Correlation of rFVIII Inactivation with Aggregation in Solution

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Purpose. This study was designed to investigate the stability of recombinant FVIII (rFVIII) in solution at different pHs and to probe the cause(s) of rFVIII inactivation under accelerated storage conditions.

Methods. Aqueous stability samples of full-length rFVIII at different pHs were incubated at 40°C for several days and analyzed by the one-stage clotting assay, SEC-HPLC, SDS-PAGE, and UV spectrophotometry.

Results. Incubation of liquid rFVIII at 40°C inactivated the protein rapidly and linearly with time on a semi-log scale at all pHs, suggesting a first order or pseudo first order process. A U-shaped relationship was found between the rate constant for loss of rFVIII activity and the solution pH. The minimal rate of inactivation was found between pH 6.6 and 7.0 with a half-life of approximately 4 days. The SEC-HPLC results indicated pH-dependent aggregation of rFVIII during incubation. It was found that the disappearance of monomeric rFVIII by SEC-HPLC correlated with the loss of rFVIII activity (r^2 = 0.97). Both the SDS-PAGE and UV results confirmed the aggregation pathway of rFVIII. In addition, the SDS-PAGE results suggest involvement of three aggregation mechanisms - disulfide-bond formation/exchange, non-reducible crosslinking, and physical interactions.

Conclusions. The full-length rFVIII is unstable in solution at 40°C and loses activity rapidly through a first order or pseudo first order aggregation process, which consists of both physical and chemical pathways. SEC-HPLC may be used in monitoring rFVIII stability studies in lieu of the clotting assay under the incubation conditions used in this study.

KEY WORDS: clotting; stability; SEC-HPLC, SDS-PAGE; UV.

INTRODUCTION

Factor VIII (FVIII) is an essential blood coagulation co-factor. The primary structure of FVIII was deduced based on the DNA sequence in 1984 (1,2), and the structure/function of the co-factor has been extensively reviewed (3–5). The mature FVIII molecule has 2332 amino acids with a calculated molecular weight of 265 kD. Based on sequence homology, the FVIII sequence contains three A domains, two C domains and a large B domain (2). The sequence of FVIII domains is NH_2-A1-A2-B-A3-C1-C2-COOH.

FVIII molecules isolated from plasma are mixtures of heterodimers consisting of 90–210 kD heavy chain (HC; A1-A2-B) and 80 kD light chain (LC; A3-C1-C2) (6,7). The heterogeneous heavy chain polypeptides, minimally represented by A1-A2, are derived from the 210 kD polypeptide through proteolysis of the A3-B junction and within the B-domain. The FVIII heterodimer is linked by a metal ion bridge (8,9) and the HC/LC interaction is enhanced by hydrophobic interactions (10). Although the identity and number of metal ion(s) in the FVIII molecule has not been definitively demonstrated (11,12), the latest results by electron paramagnetic resonance spectroscopy (EPR) (13) suggest that in addition to the calcium ion that seems to be involved in the HC/LC metal bridge, one reduced copper ion is likely present that may be required for high affinity HC/LC interaction.

FVIII molecules contain 23 cysteine residues, 19 of which are in the A and C domains (2). Three of the 19 are free cysteines, and the rest are disulfide bonded (14). Therefore, it is expected that disulfide bond formation/exchange may occur during long-term storage of FVIII.

A limited number of studies have been conducted to examine the stability of FVIII in solution. These studies include the evaluation of the stability of reconstituted FVIII products during storage (15,16) or during infusion (15,17,18) and the effect of a variety of factors, such as temperature, salts, metal ions, or lipids on the stability of rFVIII (19) or B domain-deleted rFVIII (20,21). Although the effect of pH was examined on the stability of FVIII in plasma at 37°C (22), or plasma-derived FVIII concentrate at 4°C (23), the authors have not found any report evaluating the stability of full-length rFVIII in solution as a function of pH, and the associated mechanism of rFVIII inactivation has not been clearly delineated. Whereas the pH stability study of a protein is often the first step toward the development of a stable protein formulation, both in a liquid or solid form, understanding the inactivation mechanism is critical in achieving protein stabilization. The purpose of this study is to examine the accelerated pH stability of rFVIII in solution at 40°C and to probe the cause(s) of rFVIII inactivation under accelerated storage conditions.

MATERIALS AND METHODS

Materials

Recombinant FVIII, produced in baby hamster kidney cells, was chromatographically purified and the purified rFVIII was concentrated to approximately 1000 IU/ml (1 mg/mL = 4000–5000 IU/mL) followed by diafiltration into a buffer containing 580 mM glycine, 60 mM sucrose, 60 mM NaCl, 5 mM $CaCl_2$ at pH 7. The rFVIII solution was stored frozen at –70°C before use. All the materials were USP grade and used as received.

