA-free products is significantly compromised (Osterberg et al., 1997, 2001). An optimal formulation for lyophilized FVIII still needs to be explored with the goal of achieving a comparable stability that is afforded by HSA.

4.2. Stability of FVIII in liquid state

In contrast to lyophilized formulation, the stability of FVIII in solution is very limited. It was demonstrated by the one-stage clotting assay that as much as 10% clotting activity of the full-length rFVIII was lost in 3 days at 37 °C (Grillo et al., 2001). If rFVIII inactivation follows the Arrhenius relationship and we assume a maximum rate reduction of four times per 10 °C drop in temperature, the estimated shelf life of rFVIII at 5–8 °C is approximately 6 months, insufficient to make a commercial product. In a recent study, rFVIII SQ was shown to be stable (no loss of activity) for a year at 5 °C in a solution under nitrogen containing sucrose at 300 mg/ml and Tween 80 at 2000 ppm (Fatouros and Sjostrom, 2000). Such stability demonstrated for rFVIII SQ may result from the effects of the excipients and/or the deletion of the B-domain. The potential effect of excipients was illustrated in a study where loss of 20% FVIII activity took from less than 5 days to over 28 days at room temperature for 15 reconstituted commercial FVIII products (Martinowitz, 1994; Schulman et al., 1994). The stability difference among different FVIII products suggests strong excipient effects and that improvement of FVIII stability is feasible at least for certain products. Nonetheless, because of the limited stability of FVIII in solution, a liquid FVIII product has not been commercialized. Such a future product would greatly facilitate drug administration for patients.

4.3. Stability of FVIII during infusion

The attempt to administer FVIII by continuous infusion prompted investigations on the stability of FVIII during a simulated process. All FVIII products after reconstitution should be used within the manufacturer's recommended time period (mostly 4 h or less) for both sterility and stability reasons. However, many reconstituted products can be infused beyond this period as the stability of reconstituted FVIII is usually significantly higher than what is recommended (Martinowitz, 1994; Schulman et al., 1994; Belgaumi et al., 1999). As discussed before, FVIII stability after reconstitution varies significantly among different FVIII products. Even for the same product, the FVIII stability during infusion can be strongly affected by two additional factors—the type of delivery device and the final FVIII concentration for infusion (Martinowitz, 1994; DiMichele et al., 1996). For example, while the stability of a reconstituted FVIII concentrate (Monoclate-P®) showed no detectable degradation at room temperature in 15 days with the WalkMed 350 and CADD 1 minipump, only 70% activity was left in the Medex 2001 (Martinowitz, 1994). In another study, it was demonstrated that storing 1.4 ml of reconstituted rFVIII (Kogenate®) at 146 IU/ml in 100 ml polyvinylchloride (PVC) mini-bags for 48 h at room temperature resulted in a 42% recovery of FVIII activity but only 1.8% of activity remained when 20 ml of saline-diluted rFVIII at 2 IU/ml was stored under the same conditions (McLeod et al., 2000). The loss of FVIII activity is likely due to surface adsorption and/or surface-induced denaturation (also see Section 4.4). Therefore, inclusion of a surfactant in the reconstitution medium may be necessary to prevent the loss of protein during infusion.

4.4. Stability of FVIII in isolated plasma

Currently, there are still a significant number of pdFVIII products on the market. Therefore, proper preservation of FVIII activity in plasma is crucial for a high yield. Many stability studies have been conducted in the past but the reported stability of FVIII in plasma varied significantly. For example, approximately 54% of FVIII activity was lost in citrated plasma in 24 h at 4 °C in one study (Pepper et al., 1978), but it took the same time period at 21 °C to lose the same amount of FVIII activity in another (Cumming et al., 1987). The apparent difference in FVIII stability in plasma probably results from different concentrations of citrate used during collection, presumably due to citrate chelation of calcium ions (Krachmalnicoff and Thomas, 1983; Mikaelsson et al., 1983; Morgenthaler et al., 1985; Cumming et al., 1987; Woodhams et al., 2001). The citrate-induced detrimental effect can be reversed in a short period of time after blood collection (e.g. 4–12 h) upon recalcification (Krachmalnicoff and Thomas, 1983; Morgenthaler et al., 1985). Indeed, $CaCl_2$ stabilizes FVIII in plasma and at least 1 mM (physiological level) of $CaCl_2$ is needed to achieve a significant effect (Mikaelsson et al., 1983). Similarly, chelation of calcium by addition of >2 mM EDTA in plasma can lead to a rapid loss of activity (Krachmalnicoff and

