Solubility of Recombinant Human Tissue Factor Pathway Inhibitor

BAO-LU CHEN,* XIAORONG WU, SUSAN J. BABUKA, AND MANINDER HORA

Contribution from *Department of Formulation Development, Chiron Corporation, 4560 Horton Street, Emeryville, California 94608-2916.*

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Abstract □ Study of recombinant human tissue factor pathway inhibitor (rhTFPI) solubility shows (1) an inverted bell-shaped pH–solubility profile with a broad solubility minimum between pH 5 and 10 such that the solubility minimum midpoint is 2–3 pH units away from its isoelectric point; (2) a negative temperature–solubility coefficient; (3) a strong dependence of solubility on the valence of electrolytes, with both multivalent cations and anions enhancing this effect; and (4) a significant increase of solubility in the presence of charged polymers. At pH 6–7, rhTFPI solubility–salt profiles display typical salting-in and salting-out biphasic effects. At a slightly lower pH (pH 5), a third phase in addition to the salting-in and salting-out phases was observed at low ionic strength conditions (5 to 50 mM) where rhTFPI solubility increased as salt concentration decreased. The salting-out constant for rhTFPI in NaCl is 1.04 M^{-1} and is independent of the pH of the solution. Resolubilization of rhTFPI precipitates revealed that "insolubility precipitates" (seen during buffer exchanges) resulted from protein solute saturation and could be redissolved by "native" solvent conditions. On the other hand, "instability precipitates" (typically seen after exposure to elevated temperatures or extended storage periods) were caused by insoluble protein aggregate formation and required strongly denaturing conditions to redissolve.

Introduction

Tissue factor pathway inhibitor (TFPI), formerly known as lipoprotein associated coagulation inhibitor (LACI)[1] or extrinsic pathway inhibitor (EPI),[2] is an endogenous inhibitor of tissue factor-mediated blood coagulation. It forms a quaternary complex with tissue factor, factor VIIa, and factor Xa. This complex prevents factor Xa from participating in the formation of prothrombinase complex, thus blocking the generation of thrombin from prothrombin.[3]

Recombinant human tissue factor pathway inhibitor (rhTFPI) expressed from *Escherichia coli* is a pharmaceutically important protein. In animal studies, rhTFPI has been shown to be effective in preventing thrombosis in a rabbit model of vascular trauma.[4] In a baboon sepsis model and a rabbit focus-of-infection model, rhTFPI was associated with a marked improvement in survival rate compared with that of the placebo.[5,6]

The *E. coli*-derived rhTFPI lacks glycosylation and is sparingly soluble in physiological media (approximate solubility in aqueous media under physiological pH conditions is 0.2 mg/mL). Chaotropic agents (e.g., urea) have been employed to maintain its solubility during the purification process.[7] The low solubility of rhTFPI poses a significant challenge during process and formulation development, product manufacturing, and clinical administration of this molecule.

* To whom correspondence should be addressed. Phone: (510) 923-4294. Fax: (510) 923-4116. E-mail: bao-lu_chen@cc.chiron.com.

Protein solubility has recently been reviewed in a number of publications.[8−10] Although it is known that pH and salt conditions can have a modulatory effect, strategies for enhancing the solubility of proteins at or near physiological conditions are usually derived through trial and error, and differ considerably from one protein to the other. Low solubility is often thought to result from a combination of electrostatic and hydrophobic interactions between protein−solvent and protein−protein molecules.[11] The solubility of a protein apparently depends on subtle solvent disturbances in the distribution of charged, polar, and hydrophobic residues on the protein surface. However, because the key interactions dictating the mechanisms of insolubility and precipitation are not fully understood, an empirical approach is often taken when attempting to increase the solubility of a protein.

The solubility properties of rhTFPI are unique in being significantly influenced by the distribution of both positively and negatively charged residues in clustered domains on the polypeptide chain. The rhTFPI molecule consists of an acidic amino-terminal region, three Kunitz-type domains in tandem, and a basic carboxyl-terminal region (Figure 1).[7,12] Inter- and intramolecular electrostatic interactions between the two charged termini may confer poor solubility characteristics on the protein under physiological conditions. One goal of the present investigation was to understand the solubility behavior of rhTFPI under various pH, salt, and formulation conditions to aid in developing robust manufacturing processes and an appropriate formulation for this protein.

A second goal of this study was to understand precipitation phenomena in pharmaceutical proteins using rhTFPI as an example. Protein precipitation resulting from changes in protein solubility is a common occurrence during purification and formulation processes. It is often not differentiated from the formation of insoluble protein aggregates caused by protein instability resulting from exposure to stress conditions encountered during production processes or extended storage. For example, a temperature change during processing could either alter protein solubility, resulting in protein precipitation, or affect protein stability, leading to protein aggregation and precipitation. In this article, we have designated a protein precipitate originating from an excess amount in a saturated solution as an "insolubility precipitate". In contrast, a protein precipitate attributable to changes in stability has been labeled as an "instability precipitate". Because the mechanisms of creation of the two precipitates are different, it was hoped that the characterization of these precipitate types would aid in developing approaches to prevent their formation during processing and storage of the protein.