Preparation of Stability Samples and Accelerated Stability Study

The frozen rFVIII solution was thawed at 20°C in a water bath and diluted to about 500 IU/ml with the diafiltration buffer. Separately, a histidine solution at 40 mM was prepared and adjusted to pH 5.0, 6.0, 6.5, 7.0 and 7.5 at room temperature. Stability samples at different pHs were prepared by mixing an equal amount of histidine buffer and the diluted rFVIII solution. After gentle mixing, the pH of the final solutions were measured and found to be 5.2, 6.1, 6.6, 7.0, and 7.4 at room temperature. The final rFVIII stability samples contained approximately 200 IU/mL rFVIII, 290 mM glycine, 30 mM sucrose, 30 mM NaCl, 2.5 mM $CaCl_2$, and 20 mM histi-

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dine. These rFVIII solutions were sterile filtered through 0.2 μ filters (Low Protein Binding Acrodisc, Gelman Science) in a laminar air flow hood and 1.8 mL samples of the solutions were filled into 2 mL screw-capped, O-ring sealed, sterile polypropylene vials. These vials were incubated in an oven at 40°C for 0, 1, 2, 3, and 4 days. All the stability samples were collected and frozen at –70°C before analysis.

During the stability study, the possible evaporation of water through the wall of the sample vials or the vial caps was also examined. The average of weight loss of the stability vials was approximately 3% on day 4 at 40°C. Therefore, the evaporation-induced concentration of rFVIII was negligible.

FVIII Activity Assay

FVIII activity was determined by the one-stage clotting assay. The assays were performed on an automated coagulation instrument (MLA1600C, Medical Laboratory Automation, Inc., Pleasantville, NY). Plasma-derived Mega-1 (Office of Biologics Research and Review, Bethesda, MD) was used as FVIII standard for preparation of a calibration curve. Before determination, the stability samples were diluted at least 2000-fold to concentrations that fall within the range of the FVIII calibration curve. Each sample was analyzed in duplicate and the results were averaged.

Size Exclusion HPLC (SEC-HPLC)

SEC-HPLC analysis of rFVIII was performed on a HP 1050 HPLC (Hewlett Packard, Pleasanton, CA) at room temperature. A TSK-GEL® G4000 SWXL column (8 μ, 300X7.8 mm) (TosoHaas, Montgomeryville, PA) was used with a TSK guard column (7 μ, 40X6.0 mm). The mobile phase contained NaCl (0.3 M), imidazole (0.02 M), $CaCl_2$ (0.01 M), Tween 80 (0.005%), glycerol (5%), and isopropanol (10%) with the pH adjusted to 7.0. The flow rate of the mobile phase was at 0.5 mL/min and the eluate was monitored at 280 nm.

Due to the structural heterogeneity of rFVIII, the retention time of rFVIII heterodimer covers a wide range from 16.5 to 21 min (see Fig. 3 later). Two other regions with a retention time of <15 min and approximately 22 min contain, respectively, high-molecular-weight material (HMW, >600 kD) and low-molecular-weight species (LMW, <30 kD). The remaining peaks at the end of the chromatograms contain formulation components. Molecular weight on SEC-HPLC was calibrated using Gel Filtration Standard (cat #151-1901 from Bio-Rad, Hercules, CA). This standard contains bovine thyroglobulin (670 kD), bovine gamma globulin (158 kD), chicken ovalbumin (44 kD), horse myoglobin (17 kD), and vitamin B-12 (1.35 kD).

SDS-PAGE and Gel Scanning

SDS-PAGE was performed using 4–12% Bis-Tris NuPAGE Gels (Novex, San Diego, CA). rFVIII samples were heated under both reduced and non-reduced conditions in the vendor-provided sample buffer at 95°C for 5–10 min and 15 μL of the treated protein were loaded into the gel sample well. Mark 12™ (Novex, San Diego, CA) was used as the protein molecular weight standard and diluted 10-fold with the sample buffer before use. The loaded gels were run in MOPS buffer (Novex, San Diego, CA) at 150 V for approximately 50 min and stained with 2D-Silver Stain II kit (Daiichi Pure Chemicals, Tokyo, Japan).

To compare the relative intensity of the protein bands, the washed and dried gels were fixed on a gel holder and scanned at 600 nm with the light path slit set at 0.1 mm on a DU® 650 UV/vis spectrophotometer (Beckmann, Fullerton, CA). During scanning, as many as 10 readings were collected every 1 mm on the gel. Under this scanning condition, the authors found that the total peak area of rFVIII heterodimers was linearly proportional ($r^2 = 0.983$) to the amount of rFVIII loaded on a gel (up to the rFVIII concentration used in this study). Therefore, the scanned peak area represents the relative amount of intact proteins on a gel.