Thomas, 1983; Mikaelsson et al., 1983; Woodhams et al., 2001) and re-addition of excessive calcium (25 mM $CaCl_2$) in citrated plasma can recover the lost activity to the control level (Krachmalnicoff and Thomas, 1983). The decay of FVIII activity in citrated plasma is non-linear (Weiss, 1965; Pepper et al., 1978; Krachmalnicoff and Thomas, 1983; Mikaelsson et al., 1983), but it does not seem to involve significant participation of proteases (Mikaelsson et al., 1983). Therefore, stabilization of FVIII in plasma does not need addition of a protease inhibitor(s) but rather, careful control of the citrate and/or calcium concentration.

4.5. Factors affecting in vitro FVIII stability

Many factors have been identified affecting in vitro FVIII stability, including temperature, presence of metal ions, salts, lipids or other formulation excipients, surface adsorption, pH, shaking, light exposure, freeze-thawing/freeze-drying, and packaging conditions.

4.5.1. Temperature

Lyophilized FVIII products tolerate thermal stress reasonably well because of the presence of a stabilizing excipient(s). Due to the stabilizing effect of HSA, 99% activity of full-length rFVIII was recovered after exposure of the lyophilized product at 40 °C for 6 months and 94% at 60 °C for 2 months (Parti et al., 2000). Dry heat treatment of pdFVIII concentrates at 80 °C for 72 h did not cause significant change in FVIII structure (Gilles et al., 1997; Raut et al., 1999). In contrast, FVIII is very sensitive to temperature change in a liquid state. For example, the rate of FVIII decay at pH 9.1 in plasma increases three-fold per 10 °C increase in the temperature range between 17 and 37 °C (Weiss, 1965). Although a higher temperature generally leads to a faster degradation of proteins, the effect of temperature on the stability of FVIII in a liquid state has not been always as predicted. A few studies have demonstrated a better stability of a few reconstituted FVIII products at room temperature than under refrigerated conditions (Saxena et al., 1991; Martinowitz, 1994; Schulman et al., 1994). Although this unusual observation has not been explained, it may have to do with reduced hydrophobic interaction at a lower temperature, as hydrophobic interaction plays a critical role in the association of HC and LC subunits. Indeed, the degradation rate of rFVIII SQ at 5 °C was faster than predicted from the Arrhenius kinetics and was suggested to be due to additional low temperature-associated chain dissociation (Fatouros et al., 1997a,b).

Like other proteins, FVIII unfolds at high temperatures in solution. Differential scanning calorimetry (DSC) analysis of rFVIII showed a major transition temperature near 58 °C in addition to several small transition temperatures below that temperature with significant aggregation above 60 °C (Grillo et al., 2001). The small transitions below 58 °C could be due to partial unfolding of FVIII subunits, as the light chain alone has a T_m value of 51 °C (Sudhakar and Fay, 1998a,b). A B-domain-deleted FVIII, rFVIII SQ, exhibits a similar thermal behavior with onset of unfolding and aggregation temperatures of about 56 and 64 °C, respectively (Fatouros et al., 1997a,b). However, detailed CD analysis showed a different thermal response in the secondary structure of the two forms of FVIII. While a conformational change was observed corresponding to increased β-sheet in rFVIII at approximately 45 °C (Grillo et al., 2001), the secondary structure of rFVIII SQ was not influenced in the temperature range of 5–55 °C (Fatouros and Sjostrom, 2000). Although a storage stability for the two forms has not been compared side-by-side, rFVIII SQ seems to be more stable in a liquid state at 5 °C than what was predicted for rFVIII (see Section 4.4), suggesting a higher conformational stability as a result of the secondary structure of rFVIII SQ.

