Effect of Tween 20 on Freeze-Thawing- and Agitation-Induced Aggregation of Recombinant Human Factor XIII

LOTTE KREILGAARD,[†,‡] LATOYA S. JONES,[‡,§] THEODORE W. RANDOLPH,[§] SVEN FROKJAER,[†] JAMES M. FLINK,[||] MARK C. MANNING,[‡] AND JOHN F. CARPENTER*[,‡]

Contribution from *The Department of Pharmaceutics, Royal Danish School of Pharmacy, Copenhagen, Denmark, Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado Health Sciences Center, Denver, Colorado 80262, Department of Chemical Engineering, University of Colorado, Boulder, Colorado 80302, and Pharmaceutical Development—Technology, Biopharmaceuticals Division, Novo Nordisk A/S, 2820 Gentofte, Denmark.*

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Abstract □ Agitation- and freeze-thawing-induced aggregation of recombinant human factor XIII (rFXIII) is due to interfacial adsorption and denaturation at the air–liquid and ice–liquid interfaces. The aggregation pathway proceeds through soluble aggregates to formation of insoluble aggregates regardless of the denaturing stimuli. A nonionic surfactant, polyoxyethylene sorbitan monolaurate (Tween 20), greatly reduces the rate of formation of insoluble aggregates as a function of surfactant concentration, thereby stabilizing native rFXIII. Maximum protection occurs at concentrations close to the critical micelle concentration (cmc), independent of initial protein concentration. To study the mechanistic aspects of the surfactant-induced stabilization, a series of spectroscopic studies were conducted. Electron paramagnetic resonance spectroscopy indicates that binding is not occurring between Tween 20 and either the native state or a folding intermediate state of rFXIII. Further, circular dichroism spectroscopy suggests that Tween 20 does not prevent the secondary structural changes induced upon guanidinium hydrochloride-induced unfolding. Taken together, these results imply that Tween 20 protects rFXIII against freeze-thawing- and agitation-induced aggregation primarily by competing with stress-induced soluble aggregates for interfaces, inhibiting subsequent transition to insoluble aggregates.

Introduction

During the course of developing a stable protein formulation, accelerated stability studies are usually conducted. Two methods commonly used to stress liquid formulations are agitation[1,2] and freeze-thawing.[3−5] In addition, agitation and freezing are events that can occur during processing. Agitation is a stress factor to which liquid formulations can be subjected during shipping and handling. Similarly, freezing can occur accidentally during shipping and is also used as a planned step for intermediate processing of protein products.

Freezing induces several stresses capable of denaturing the protein such as cold denaturation,[6,7] increased solute and protein concentrations,[6−8] and potential large-scale changes in pH.[9,10] Also, proteins may denature at the ice−liquid interface.[3,11,12] Agitation-induced denaturation and aggregation are mainly due to adsorption of protein at the air−liquid interface.[13] It is thought that protein unfolding at this interface exposes the hydrophobic core, which ultimately may result in aggregation via intermolecular contact of nonpolar residues.[13,14]

Nonionic surfactants have been exploited as excipients for their ability to prevent protein denaturation and aggregation. Polyoxyethylene sorbitan monolaurate (Tween 20), a commonly used nonionic surfactant, was chosen for the current study because it has been shown to be an effective stabilizer against aggregation of proteins under a variety of conditions, such as GuHCl-induced unfolding,[15] freezing,[5] agitation,[1] reconstitution,[16] and nebulization.[17] In addition, Tween 20 is used in FDA-approved parenterals.[18]

Two predominant mechanisms have been proposed for protein stabilization by surfactants. First, it has been found with certain proteins (e.g., human growth hormone[19]) that stabilization is due to a direct binding of surfactant to the protein, a property that is highly dependent on the protein.[20,21] Binding to the native protein inhibits the intermolecular contacts leading to aggregation. In addition, surfactants can bind to a folding intermediate state and, thus, act as a molecular chaperone and foster refolding over aggregation.[19,22]

Second, surfactants can compete with proteins for adsorption on denaturing interfaces, a property that should be manifested with most proteins. It has been shown that surfactants are able to minimize or prevent protein adsorption and denaturation at air−liquid or solid−liquid interfaces through inhibition of adsorption, desorption, exchange, and/or complex formation with the protein at these interfaces.[23,24]

Recombinant human factor XIII (rFXIII) is a 166 kDa homodimeric protein[25] which is used as a model protein because of its propensity to aggregate readily.[26,27] The present study was conducted to determine the capacity of Tween 20 to stabilize rFXIII during agitation and freeze-thawing and to discern whether protection is due to direct protein−surfactant binding, a general inhibition of protein surface adsorption by the surfactant, or both mechanisms.