Determination of Protein Aggregation

rFVIII aggregation was monitored by measuring the optical density at 280 and 350 nm for all the stability samples. The aggregation index (A.I.), in percentage, a relative measurement of the degree of protein aggregation, was calculated using the following equation:

$$\text{A.I.} = \frac{OD_{350}}{OD_{280} - OD_{350}} \times 100$$

Since proteins exhibit negligible UV absorption at 350 nm, this index reflects the relative light-scattering intensity of the protein aggregates. It should be noted that the denominator in the equation does not correct any light-scattering contribution due to change in wavelength.

RESULTS

The solution stability studies of rFVIII were conducted at different pHs at 40°C. Stability samples were monitored by four different assays: the one-stage clotting assay, SEC-HPLC, SDS-PAGE and UV spectroscopy (280 and 350 nm). Although the clotting assay is essential for determination of rFVIIII activity, SEC-HPLC and SDS-PAGE were used to determine potential formation of FVIII soluble aggregates or cleaved fragments during storage. UV spectroscopy was used to determine the overall formation of both soluble and insoluble protein aggregates.

rFVIII Stability by Clotting Assay

During incubation at 40°C, the rFVIII clotting activity dropped linearly with time on a semi-log scale (Fig. 1). The majority of rFVIII activity was lost after 4 days at most pHs, whereas rFVIII at pH 5.2 lost its activity within 1 day (non-detectable activity on and after day 2). The linear drop in rFVIII activity with time suggests a first-order or pseudo first-order inactivation process (the authors also tried to fit the data to a second-order kinetics but non of the curves fit better). Upon linear regression, the rate constant of rFVIII inactivation at 40°C was obtained (Table I). Plotting the rate constant against the formulation pH revealed a U-shaped relationship (Fig. 2). The most stable pH for rFVIII in solution is between 6.6 and 7.0. The extrapolated pH of minimal inactivation is approximately 6.8.

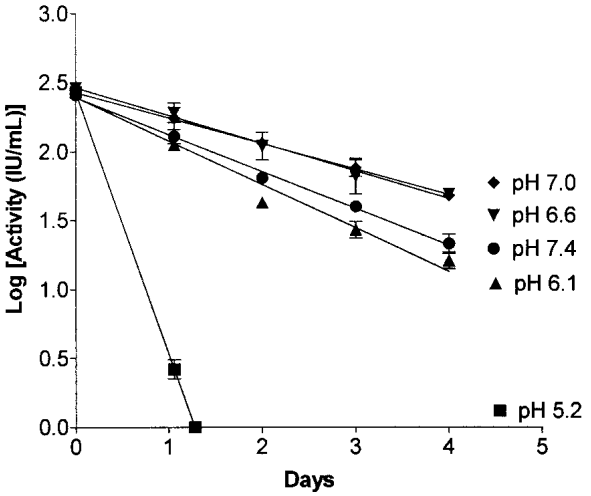

Fig. 1. Stability of rFVIII in solution during incubation at 40°C at different pHs. The composition of the solution is described in the Method section. rFVIII activity was monitored by the one-stage clotting assay. The data are mean ± standard deviation of three samples.

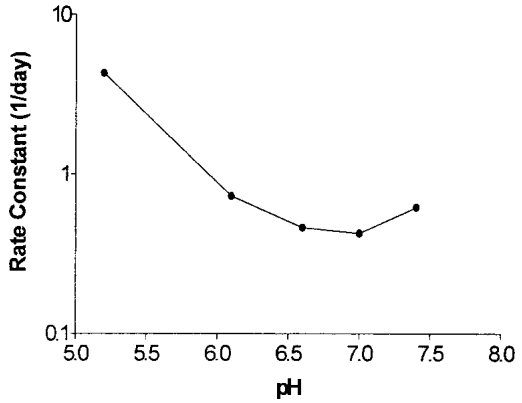

Fig. 2. Stability of rFVIII in solution during incubation at 40°C at different pHs. The composition of the solution is described in the Methods section. rFVIII activity was monitored by the one-stage clotting assay. The rate constant was estimated by linear regression of log activity-vs-time curve.

rFVIII Stability by SEC-HPLC

Representative SEC-HPLC chromatograms for rFVIII stability samples at pH 5.2 and 6.6 are shown in Fig. 3. On incubation at 40°C, rFVIII formed HMW aggregates. Formation of HMW aggregates was almost complete in 24 h at pH 5.2 and the formation of rFVIII aggregates was accompanied by loss of most rFVIII activity (Fig. 1), suggesting that the aggregates have little or no clotting activity. On day 4, the peak area of the aggregates decreased dramatically at pH 5.2, presumably due to the formation of large or insoluble aggregates. Visual observation confirmed the presence of minute particulates in the stability sample. At pH 6.6, the formation of FVIII aggregates was at a slower rate and the hydrodynamic radius of the aggregates gradually increased as the retention time of the aggregates was reduced from 13.4 min on day 1 to 12.1 min on day 4. The peak area of low-molecular species did not change significantly during the 4-day incubation period, suggesting minimal fragmentation.