4.5.2. Effect of metal ions

The positive effect of metal ions such as calcium on the stability of FVIII was identified in plasma (Weiss, 1965) and in a liquid or lyophilized state (Foster et al., 1988). The stabilizing effect of calcium was also observed in rapid thermal stability studies on rFVIII (Grillo et al., 2001) and storage stability studies on other FVIII forms such as rFVIII SQ (Fatouros et al., 1997a,b). Similar to Ca^{2+}, Sr^{2+} was found to stabilize FVIII in plasma (Mikaelsson et al., 1983) and rFVIII SQ in solution (Fatouros et al., 1997a,b). Other metal ions, including Cu^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Ba^{2+}, or Ni^{2+} could not protect FVIII in plasma (Mikaelsson et al., 1983) or rFVIII SQ in solution (Fatouros et al., 1997a,b). Fe^{2+} was detrimental to FVIII SQ activity.

Although calcium ion stabilizes FVIII, excessive amounts (e.g. >50 mM Ca^{2+}) may eventually lead to destabilization of FVIII in a liquid state (Fatouros et al., 1997a,b). This is because a high calcium concentration enhances the decay of FVIIIa (Fay et al., 1993), or specifically, the dissociation of A2 and A1/A3-C1-C2 subunits (Persson et al., 1995). It was found that Ca^{2+} at 10 mM was apparently most effective in the protection of FVIII SQ in a liquid state (Fatouros et al., 1997a,b). The optimum concentration of calcium for lyophilized FVIII had not been determined.

4.5.3. Effect of salts

Salt plays a critical role in controlling both FVIII stability and solubility. Reducing the salt concentration from 58 to 18 mg/ml increased the formation of rFVIII SQ aggregates in a solution during incubation at 7 °C as determined by SEC-HPLC and also reduced its thermal aggregation temperature (Fatouros et al., 1997a,b), probably because a high ionic strength stabilizes the FVIII heterodimer (Donath et al., 1995). When the salt concentration was below 5 mg/ml (0.1 M) reversible precipitation of rFVIII SQ was observed at 125 IU/ml at pH 7, and precipitation of rFVIII SQ at a higher concentration of >1500 IU/ml occurred at a salt concentration of 9 mg/ml (0.15 M) or below (Fatouros et al., 1997a,b). Apparently, the solubility of FVIII SQ requires the presence of protein concentration-dependent amounts of salt (Osterberg et al., 2001). Probably because of this effect, increasing NaCl concentration from 9 to 19 or 35 mg/ml increased the recovery of rFVIII SQ upon freeze-drying (Osterberg and Wadsten, 1999). Since salt generally reduces the glass transition temperature of a protein formulation and inhibits the formation of hydrogen bonds with a protein during lyophilization, a mechanism is required for FVIII stabilization in a lyophilized state. The effect of salt on the stability of FVIII in a lyophilized state has not been fully elucidated.

4.5.4. Interaction with lipids

FVIII binds to phospholipids rapidly and reversibly via a multistep process (Gilbert et al., 1990; Bardelle et al., 1993). Among the phospholipids, negatively-charged phosphatidylserine (PS) and phosphatidic acid (PA) are the major FVIII-binding species (Kemball-Cook and Barrowcliffe, 1992). The interaction increases FVIII activity. For example, incubation of FVIII/vWF complex with PS and phosphatidylethanolamine (1:1) preparation can induce a two- to three-fold increase in apparent FVIII activity (Broden et al., 1983). At least 30-fold increase in the catalytic conversion of FX was observed in the presence of phospholipids (Fay et al., 2001). In addition, the interaction offers FVIII in vitro stability by inhibiting the chain dissociation (Curtis et al., 1994) or specifically, the salt-sensitive dissociation of A1 and A2 subunits (Fay et al., 2001). In a recent study, PS-containing liposomes have been shown to protect rFVIII SQ from both non-proteolytic and proteolytic degradation in homogenates of human subcutaneous tissue (Fatouros et al., 2000). Therefore, although phospholipids are unlikely to be used in a traditional FVIII product simply for stability improvement due to their limited aqueous solubility, they can be considered in designing a controlled release FVIII delivery system.