Materials and Methods

Materials—Recombinant human TFPI was expressed in *E. coli* and purified by a series of chromatographic, diafiltration, and associated procedures described previously.[7,12,13] The purified

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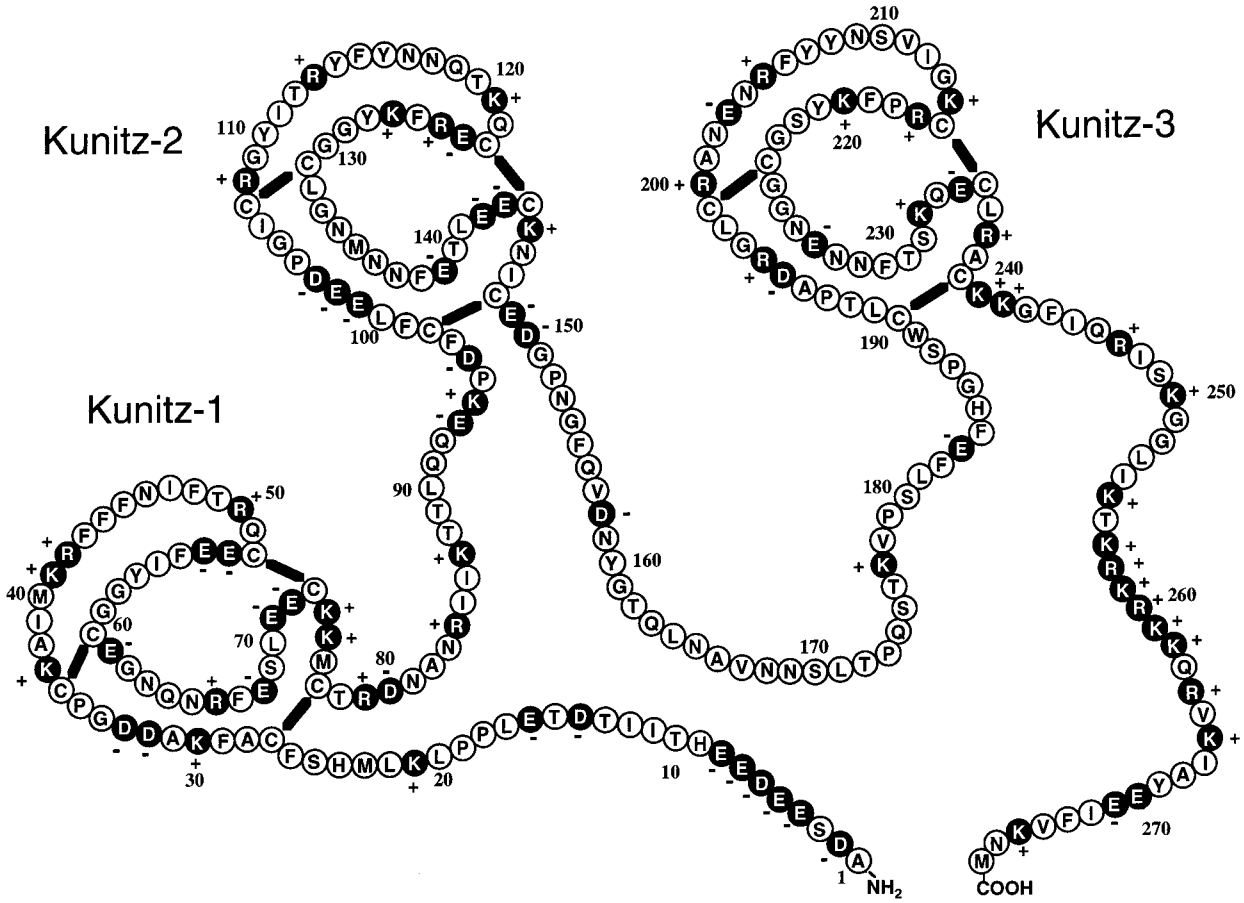

Figure 1—Structure of rhTFPI with the charged amino acid side chains marked. The three Kunitz-type domains are also shown.

protein, 10 mg/mL in a solution containing 150 mM NaCl and 2 M urea buffered with 20 mM sodium phosphate at pH 7, was stored at −70 °C as a frozen bulk. This protein was used as the starting material for all solubility studies. Dextran (MW 10 000), dextran sulfate (MW 10 000), poly-L-arginine hydrochloride (MW 11 800), poly-L-glutamine (MW 10 100), and poly-L-glutamic acid sodium salt (MW 1000) were purchased from Sigma Chemical Co. Poly(acrylic acid) (MW 10 000) was purchased from Polysciences, Inc. All buffer solutions for solubility screening and resolubilization experiments were prepared by Chiron Media Service Groups using reagent grade chemicals.