Figure 4 summarizes the changes in peak areas of the three regions at all pHs. As the content of rFVIII heterodimer dropped, the HMW peak area increased significantly, whereas the LMW peak area remained relatively constant. This suggests that rFVIII aggregation is the major degradation pathway in the pH range studied. The formation of rFVIII aggregates is most rapid at pH 5.2. The peak area of HMW aggregates gradually increased to a level, which is higher than the initial peak area of rFVIII heterodimer at all

Table I. Kinetic Parameters for FVIII Inactivation at Different pHs

pH	5.2	6.1	6.6	7.0	7.4
k[a] (1/day)	–1.83[c]	–0.316	–0.201	–0.185	–0.269
$T_{1/2}$[b] (day)	0.38	2.2	3.4	3.7	2.6
r^2 (correlation coefficient)	N/A	0.971	0.990	0.999	0.996

[a] k is the first-order rate constant.
[b] $T_{1/2}$ is half-life.
[c] k was estimated based on the FVIII activity difference between day 0 and day 1.

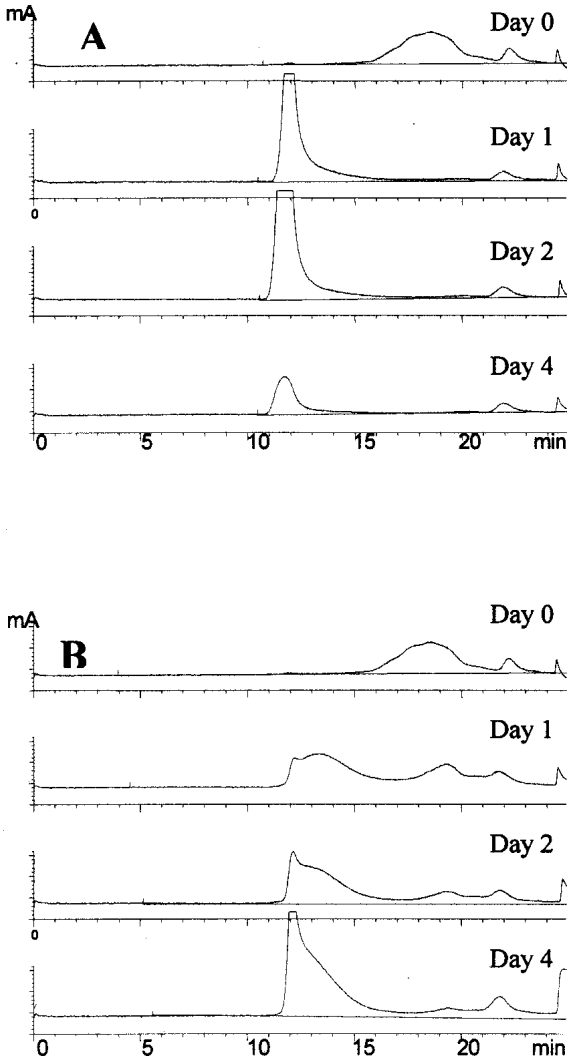

Fig. 3. SEC-HPLC analysis of rFVIII stability samples at pH 5.2 (panel A) and 6.6 (panel B). The stability samples were incubated at 40°C for 0, 1, 2, and 4 days (top to bottom). The retention time of rFVIII is around 19′ and the aggregates around 12′.

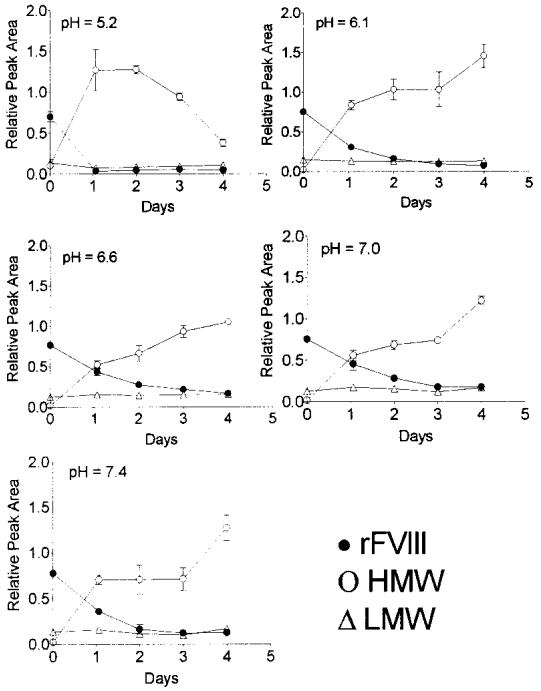

Fig. 4. Stability of rFVIII in solution at different pHs during incubation at 40°C. Samples were analyzed by SEC-HPLC. rFVIII peaks are divided into three regions: rFVIII heterodimer (●), aggregates (○), and low-molecular species (Δ). The data are mean ± standard deviation of three samples.

pHs. This is likely due to an increasing light scattering contribution of aggregates to the A_{280} signal. The peak area of rFVIII aggregates did not drop in all stability samples except those at pH 5.2. As suggested previously, the steep drop in the HMW peak area observed after day 2 at pH 5.2 is likely due to loss of protein material through the formation of large or insoluble aggregates.