4.5.5. Surface adsorption/surfactants

FVIII adsorbs to a variety of surfaces, including glass (Martinowitz, 1994) and plastics such as PVC (DiMichele et al., 1996; Hurst et al., 1998), and polyethylene (Hurst et al., 1998). Surface adsorption of FVIII is rapid and an equilibrium can be reached within hours (DiMichele et al., 1996; Hurst et al., 1998). The amount adsorbed to a surface depends on the surface area and does not seem to be concentration-dependent (Hurst et al., 1998; McLeod et al., 2000). Therefore, the percent loss of FVIII activity would be higher when storing the same volume of FVIII solution at a lower concentration. In addition, the adsorption loss of FVIII varies depending on the composition of the FVIII product and the diluent (DiMichele et al., 1996; Parti et al., 2000). Probably because of the stabilizing effect of vWF, no loss of FVIII (recovery >95%) was observed during infusion of pdFVIII/vWF concentrate (IMMUNATE) at 50 and 100 IU/ml for 48 h through infusion pumps equipped with polyethylene, polypropylene or PVC plastic components (Thomas et al., 1999). Since the remaining FVIII activity is the only indicator for surface adsorption in these studies, any loss of FVIII activity could be not only due to surface adsorption but also to surface-induced protein denaturation. An example of surface-induced FVIII denaturation

was the rapid loss of rFVIII SQ activity at 25 °C by agitation (Fatouros and Sjostrom, 2000).

Surfactants are generally effective in reducing protein surface adsorption. It was demonstrated that polysorbate 80, a non-ionic surfactant, minimized significantly adsorption-induced loss of rFVIII SQ (Osterberg and Fatouros, 1999) and surface-induced rFVIII SQ denaturation (Fatouros and Sjostrom, 2000). In this aspect, serum albumin at 1 or 2% was not as effective as polysorbate 80 in preventing surface loss of diluted rFVIII (Recombinate) in PVC bags (Parti et al., 2000) or surface-induced rFVIII SQ denaturation (Fatouros and Sjostrom, 2000). In addition to the surface effect, limited data suggest that polysorbate 80 may stabilize FVIII in solution (Osterberg and Fatouros, 1999) and during lyophilization (Osterberg et al., 2001). Nevertheless, the role of polysorbate 80 as a FVIII stabilizer during long-term storage has not been clearly established, at least in a lyophilized state. The peroxide content in polysorbate 80 needs to be carefully controlled in future stability studies as it may adversely affect protein stability (Ha et al., 2002).

4.5.6. *Effect of other formulation excipients*

Many formulation excipients have been examined and a few of them clearly stabilize FVIII in a liquid state, including sucrose, sorbitol, mannitol (Fatouros et al., 1997a,b), histidine (Sandberg et al., 2001), glycine (Brodniewicz-Proba and Beauregard, 1987), and some other amino acids (Margolis and Eisen, 1984). Glycine (Brodniewicz-Proba and Beauregard, 1987), sucrose and raffinose (Besman et al., 2000) also stabilize FVIII in a lyophilized state. On the other hand, trehalose, a commonly-used protein stabilizer, failed to stabilize rFVIII SQ in solution (Fatouros et al., 1997a,b). Citrate and phosphate are detrimental to FVIII, respectively, during storage at 20 °C (Foster et al., 1988) and during the freeze-thaw process (Hynes et al., 1969), presumably due to the chelation effect and buffer-induced pH shift during freeze-thaw, respectively (Anchordoquy and Carpenter, 1996). Several antioxidants have been shown to stabilize FVIII SQ in a solution, including glutathione, acetylcysteine, methionine (Osterberg and Fatouros, 1994, 1996). Glutathione also stabilizes FVIII in a lyophilized albumin-free formulation (Besman et al., 2000).

The identification of these FVIII stabilizers has undoubtedly facilitated and will continue to facilitate the design of a stable HSA-free FVIII product. The remaining tasks are to determine the optimal stabilizing concentration of individual excipients, possible existence of any synergistic effect, and the optimal combination of these excipients.