Experimental Section

Materials—Recombinant human factor XIII (rFXIII), expressed in yeast (*Saccharomyces cerevisiae*),[25] was a generous gift from ZymoGenetics, Inc. (Seattle, WA). The material was obtained as a freeze-dried formulation which contained 10 mg/mL protein (>99% purity by high performance liquid chromatography), 2% sucrose, 10 mM glycine, and 1 mM disodium ethylenediaminetetraacetate (EDTA). Tris base was obtained from Boehringer Mannheim (Indianapolis, IN). EDTA, polyoxyethylene sorbitan monolaurate (Tween 20), guanidinium hydrochloride (GuHCl), 4,4-dimethyloxazolidine-16-oxyl stearic acid (16-doxyl stearic acid), and 8-anilinonaphthalene-1-sulfonic acid (1,8-ANS) were obtained from Sigma Chemical Co. (St. Louis, MO). Gel filtration chromatography standards were purchased from Bio-Rad Laboratories,

* Corresponding author. Phone: (303) 315-6075. Fax: (303) 315-6281. E-mail: john.carpenter@uchsc.edu.
[†] Royal Danish School of Pharmacy.
[‡] University of Colorado Health Sciences Center.
[§] University of Colorado.
[||] Novo Nordisk A/S.

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Inc. Bicinchoninic acid (BCA) reagent was obtained from Pierce (Rockford, IL) and used according to directions for the microtiter plate assay.

Prior to each experiment, the lyophilized protein formulation was dissolved in distilled, deionized water and dialyzed against a 10 mM Tris, 0.1 mM EDTA (pH 8 at 23 °C) buffer (formulation buffer) at 4 °C for 15 h. Tris was chosen as a buffer salt because it does not lead to pH changes upon freezing.[28] Insoluble material was removed by centrifugation at 12000*g* for 10 min at 4 °C, and the protein concentration in the supernatant was determined spectrophotometrically at 280 nm ($\epsilon = 1.38$ cm^{-1} (mg/mL)$^{-1}$).[29] Solutions of Tween 20 were prepared using the formulation buffer.

Agitation Study—Samples containing 1, 5, or 10 mg/mL rFXIII were prepared with 0−120 μM Tween 20. Aliquots (0.4 mL) of each formulation were placed into 1.5 mL polypropylene microcentrifuge vials (diameter = 9 mm), which allowed ample room for the air−liquid interface to form during agitation. In addition, samples with a greatly minimized air−liquid interface were prepared by filling vials to capacity with surfactant-free rFXIII solution (1 mg/mL). Upon closure of the vials, a small air bubble formed corresponding to an air:liquid ratio approximately 50 times smaller than that in vials filled with 0.4 mL of solution.

Vials were agitated horizontally at 400 rpm on an orbital shaker at room temperature (23 ± 2 °C) for 117 h. Vials were removed for testing at 0, 2, 4, 6, 24, 48, 72, and 117 h. In addition, nonagitated control samples (0.4 mL in 1.5 mL polypropylene microcentrifuge vials) containing 1, 5, and 10 mg/mL rFXIII were assayed following storage for 117 h at room temperature.

Prior to testing, vials were centrifuged at 12000*g* for 10 min using a refrigerated (4 °C) μSpeedFuge SFR 13K (Savant, Holbrook, NY). The supernate was assayed for native protein and soluble aggregates by size exclusion high-performance liquid chromatography (HPLC-SEC) using an HPLC unit from Dionex (Sunnyvale, CA). At neutral pH, rFXIII exists as a dimer with a molecular mass of 166 kDa.[25] The amounts of native dimers (166 kD) (termed "native protein" hereafter) and non-native soluble aggregates (termed "soluble aggregates" hereafter) were quantitated using a detection wavelength of 215 nm and a $G2000_{SW}$ column (TosoHaas, Montgomeryville, PA) eluted with phosphate-buffered saline solution (120 mM sodium chloride, 2.7 mM potassium chloride, 10 mM monobasic potassium phosphate) (pH 7.4 at 23 °C) at a flow rate of 0.4 mL/min. Between 1 and 20 μg of protein was injected onto the column. The masses of native rFXIII and soluble aggregates were calculated on the basis of the peak areas of protein absorbance at 16.7 min and between 13.8 and 15.1 min, respectively. Results are expressed as percentage of the original native protein mass prior to agitation.

The amount of the sample forming insoluble aggregates was calculated as the difference between the original mass of native protein prior to agitation and the mass of soluble protein recovered off the HPLC column. Thus, insoluble aggregates constitute the protein fraction pelleted during the centrifugation step described above. The results are expressed as percentage of the original native protein mass prior to agitation. To test whether insoluble aggregated protein also included protein lost from solution due to adsorption to the vial surface, a Bradford assay using bicinchoninic acid (BCA) as a reagent was conducted. For all samples, <0.1% of the original protein mass was found to adsorb to the vial surface, using the following procedure: After agitation, the protein solutions were removed from the polypropylene microcentrifuge vials and the vials were rinsed with 3 × 1 mL of distilled water. A 210 μL portion of BCA reagent was added to each vial and allowed to equilibrate with the vial surface for 30 min. The equilibrated reagent solutions were subsequently transferred to a 96-well microtiter plate and incubated at 37 °C for 30 min, and total protein content was determined by absorption at 550 nm using a microtiter plate reader.