To determine whether the loss of rFVIII heterodimer (through the formation of aggregates) corresponded to the drop in rFVIII activity, the remaining peak area (in percentage) of rFVIII heterodimer (that of HMW was not used as it also contained contribution from light scattering) was plotted against the remaining rFVIII activity in percentage in all the stability samples (Fig. 5). There was a linear relationship with a correlation coefficient (r^2) of 0.968 and slope of 0.870. This correlation not only suggests that rFVIII aggregation is mainly responsible for rFVIII inactivation in solution at 40°C but also indicates that SEC-HPLC may be used to monitor rFVIII stability in lieu of the clotting assay for samples incubated at 40°C under the conditions described. The curve in Fig. 5 does not pass the origin, suggesting the presence of inactive heterodimeric rFVIII or an inactivation pathway(s) that does not involve aggregation.

rFVIII Stability by SDS-PAGE

To further probe the aggregation mechanism(s) of rFVIII, representative stability samples at low and high pHs (5.2 and 7.4) were analyzed by SDS-PAGE both under non-reduced and reduced conditions. The scanning results of these gels are shown in Figs. 6 and 7. Under non-reduced conditions, the time zero samples showed a 80 kD peak—the light

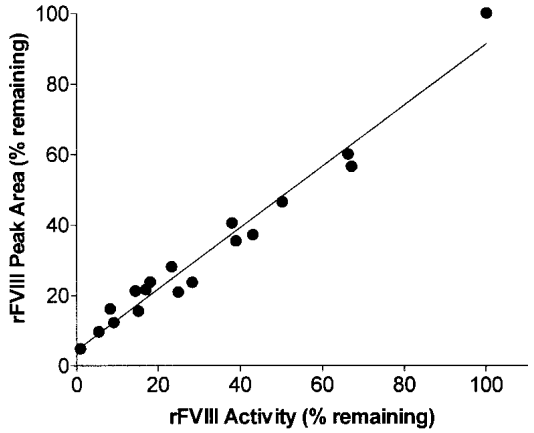

Fig. 5. Correlation of loss of rFVIII activity (by the one-stage clotting assay) with loss of rFVIII heterodimer (by SEC-HPLC assay) for stability samples at 40°C.

chain of rFVIII and a group of peaks from 100–210 kD—the heterogeneous heavy chain of rFVIII. An extra small peak of 50 kD was present at pH 5.2, which is likely a rFVIII hydrolytic fragment generated during the sample preparation process.

During incubation at 40°C, the peak area of rFVIII heterodimers (80–210 kD) decreased with the baseline increased

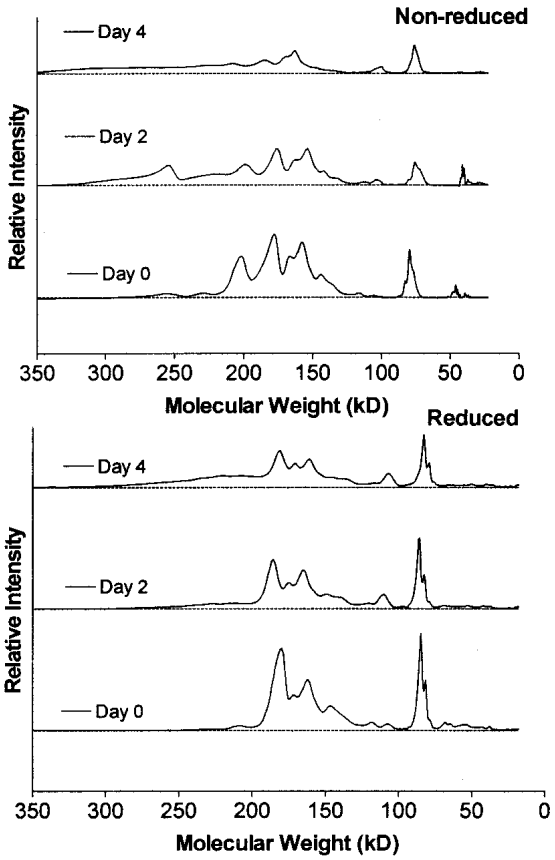

Fig. 6. Changes in relative intensity and distribution of different rFVIII bands for stability samples at pH 5.2 during incubation at 40°C. rFVIII samples were analyzed under non-reduced (upper panel) and reduced (lower panel) conditions. Gels were scanned on a UV/visible spectrophotometer.