4.5.7. *Effect of pH*

The solution pH strongly affects the stability of FVIII in a liquid state. The pH effect depends on the composition of the FVIII solution. While the most stable pH for FVIII in plasma was found between 6.2 and 6.7 at 37 °C (Weiss, 1965), that for pdFVIII concentrate at 4 °C was between 6.2 and 7.0 (Wolf, 1959). The most stable pH range for rFVIII SQ at 7 °C was between 6.0 and 7.0 in a solution containing 58 mg/ml NaCl and shifted slightly to 6.5–7.0 at a reduced salt concentration of 5.8 mg/ml (Fatouros et al., 1997a,b). Outside this optimal pH range, rFVIII SQ not only aggregates but also forms fragments (Fatouros et al., 1997a,b). We recently found that the optimal pH range for full-length rFVIII in a solution at 40 °C was between 6.6 and 7.0 (Wang and Kelner, 2003). Since the solution pH can strongly affect the stability of a lyophilized protein (Chang et al., 1996), it would be necessary to determine whether the optimal pH range in a solution would hold true in storing lyophilized FVIII.

4.5.8. *Effect of packaging conditions*

Packaging conditions may affect the stability of a protein product. This is also true for FVIII (Woodhams et al., 2001). In the evaluation of the stability at room temperature of 15 reconstituted FVIII concentrates, 6 of these showed superior stability in plastic containers, whereas 3 of these showed superior stability in glass containers (Martinowitz, 1994). These results suggest several possibilities—(1) variation in surface composition and/or properties even for the same type of containers; (2) variation in the amount and composition of container leachables; and (3) variation in the degree of FVIII/surface interaction due to different product excipients. The effect of container on FVIII stability was shown to be temperature-dependent. At 4–8 °C, 2 of 15 reconstituted FVIII concentrates showed better stability in plastic containers and 2 concentrates showed better stability in glass containers;

at 20–23 °C, most concentrates showed better stability in plastic containers; and at 37 °C, all concentrates showed equal or better stability in plastic containers (Schulman et al., 1994). Therefore, stability studies comparing different types of containers should be conducted at the product storage temperature. However, due to the limited diffusion of molecules in a solid state, containers would not be expected to play a critical role in storing lyophilized FVIII products, unless the air permeation through the container and/or container stoppers is significantly different. This is because air has been shown to accelerate the inactivation of rFVIII SQ not only in a solution (Osterberg and Fatouros, 1996; Fatouros et al., 1997a,b) but also in a lyophilized state (Osterberg et al., 2001).

4.5.9. Freeze-thawing/drying

Freeze-thaw does not seem to cause significant inactivation of FVIII in a non-phosphate buffer. It was demonstrated that freeze-thawing of rFVIII three times does not cause aggregation or loss of activity (Grillo et al., 2001). FVIII SQ was stable after ten freeze-thaw cycles in a formulation containing 65 mM His, 300 mM NaCl, 4 mM $CaCl_2$ and 250 ppm Tween 80 (Osterberg and Fatouros, 1998). However, freeze-drying caused a 10% loss of rFVIII SQ (ReFacto) activity, regardless of the formulation pH (6.0–8.0) (Osterberg et al., 2001). The results suggest that inactivation of rFVIII SQ occurred in the drying step. One possible solution is to add specific monosaccharides and/or disaccharides, which can adequately form hydrogen bonds with FVIII for possible protection against dehydration-induced protein conformation change and/or denaturation.

4.5.10. Effect of light exposure

A recent study demonstrated that lyophilized FVIII (Recombinate) both at 28 IU/ml and 103 U/ml was stable upon exposure to accelerated simulated natural light for 10 h, but the activity of reconstituted FVIII was reduced by 31 and 29% at 26 and 106 IU/ml, respectively, under the same lighting conditions (Parti et al., 2000). Light promotes formation of free radicals, which can initiate oxidative damage of proteins (Hovorka and Schoneich, 2001). Since radical-induced reaction is diffusion-controlled (Maillard et al., 1983), the rate of such a reaction in solution would be expected to be much faster than in a solid state.