Freeze-Thawing Study—Samples were prepared at 1, 5, and 10 mg/mL rFXIII in the presence of various concentrations of Tween 20 ranging from 0 to 240 μM. A 0.7 mL portion of each formulation was added to 1.5 mL polypropylene microcentrifuge vials. Samples were freeze-thawed 10 times. Each cycle consisted of freezing by immersion in liquid nitrogen for 10 min followed by thawing at 20 °C in a water bath for 20 min. Samples were subsequently filtered through a low-protein-binding 0.2 μm syringe filter, which was prewetted with the formulation buffer. The filtered samples were then assayed for native protein and soluble aggregates by HPLC-SEC using an HP1090 instrument (Hewlett-Packard, Wilmington, DE), a detection wavelength of 215 nm, and a Zorbax G250 column (MacMod Analytical Inc., Chadds Ford, PA), which was eluted with 1 M sodium chloride and phosphate-buffered saline solution (120 mM sodium chloride, 2.7 mM potassium chloride, 10 mM monobasic potassium phosphate) (pH 7.4) at a flow rate of 2.0 mL/min. Between 1 and 20 μg of protein was injected onto the column. The masses of native rFXIII and soluble aggregates were calculated on the basis of the peak areas of protein absorption at 5.1 min and between 3.9 and 4.9 min, respectively. Results are expressed as percentage of the original native protein mass prior to freeze-thawing. The masses of insoluble aggregates were calculated as described above.

To study the loss of native protein following one freeze−thaw cycle, surfactant-free solutions containing 0.25−5.0 mg/mL rFXIII were prepared and 0.5 mL aliquots were dispensed into 1.5 mL polypropylene microcentrifuge vials, using the same freeze−thaw protocol as described above. Identical rFXIII concentrations, volumes, vials, and freeze−thaw procedures were used for degassed solutions. Solutions were degassed by purging the formulation buffer for 1 h with He and subsequently using the degassed buffer solution to dilute concentrated rFXIII stock solution (20 mg/mL) to obtain the desired final rFXIII concentration.

Electron Paramagnetic Resonance (EPR) Spectroscopy—A spin probe solution was prepared by dissolving 1.6 mg of 16-doxyl stearic acid in 40 μL of 2-propanol and then diluting to 1 mL with the formulation buffer. The solution was vortexed and filtered through a 0.2 μm syringe filter to remove insoluble material. Samples were prepared by adding various amounts of Tween 20 to a protein-containing and a protein-free solution. A 10 μL spin probe solution was added, and the total volume was brought to 100 μL with the formulation buffer. This resulted in a final spin probe concentration of less than 10 μM and a final protein concentration of 4 mg/mL. Samples containing guanidinium hydrochloride (GuHCl) were prepared as described above by adding GuHCl (8 M) stock solution and the formulation buffer to obtain a final volume of 100 μL, a GuHCl concentration of 1.3 M, and a final protein concentration of 1 mg/mL. Since rFXIII is unstable in the presence of 1.3 M GuHCl, it was necessary to decrease the protein concentration to 1 mg/mL to prevent precipitation from occurring during the experiment. GuHCl was added carefully to avoid introduction of any air−liquid interface, which might have caused the protein to denature and aggregate. The samples were subsequently allowed to equilibrate for 1 h at room temperature prior to recording the EPR spectra.

An ESP 300 spectrometer (Bruker, Billerica, MA) was used with a field modulation frequency of 100 kHz, a modulation amplitude of 1 G, a scan time of 42 s, a scan width of 75 G divided into 2048 intervals, 10 mW microwave power, a sample temperature of 20 °C, and 20−40 scans per sample. An HP735 workstation (Hewlett-Packard, Wilmington, DE) was used to analyze further the digitized spectra obtained on the EPR system. Spectra were deconvoluted, and the binding stoichiometry was determined by the maximum difference between two titration curves with and without protein corresponding to the saturation of the protein surface with surfactant molecules, according to the method described by Bam and colleagues.[30]

Fluorescence Spectroscopy—Fluorescence measurements were performed at room temperature using an SLM 48000 spectrofluorimeter with excitation and emission wavelengths of 350 and 500 nm, respectively. rFXIII was assayed at 0.04 mg/mL. To monitor GuHCl-induced unfolding, an extrinsic fluorescence probe, 8-anilino-1-naphthalenesulfonic acid (1,8-ANS), was used in 100-fold molar excess over the protein concentration. Samples were prepared by adding increasing amounts of GuHCl (8 M) to a rFXIII/1,8-ANS, a rFXIII, or a 1,8-ANS solution and bringing the final volume to 1 mL of formulation buffer. Samples were equilibrated for 1 h at room temperature prior to measurements. Final results are shown as rFXIII/1,8-ANS fluorescence minus rFXIII and 1,8-ANS fluorescence.