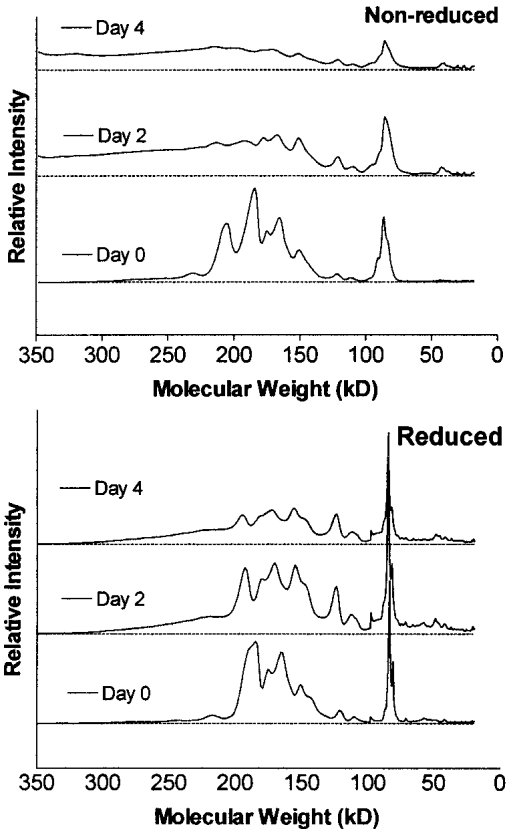

Fig. 7. Changes in relative intensity and distribution of different rFVIII bands for stability samples at pH 7.4 during incubation at 40°C. rFVIII samples were analyzed under non-reduced (upper panel) and reduced (lower panel) conditions. Gels were scanned on a UV/visible spectrophotometer.

in the region of higher molecular weights (>210 kD) at both pHs. The drop in the peak area of rFVIII heterodimers indicates loss of rFVIII heterodimers with time. The loss may be attributable to rFVIII aggregation, as no significant amount of rFVIII fragments was detected. This conclusion matched the SEC-HPLC results. However, the peak area of rFVIII heterodimers dropped by 53% on day 4 at pH 5.2 under non-reduced conditions, compared with a 94% drop by SEC-HPLC. Such a difference in protein recovery by the two methods was also observed at pH 7.4. Therefore, the amount of rFVIII heterodimers in the stability samples was apparently overestimated by SDS-PAGE relative to SEC-HPLC. An obvious explanation is that the rFVIII aggregates are, in part, non-covalent in nature and are disaggregated in SDS-PAGE sample buffer during the sample preparation process. Therefore, higher levels of disaggregated rFVIII were detected by SDS-PAGE that could not be detected by the non-denaturing SEC-HPLC method.

The total area under the curve (AUC) of the stability sample on day 4 dropped by 50% at pH 5.2 and 4% at pH 7.4 under non-reduced conditions relative to that of the initial samples. Assuming the protein aggregates were equally sensitive to silver staining, the drop in AUC indicates that less protein was present on the gel on day 4 relative to the initial sample. This suggests that some rFVIII aggregates were too large to diffuse into the gel or insoluble in the SDS sample buffer. The higher percentage drop at pH 5.2 suggests the presence of a greater amount of large or insoluble aggregates at this pH than at pH 7.4, consistent with the SEC-HPLC results. However, the aggregates above 210 kD on the gel were stained more intensely at pH 7.4 to an extent that the heavy chain bands were not distinguishable on day 2 whereas those at pH 5.2 were weakly existent on day 4. In addition, some staining was clearly observed on the bottom edge of the gel sample wells at pH 7.4 whereas minimal sample well staining was observed at pH 5.2. These differences between the two pHs suggest (a) pH-dependent rFVIII aggregation mechanism(s).

Under reduced conditions, the intensity of the stained aggregates above 210 kD was significantly less than under non-reduced conditions at both pHs, and no stained aggregates were observed on the bottom edge of the gel sample wells. These results suggest that some aggregates are disulfide-bonded in nature. The small amount of aggregates above 210 kD under reduced conditions suggest that some aggregates were formed by non-disulfide bond-mediated cross-linking or strong non-covalent interactions that are not fully reversed under SDS-PAGE conditions.