4.6. Mechanisms of in vitro instability

Limited stability studies so far suggest that multiple mechanisms may be involved in the inactivation of FVIII in vitro. One of them is aggregation. It was demonstrated that incubation of rFVIII at 0.5 mg/ml in a solution at 37 °C led to formation of soluble aggregates by both SEC-HPLC (26% aggregates at day 7 with 15% loss in activity) and dynamic light scattering (size increase) (Grillo et al., 2001). The aggregation process is conformation-induced and has a 6-h lag time (nucleation process). This aggregation behavior was also demonstrated for rFVIII SQ, which underwent an initial chain dissociation (nucleation process), followed by aggregation of the dissociated heavy chain (Fatouros et al., 1997a,b). In addition, the dissociation of the LC and HC subunits of rFVIII SQ by SDS–PAGE correlated well with the loss of its activity. A FVIII mutein, $FVIII_{des\text{-}794–1689}$, devoid of one thrombin cleavage site, has enhanced in vitro stability due to its resistance to subunit dissociation (Pipe and Kaufman, 1997). Recently, we found that rFVIII aggregation was apparently the major mechanism of inactivation in solution at 40 °C and both non-covalent and covalent (disulfide-bonded) pathways of FVIII aggregation were involved (Wang and Kelner, 2003).

Kinetically, the loss of rFVIII activity was demonstrated to be first or pseudo-first order (Manning et al., 1995; Fatouros et al., 1997a,b; Wang and Kelner, 2003). Since protein aggregation arising from physical protein–protein interactions generally exhibits an apparent reaction order of ≥ 2 (Fink, 1998; De Bernardez Clark and Schwarz, 1999), the major mechanism of rFVIII aggregation is probably not a simple physical protein–protein interaction process; rather it is probably initiated and controlled by a protein conformational change (Grillo et al., 2001).

Oxidation is clearly involved in FVIII inactivation. First of all, treatment of FVIII with hydrogen peroxide inactivates FVIII rapidly in several studies (Austen, 1970; Manning et al., 1995; Stief et al., 2000). Other oxidizing and inactivating agents include iodine (Austen, 1970), sodium hypochlorite and chloramines (Stief et al., 2000). Even air could significantly accelerate the inactivation of rFVIII SQ both in a solution (Osterberg and Fatouros, 1996; Fatouros et al., 1997a,b) and in a lyophilized state (Osterberg et al., 2001). Although the oxidation sites have not

been identified, cysteine and methionine residues are likely to be involved, as air-induced oxidation can be inhibited either by methionine, glutathione, or acetylcysteine (Osterberg and Fatouros, 1996) and chloroamine T-induced oxidation of FVIII can be inhibited significantly also by methionine or cysteine in 10-fold molar excess (Stief et al., 2000). In fact, significant amount of FVIII aggregates were found to be reducible, suggesting possible oxidation of free cysteines (or disulfide exchange) (Wang and Kelner, 2003). Another line of supporting evidence for the possible involvement of free cysteines in FVIII inactivation is the rapid loss of FVIII activity upon modification of the free cysteine groups (Austen, 1970). However, not all the free cysteines are required for full FVIII activity (Manning et al., 1995).

The FVIII molecule is large and complex. Limited data suggest that residues 721–729 (Kjalke et al., 1995), and some amino and lysine groups (Manning et al., 1995) are essential for FVIII activity (Manning et al., 1995). The hemophilia database (http://europium.csc.mrc.ac.uk) reveals that mutation of FVIII can occur on a single amino acid at more than 200 sites, and the mutated FVIII is partially or completely non-functioning. Therefore, loss of FVIII activity in vitro could result from a modification of a critical sequence or even a single amino acid. Therefore, complete delineation of FVIII inactivation mechanism is a daunting task.

5. Conclusions

Although significant progress has been made in the past several decades in understanding FVIII structure and stability, our knowledge can be enhanced by further investigations. The major areas of future research include: (1) evaluation of the high-resolution three-dimensional structure of the protein and the ternary pre-coagulant complex; (2) stabilization mechanism of FVIII in both the liquid and solid states, and (3) the role of the multiple mechanisms that appear to be involved in degradation of the co-factor both in vivo and in vitro. Through further efforts, we may achieve the goal of developing a more stable, convenient, economical, and safe FVIII product to further improve the quality of life for hemophilia patients.

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