Circular Dichroism (CD) Spectroscopy—CD spectra were recorded with an AVIV 62DS circular dichroism spectrophotometer using a rFXIII concentration of 0.1 mg/mL in a 2 mm path length cell. Each far-UV spectrum ((260−200 nm (formulation buffer); 260−214 nm (GuHCl-containing solutions)) was recorded at 25 °C with a step width of 2.0 nm, a bandwidth of 1.5 nm, and an averaging time of 12 s^{-1}. Samples were prepared by slowly titrating increasing amounts of GuHCl into protein and protein-free solutions, bringing the final volume to 1.0 mL with the

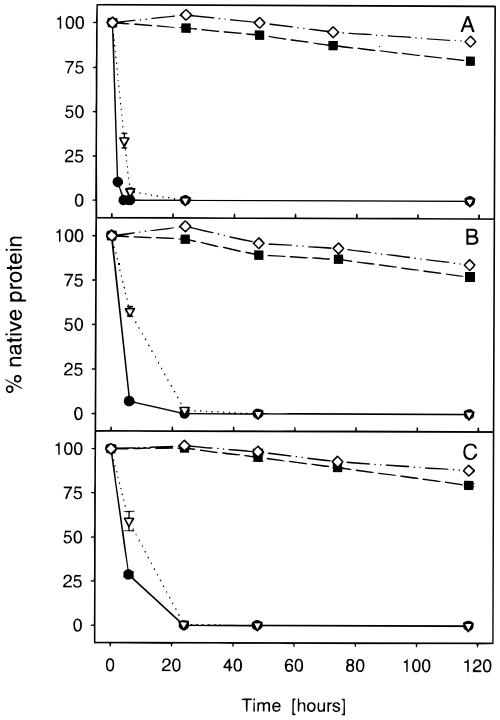

Figure 1—Effect of Tween 20 (0 μM, ●; 6 μM, ▽; 60 μM, ■; 120 μM, ◇) on the recovery of native rFXIII following agitation at 25 °C of solutions containing 1 mg/mL (A), 5 mg/mL (B), and 10 mg/mL rFXIII (C). Results are plotted as mean ± standard deviation for duplicate samples.

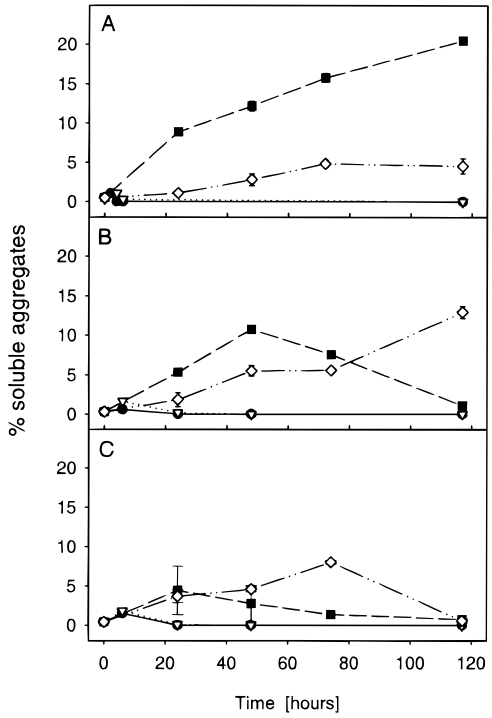

Figure 2—Effect of Tween 20 (0 μM, ●; 6 μM, ▽; 60 μM, ■; 120 μM, ◇) on the formation of soluble aggregates following agitation at 25 °C of solutions containing 1 mg/mL (A), 5 mg/mL (B), and 10 mg/mL rFXIII (C). Results are plotted as mean ± standard deviation for duplicate samples.

formulation buffer and allowing the samples to equilibrate for 1 h at room temperature prior to measurements. Samples were prepared with and without 60 μM Tween 20, since this is the minimum surfactant concentration at which maximum stabilization is achieved upon freeze-thawing and agitation (see Results and Discussion).

Results and Discussion

Agitation Study—Continuous agitation of surfactant-free rFXIII solutions in the presence of an air−liquid interface leads to rapid loss of native rFXIII, as shown in Figure 1. At each of the three protein concentrations tested (1, 5, and 10 mg/mL), native protein is completely lost within 24 h. To ensure that aggregation was not simply induced during storage of samples at 25 °C, nonagitated controls without Tween 20 were stored at 25 °C for the duration of the agitation study. Samples containing 1 mg/mL rFXIII show a 7% loss of native protein whereas samples with 5 mg/mL and 10 mg/mL rFXIII both show a 5% loss of native protein due to insoluble aggregate formation (not shown). This indicates that storage only accounts for a small part of the loss of protein seen over the time course of the study.

In the absence of Tween 20, no soluble aggregates could be detected (Figure 2). Rather, the loss of native protein could be completely accounted for by the formation of insoluble aggregates (Figure 3). Addition of Tween 20 reduces the loss of native protein (Figure 1). At all three protein concentrations tested, this protective effect of Tween 20 is saturated at Tween 20 concentrations between 60 μM, a concentration near the critical micelle concentration (cmc),[31] and 120 μM.