The AUC of the stability sample at pH 5.2 under reduced conditions dropped by 8% on day 4 (50% under non-reduced conditions) and no drop was observed in the reduced sample at pH 7.4 (4% under non-reduced conditions). The difference in total protein recovery between non-reduced and reduced conditions suggests that most aggregates at pH 5.2, either large or insoluble, were reducible to sizes below 300 kD. Because of the incomplete recovery of the protein on the gel under reduced conditions and the possibility of facilitated disaggregation by disulfide reduction, the relative amounts of the reducible and non-reducible aggregates could not be determined by calculating the difference in the areas under the aggregate curve (>210 kD). The available evidence suggests that disulfide-induced aggregation, non-disulfide-induced aggregation, and non-covalent aggregation likely contribute to the aggregation-induced inactivation of rFVIII during incubation at 40°C.

Determination of rFVIII Aggregation by UV

During incubation at 40°C, the aggregation index (A.I.) increased in all the stability samples and the average rate of increase is shown in Fig. 8. The increase in A.I. confirms rFVIII aggregation during incubation. The maximum increase was observed at pH 5.2, which corresponded to the most rapid loss of rFVIII activity and fastest formation of the rFVIII aggregates as monitored by SEC-HPLC at this pH. However, the minimum increase in A.I. was found between pH 6.1 and 6.6, which did not match the most stable pH range based on the activity assay. The potential cause of this discrepancy is discussed in the following section.

DISCUSSION

In this study, the authors examined the pH stability of purified rFVIII in solution at 40°C. A U-shaped relationship between the rate constant for the loss of rFVIII activity and pH was found, and the most stable pH range was between 6.6 and 7.0. Similar pH ranges have been reported in several relevant studies. The most stable pH range for plasma-derived FVIII was found between 6.2 and 6.7 in plasma at

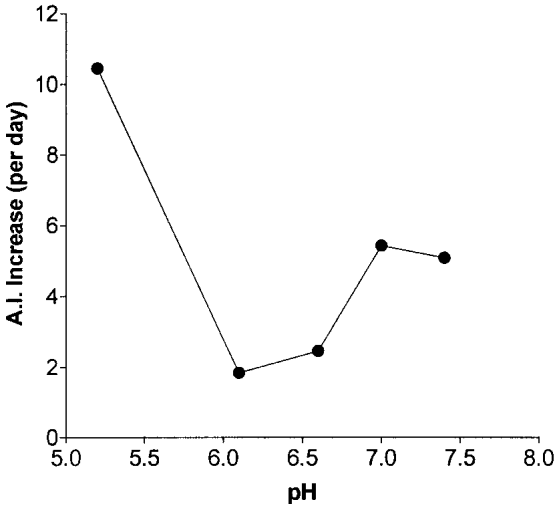

Fig. 8. Increase in aggregation index (A.I.) of rFVIII stability samples at different pHs during incubation at 40°C. The rate of A.I. increase was estimated by linear regression of the A.I.-vs-time curve.

37°C (22) and between 6.2 and 7 in FVIII concentrate at 4°C (23). The most stable pH range for the B domain-deleted rFVIII (rFVIII SQ) at 7°C was between 6.0 and 7.0 in a solution containing 1 M NaCl and between 6.5 and 7.0 at a reduced salt concentration of 0.1 M (24). The stable pH range for rFVIII SQ at a lower salt concentration matched what was observed for rFVIII in this study, suggesting that the deletion of B domain may not affect the pH effect on the stability of rFVIII.

The longest half-life of rFVIII in this study was determined to be 3.7 days at 40°C at pH 7.0 (Table I). However, a recent stability study on full-length rFVIII in solution showed that approximately 10% of protein activity was lost after 4 days at 37°C (19), which is much more stable than observed. There are two possible interpretations. First, Grillo *et al.* (2001) demonstrated that the exposure of hydrophobic surfaces in rFVIII started to increase at approximately 40°C, as monitored by increased binding of the fluorescent probe, bis-anilinonaphthalene sulfonic acid (bis-ANS). It is possible that rFVIII aggregation is dramatically facilitated by the increased hydrophobic surfaces of partially-unfolded rFVIII at 40°C. This is because protein folding/unfolding intermediates generally initiate the protein aggregation process (25,26). Second, the rFVIII solution in that study contained an unidentified amount of Tween 80, which has been shown to improve rFVIII SQ stability in solution (27).

The SEC-HPLC results, along with the excellent correlation between loss of the rFVIII activity and loss of the rFVIII heterodimer, showed that rFVIII aggregation was the major inactivation pathway in solution at 40°C. The authors believe that this is the first study leading to this conclusion. Although a previous study reported aggregation of full-length rFVIII in solution at 37°C, the relationship between rFVIII aggregation and loss of the clotting activity was not established (19). In comparison, the B-domain deleted rFVIII was shown to lose the clotting activity in solution apparently both by fragmentation and aggregation at 7°C (24). The mechanistic difference in FVIII inactivation may reflect possibly one or more of the following effects: presence of B-domain, interaction of formulation excipient(s) with the protein, temperature and protein concentration dependency, and difference in ionic strength.