Interestingly, in the presence of 60 μM Tween 20, a progressive increase in soluble aggregate is seen with time in samples containing 1 mg/mL protein (Figure 2A), but insoluble aggregates are not detected (Figure 3A). With 5 mg/mL protein, soluble aggregates accumulate for 50 h and then diminish. Insoluble aggregates begin to appear as

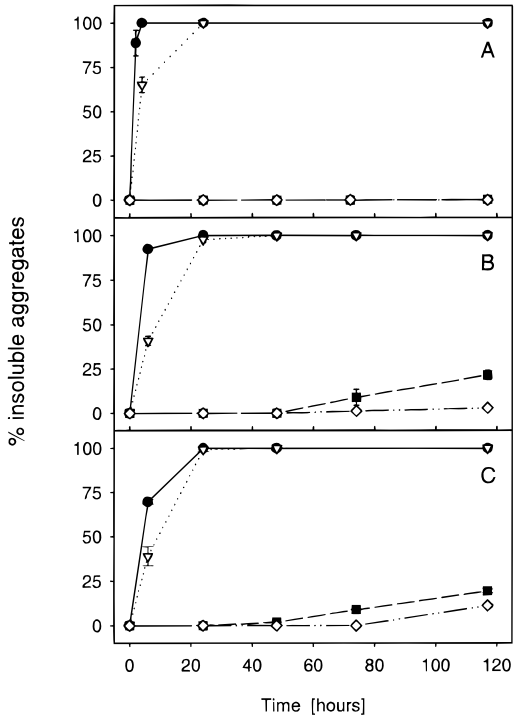

Figure 3—Effect of Tween 20 (0 μM, ●; 6 μM, ▽; 60 μM, ■; 120 μM, ◇) on formation of insoluble aggregates following agitation at 25 °C of solutions containing 1 mg/mL (A), 5 mg/mL (B) and 10 mg/mL rFXIII (C). Results are plotted as mean ± standard deviation for duplicate samples.

soluble aggregates are lost (Figures 2B and 3B). With 10 mg/mL protein, a similar trend is seen, but the maximum amounts of both kinds of aggregates are reduced (Figures 2C and 3C). In the presence of 120 μM Tween 20, less native protein is lost, but the trends for aggregation are similar to those seen in the presence of 60 μM Tween 20.

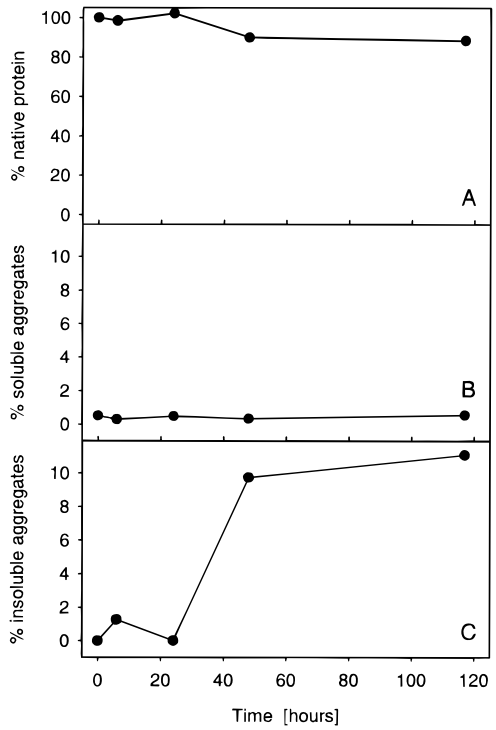

Figure 4—Recovery of native rFXIII (A) and formation of soluble (B) and insoluble aggregates (C) following agitation of 1 mg/mL rFXIII (25 °C) in the presence of a greatly minimized air–water interface (see Experimental Section).

At Tween 20 concentrations of 60 and 120 μM, the loss of native protein approximately follows first-order kinetics (Figure 1). This observation, combined with the apparent formation of insoluble aggregates from soluble aggregates, suggests that aggregation in the presence of Tween 20 can be described by the simplified pathway shown in Scheme 1.

$$\text{native} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{soluble aggregate} \xrightarrow{k_2} \text{insoluble aggregate}$$

Scheme 1

Formation of soluble aggregates may proceed from the native state via formation of one or several unfolding intermediates and may be reversible.

Agitation-induced aggregation of various proteins has previously been attributed primarily to adsorption and denaturation at the air−liquid interface rather than to shear.[2,32] To determine if the air−liquid interface also accounts for agitation-induced aggregation of rFXIII, a greatly minimized air−liquid interfacial area was created by filling vials to capacity with 1 mg/mL rFXIII solutions. Upon closure of the vials, a small air−liquid interface was created which was approximately 50 times smaller than that found in the partially filled vials used above. No detectable loss of native protein was seen upon agitation for 24 h and only 10% loss due to insoluble aggregate formation was seen after 117 h (Figure 4). As described above a comparable loss was seen in nonagitated control samples (not shown).

Taken together, the results indicate that the air−liquid interface plays a significant role in agitation-induced aggregation of rFXIII. The surfactant concentration needed to inhibit loss of native protein is similar to the cmc of Tween 20.[31] Furthermore, the concentration of Tween 20 required for maximum inhibition of native protein loss is independent of initial protein concentration. Thus, a plausible mechanism by which Tween 20 may act to protect

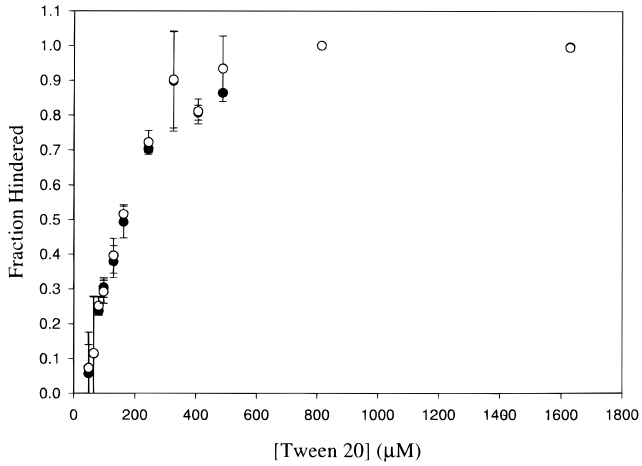

Figure 5—Spin probe partitioning curves in the absence (●) and in the presence (○) of native rFXIII at 4 mg/mL.

the protein is competition with the protein for the air−water interface. However, this competition alone cannot explain the apparent stabilization of soluble aggregates by Tween 20 (Figure 2). Tween 20 appears to reduce the rate of formation of insoluble aggregates but not soluble aggregates (Figures 2 and 3). Within the framework of Scheme 1, Tween 20 may reduce k_2 but appears to have little effect on k_1. Formation of insoluble aggregates from soluble aggregates may be surface-mediated, and Tween 20 could compete for the surface. Alternatively, Tween 20 may reduce the rate of formation of insoluble aggregates via specific binding to a soluble intermediate aggregate. Either of these mechanisms may ultimately stabilize the native conformation by simple mass action, assuming that formation of soluble aggregates is a reversible reaction.

Interaction between the Native State of rFXIII and Tween 20—As described above, one of the proposed mechanisms by which surfactants can stabilize proteins in solution is binding to the surface of the native protein.[19,20] Electron paramagnetic resonance (EPR) spectroscopy was employed to determine potential binding stoichiometries between Tween 20 and native rFXIII. With this method, the local environment of a nitroxide radical (spin probe) is measured by EPR spectroscopy. In the absence of surfactant micelles or protein−surfactant complexes, the spin probe rotates freely in solution. Upon formation of either of these complexes, a hydrophobic environment with increased viscosity forms. The spin probe preferentially partitions into the hydrophobic environment, leading to decreased spin probe rotational mobility, reflected by a partially "hindered" EPR spectrum. The fraction of rotationally hindered probe is measured as a function of surfactant concentration in the absence and in the presence of protein. The maximum difference between the two titration curves corresponds to the saturation of the protein surface with surfactant molecules, i.e., the surfactant: protein binding stoichiometry.[30]

Figure 5 shows that there is no significant difference in the fraction of hindered spin probe in the presence and absence of protein as a function of Tween 20 concentration, indicating weak or nonexistent binding of Tween 20 to the native protein. This result is not surprising considering that (1) nonionic surfactants are known to bind to proteins primarily through hydrophobic interactions[30,33] and (2) the surface of the native rFXIII dimer is primarily hydrophilic, as shown in the ANS-binding studies described below.

Interaction between a Folding Intermediate State of rFXIII and Tween 20—Surfactants can also inhibit aggregation by acting as molecular chaperones.[19] The surfactant binds to a protein folding intermediate state

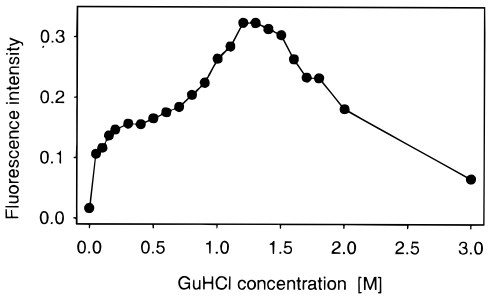

Figure 6—Fluorescence intensity of the extrinsic fluorescent probe 1,8-ANS (48 μM) upon GuHCl-induced denaturation of rFXIII (0.04 mg/mL) (excitation at 350 nm and emission at 500 nm).

(molten globule), favoring refolding over aggregation. To determine if Tween 20 inhibits agitation-induced rFXIII aggregation via this mechanism, it was necessary to verify the existence of a possible folding intermediate state of rFXIII. The fluorescence of the extrinsic probe, 1,8-ANS, was measured as a function of GuHCl concentration in the presence of rFXIII. 1,8-ANS binds to solvent-accessible nonpolar regions in proteins,[34] resulting in a large increase in fluorescence.[35] Minimal fluorescence is seen in the absence of denaturant, indicating that the native protein has a hydrophilic surface (Figure 6). Maximum fluorescence is seen in the presence of 1.2−1.3 M GuHCl, indicating the existence of a folding intermediate state (Figure 6). This result is consistent with results obtained by Kurochkin and colleagues,[27] who studied unfolding of rFXIII by monitoring GuHCl-induced changes in intrinsic fluorescence. They found two transitions with midpoints of approximately 0.9 and 1.3 M GuHCl and explained the transitions in terms of stepwise unfolding of subunit domains in the protein. Dissociation of the subunits requires the presence of >6 M GuHCl,[36] and it is therefore assumed that the folding intermediate state detected in our study is a dimer.