In this study, the peak area of rFVIII aggregates on SEC-HPLC reached a level that was significantly greater than that of rFVIII heterodimer at all pHs. Since the elution peaks were monitored at 280 nm, the increased peak area is likely due to light scattering contribution of rFVIII aggregates to the UV signal. The light scattering property of the stability samples was confirmed by the significant increase in A.I. (Fig. 8). The higher-than-expected aggregate peak in this study may explain a similar observation reported in a different study, where the loss of rFVIII activity was accompanied by twice as much formation of rFVIII aggregates by SEC-HPLC (19).

The aggregate peak by SEC-HPLC, after reaching a maximum, started to decrease on day 3 at pH 5.2, probably due to the formation of large or insoluble aggregates. Since the pore size of the guard column is 0.25 μ, any aggregates larger than 0.25 μ would be filtered without going through the SEC-HPLC column. Loss of rFVIII as large or insoluble aggregates was evidenced in the SDS-PAGE analysis, where the AUC of the stability sample at pH 5.2 under non-reduced conditions dropped by 50% on day 4 relative to that of the initial sample. The highest rate of A.I. increase at pH 5.2 also suggests the formation of maximum amount of aggregates, assuming that the aggregates have the same size distribution at different pHs. In reality, the solubility or size distribution of protein aggregates may be pH-dependent (28). In this study, the intensity of stained rFVIII aggregates on the gel at pH 7.4 was significantly higher than that at pH 5.2 under non-reduced conditions, although rFVIII aggregation was faster at pH 5.2, suggesting that the aggregates generated at pH 5.2 were larger or more in insoluble form than at pH 7.2. In this case the relative A.I. value may not reflect the total amount of rFVIII aggregates in solution at different pHs because the light scattering signal is dependent on both protein concentration and size of protein aggregates. Possibly due to a difference in solubility or size distribution of rFVIII aggregates at different pHs. The pH-vs-A.I. profile (Fig. 8) did not completely match the pH-vs-rate relationship (Fig. 2), even though aggregation was mainly responsible for the inactivation of rFVIII. Because of this, UV measurement is not a reliable tool to compare rFVIII aggregation at different pHs.

The SDS-PAGE results confirmed that rFVIII aggregation in solution is the major pathway of rFVIII inactivation at 40°C. In addition, the results suggest that the aggregation of rFVIII may involve three different mechanisms—disulfide-bond formation/exchange, non-reducible crosslinking, and physical interactions. Because of the incomplete protein recovery on the gel, possibility of facilitated disaggregation by disulfide reduction, and possibility of differential staining sensitivity of aggregates, the relative contribution of these mechanisms could not be determined. The SDS-PAGE results also suggest a pH-dependency of aggregation mechanisms. This conclusion is mainly based on the observation of different protein recoveries and different staining patterns of rFVIII and aggregates on the gels at different pHs. Proteins aggregate in a pH-dependent manner as the pH determines the type and density of surface charge on the protein; the charge distribution is critical in controlling protein aggregation. Since a higher pH favors the formation of the thiolate ion, the initiating reaction species for disulfide bond ex-

change, this chemical aggregation mechanism should be favored at a higher pH.

Protein aggregation arising from physical protein-protein interactions generally exhibits an apparent reaction order of ≥2 (26,29). This study demonstrated a first-order (or pseudo first-order) inactivation (aggregation) kinetics of rFVIII during incubation at 40°C. The same order of reaction was also observed for the loss of rFVIII activity at 55°C in a solution (30) and for the loss of rFVIII SQ activity in a solution at different temperatures (20). Such an order of aggregation was observed for several other proteins, including dimerization of IL-1ra in aqueous solution during storage (31), and aggregation of recombinant human interferon-γ (IFN-γ) under a variety of conditions (32,33). This order of reaction suggests that the major mechanism or the rate-controlling step of overall rFVIII aggregation is probably not a simple physical protein-protein interaction process; rather, that it is initiated and controlled by a protein conformational change (19). Nevertheless, since physical protein aggregation is related to the surface content of hydrophobic groups (34) and rFVIII does contain exposed hydrophobic binding sites (10), protein aggregation from simple protein-protein interaction could not be ruled out.

CONCLUSIONS

Incubation of rFVIII in solution at 40°C inactivates the protein rapidly, especially at an acidic pH. rFVIII in solution was most stable between pH 6.6 and 7.0 at 40°C with a half-life of approximately 4 days. The rFVIII inactivation process at this temperature was shown to be first order or pseudo first order. Aggregation of rFVIII was mainly responsible for its inactivation during incubation at 40°C and apparently involved three different mechanisms—disulfide-bond formation/exchange, non-reducible crosslinking, and physical interactions. SEC-HPLC was found to be an excellent tool for monitoring rFVIII stability in lieu of the one-stage clotting assay for samples incubated at 40°C under the conditions described.

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