On the basis of the above results, an additional EPR study was performed in which potential interaction between the folding intermediate state of rFXIII and Tween 20 was investigated. The EPR spin probe studies described above were repeated in the presence of 1.3 M GuHCl. Differences between the fractions of hindered spin label in the absence and in the presence of rFXIII were insignificant, indicating that binding of Tween to rFXIII in the presence of 1.3M GuHCl was absent or undetectable by the EPR technique (data not shown). It has been shown that refolding yields of rFXIII are very low in the presence of a variety of different excipients including Tween 80,[36] further suggesting that Tween type surfactants do not bind to rFXIII folding intermediates. A caveat for all of these studies is that GuHCl may interfere with any putative Tween interactions with folding intermediates. Thus, we cannot rule out the possibility that Tween might interact with an agitation-induced folding intermediate in the absence of guanidine.

Circular dichroism was used to study secondary structural changes induced by 1.3 M GuHCl in the absence or presence of 60 mM Tween 20. Tween 20 has no effect on the far-UV CD spectrum of rFXIII in the absence or in the presence of 1.3 M GuHCl (Figure 7). Thus, no Tween 20-stabilized intermediate could be detected by CD spectroscopy.

Freeze-Thawing Study—To investigate further the role of Tween 20 in stabilizing rFXIII against stress-induced aggregation, a freeze-thawing study was conducted. A preliminary experiment was conducted to monitor the loss of native rFXIII (1 mg/mL) in the absence of Tween 20 as a function of the number of freeze−thaw cycles. The loss

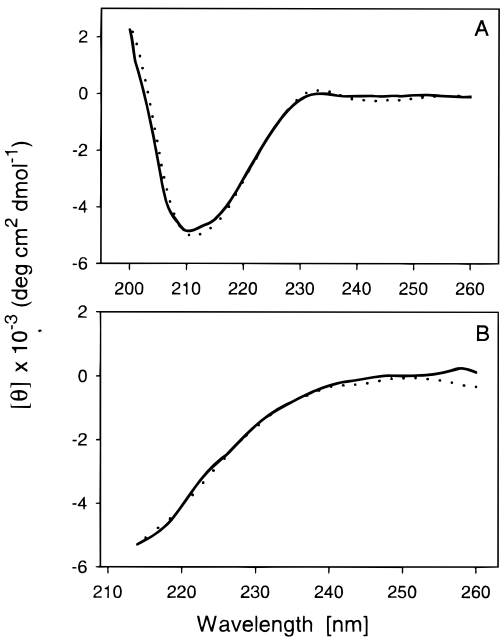

Figure 7—Far-UV CD spectra of native rFXIII (A) and a folding intermediate state of rFXIII (0.1 mg/mL) (B) in the absence (—) and in the presence (· · ·) of 60 μM Tween 20.

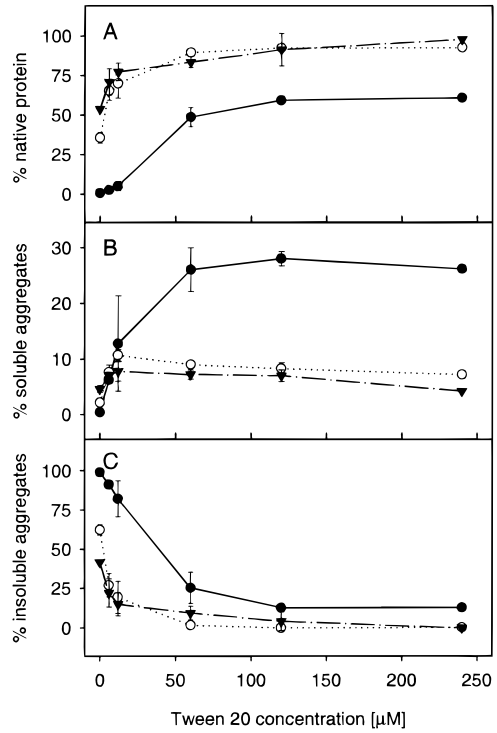

Figure 8—Recovery of native rFXIII (A) and formation of soluble (B) and insoluble aggregates (C) following 10 freeze–thaw cycles of 1 mg/mL (●), 5 mg/mL (○), and 10 mg/mL rFXIII (▼) as a function of Tween 20. Results are plotted as mean ± standard deviation for duplicate samples.

of native protein increases linearly with freeze−thaw cycles. Ten cycles result in greater than 98% loss of native protein (not shown). A standard protocol of 10 freeze−thaw cycles was therefore chosen for the current study.

Figure 8 shows that, similar to results obtained in the agitation study, in the absence of Tween 20 the relative recovery of native protein increases with increasing initial protein concentration. To determine if the specific amount of native protein lost after one freeze−thaw cycle is independent of initial protein concentration, various con-

Table 1—Loss of Native rFXIII following One Freeze–Thaw Cycle for Surfactant-Free Solutions of Various Initial rFXIII Concentrations[a]

rFXIII concn (μg/mL)	amt of native rFXIII lost (μg)	
	regular solns	degassed solns
250	72 ± 1	72 ± 3
500	73 ± 12	73 ± 12
1000	72 ± 1	80 ± 1
2500	50 ± 7	69 ± 20
5000	97 ± 12	100 ± 27

[a] Results are presented as mean ± standard deviation of three samples.

centrations of rFXIII (without Tween 20) were freeze-thawed one time. Table 1 shows that a fairly constant amount of native protein, corresponding to an average of approximately 0.07−0.08 mg, is lost, mainly due to insoluble aggregate formation, over a 20-fold range of initial rFXIII concentrations. The losses of protein in a single cycle are smaller than the average amounts lost per cycle after 10 freeze−thaw cycles (Figure 8). These results suggest that the protein aggregates due to adsorption and denaturation at one or more interfaces during freeze-thawing. rFXIII may adsorb to the ice−liquid interface,[11,12] the vial surface,[23] the air−liquid interface[13] that appears when air bubbles form during freeze-thawing,[11] or, in the case of multiple freeze−thaw cycles, the surface of insoluble protein aggregates. Analysis of the protein adsorbed on the vial surface after freeze-thawing (<0.2% as estimated by BCA assay) was insufficient to account for all of the protein damage. Likewise, elimination of the appearance of air bubbles by degassing samples prior to freezing did not reduce the loss of native protein (Table 1). While we are unable to eliminate the possibility that protein denatures during freezing and then desorbs from the vial interface during thawing, the above results suggest by a process of elimination that the ice−water interface may play a role in the loss of native protein, as has been previously suggested.[11,12]

In addition to aggregation due to adsorption and denaturation at the ice−liquid interface, rFXIII may also aggregate upon freezing due to cold denaturation,[6,7] due to increased solute concentrations,[6−8] and due to pH changes.[9,10] None of these mechanisms would be expected to give the same absolute loss of native protein independent of initial protein concentration. Additionally, pH changes may be excluded as a significant factor since the pH of Tris-buffered solutions is not altered by freezing.[28]

Freeze-thawing (10 cycles) in the presence of increasing concentrations of Tween 20 results in increased recovery of native protein (Figure 8A), accompanied by decreased formation of insoluble aggregates (Figure 8C). However, the relative amount of soluble aggregates increases with increasing Tween 20 concentration (Figure 8B). This effect is consistent with Scheme 1. Similar to agitation-induced damage, during freeze-thawing Tween 20 may decrease k_2 but have little effect on k_1 and thus prevent formation of insoluble aggregates but not soluble aggregates. Furthermore, as with agitation, maximum recovery of native protein, independent of initial protein concentration, is obtained at and above 60 μM Tween 20 (Figure 8), the cmc of Tween 20.

To ascertain that Tween 20 stabilizes rFXIII during the actual freeze-thawing process and not by simply dissolving the aggregates after thawing, the following experiment was conducted. Samples (1 mg/mL rFXIII, no Tween 20) were freeze-thawed 10 times, mixed with small aliquots of either the formulation buffer or the formulation buffer containing Tween 20 to obtain final surfactant concentrations of 0−60 μM Tween 20. No difference in degree of aggregation was noted between samples mixed with buffer and samples mixed with Tween 20 (not shown), eliminating the possibility that Tween 20 dissolves aggregates after thawing.

It is curious that the Tween 20 concentration needed to saturate the freeze-thawing protective effect corresponds to Tween 20's cmc. Once freezing begins, solutes concentrate due to water being converted to ice, and assuming that the surfactant remains in the amorphous phase with the protein, at least a 5-fold increase in solute concentration may occur due to ice crystal formation.[8] Thus, if Tween 20 concentrations corresponding to the cmc were required to prevent damage in the frozen state, one would expect that initial concentrations lower than 60 mM would be maximally effective. However, if Tween 20 protected mainly during thawing, a minimal initial concentration of 60 mM would be required to maintain surfactant concentrations at or above the cmc for the thawing process. This presents a quandary. The amount of native protein lost (in the absence Tween 20) following one freeze−thaw cycle is mainly due to formation of insoluble aggregates and is independent of initial protein concentration. This suggests that protein damage is due to surface-induced denaturation—with the ice−water interface a likely candidate. Tween 20 stabilizes native rFXIII by minimizing the formation of insoluble aggregates (Scheme 1). Thus, if insoluble aggregates were formed during freezing, initial Tween 20 concentrations lower than the cmc would have been expected to stabilize rFXIII during freezing. However, Tween 20 does not prevent formation of soluble aggregates, suggesting that these aggregates may form due to ice−water interfacial denaturation during freezing. Conversely, the insoluble aggregates may form due to intermolecular soluble aggregate interactions at the ice−water interface during thawing. Tween 20 may thus prevent insoluble aggregate formation by competing with the soluble aggregates for the ice−water interface during thawing.

Conclusion

Interfacial adsorption and concomitant denaturation account for agitation- and freeze-thawing-induced aggregation of rFXIII. The aggregation pathway proceeds via soluble aggregates to the formation of insoluble aggregates. Tween 20, at concentrations corresponding to the cmc (60 μM or 0.007% (w/v)), is maximally effective at stabilizing rFXIII against both agitation- and freeze-thawing-induced aggregation, independent of protein concentration. Spectroscopic studies showed that Tween 20 does not bind to either the native or a folding intermediate state of rFXIII. Together, these observations suggest that Tween 20 stabilizes rFXIII primarily by competing with stress-induced soluble aggregates for interfaces, inhibiting subsequent transition to insoluble aggregates.

References and Notes

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