Solubility Screening—A dialysis method was used for the solubility screening experiments. Approximately 2−3 mL of rhTFPI bulk was loaded into a dialysis tubing (Spectra/Por 7 from Spectrum Inc., MWCO 3500) and dialyzed against 50- to 100-fold excess buffer solution (three exchanges per run) at either 4 °C or ambient temperature for 12 to 24 h. The dialysate was filtered through a 0.2-μm filter (Spin-X centrifuge tube filters from Corning Costar Corp.) to remove visible precipitate, and the resulting clear protein solution was analyzed for concentration of remaining soluble rhTFPI by ultraviolet (UV) absorbance and/or one of two high-performance liquid chromatography (HPLC) methods. Bioactivity, based on the anticoagulation role of TFPI, was assessed by measuring in vitro prothrombin time.

UV Absorbance Measurement—UV absorbance of protein solutions was measured using a Hewlett-Packard Diode Array spectrometer (Model 8452). Absorbance at 278 and 400 nm for protein solutions (P_{278} and P_{400}) and buffer solutions (B_{278} and B_{400}) was recorded using a 1.0-cm path length quartz cuvette. The concentration of rhTFPI in mg/mL was calculated using an extinction coefficient of 0.68 $(mg/mL)^{-1}cm^{-1}$ according to the following expression:

$$[(P_{278} - P_{400}) - (B_{278} - B_{400})]/0.68 \quad (1)$$

High-Performance Liquid Chromatography (HPLC)—Cation-exchange HPLC (CEX-HPLC) was performed on a Pharmacia Mono-S HR 5/5 glass column using a Waters 626 LC system with a 717 heater/cooler autosampler. The column was equilibrated with 80% mobile phase A (70:30 v/v, 20 mM sodium acetate: acetonitrile at pH 5.4) and 20% mobile phase B (70:30 v/v, 20 mM sodium acetate and 1 M ammonium chloride:acetonitrile at pH 5.4). After injection, the protein was eluted by increasing mobile phase B to 85% in 21 min at a flow rate of 0.7 mL/min. The rhTFPI eluted at ~16.5 min as a single peak and was detected by UV absorbance at 280 nm with a Waters 486 absorbance detector. Data acquisition and processing were performed on a Perkin-Elmer Turbochrom system. Protein concentration was estimated by integrating the peak area and comparing it with a standard curve generated from samples of known concentrations.

Reversed-phase HPLC (RP-HPLC) was performed on a Rainin Microsorb-MVC8 column (5 cm × 4.6 mm, 5 μm, 300 Å) using a Waters 626 LC system with a 717 heater/cooler autosampler. The column was equilibrated with 86% mobile phase A (30% acetonitrile/0.45% TFA) and 14% mobile phase B (60% acetonitrile/0.45% TFA). After injection, the protein was eluted by increasing mobile phase B to 20% in 6.5 min, then to 68% in 15.5 min, and finally to 100% in 17 min at a flow rate of 1 mL/min. Eluted protein was detected by UV absorbance at 280 nm with a Waters 486 absorbance detector. Data acquisition and processing were performed on a Perkin-Elmer Turbochrom system.

The Prothrombin Time Bioassay—The in vitro Prothrombin Time bioassay (PT assay) was performed on a Coag-A-Mate RA4 instrument (Organon, Teknika). Samples were first diluted to 150 μg/mL with a urea buffer (2 M urea, 20 mM sodium phosphate, 250 mM NaCl, pH 7.2) and then to 30 μg/mL with a Tris buffer (50 mM Tris, 100 mM NaCl, 1 mg/mL bovine serum albumin, pH 7.5). Finally, standards of known rhTFPI concentrations were diluted to 18, 15, 12 and 9 μg/mL and test samples to ~15 and 12 μg/mL by the Tris buffer. A 10-μL aliquot of diluted sample was first mixed with 90 μL of Verify I (Organon Teknika) in a plastic test tray (Organon Teknika) and the tray was then placed into the Coag-A-Mate instrument. A 200-μL aliquot of Simplastin Excel (Organon Teknika) was added to each well to initiate the clotting process. The clotting times for test samples and standards were recorded. The clotting time for the test sample was converted to

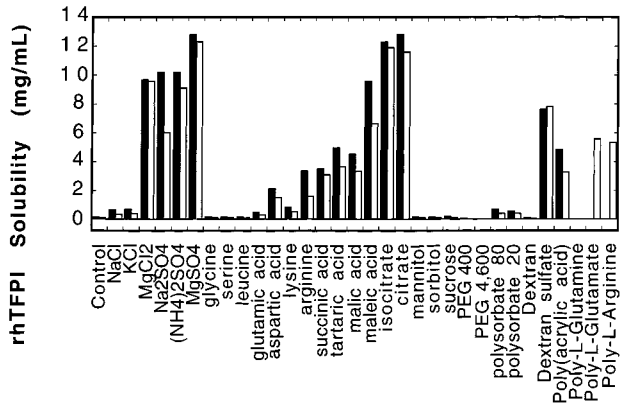

Figure 2—rhTFPI solubility in various solvent systems at 4 °C (shaded) and ambient temperature (open). All solutions were buffered at pH 7 by 10 mM sodium phosphate, which is also shown as the control. Concentrations of tested compounds were 150 mM for salts and amino acids, 5% (w/v) for mannitol and sorbitol, 9% (w/v) for sucrose, 0.1% (w/v) for polysorbates, 0.5% (w/v) for dextran, dextran sulfate, and poly(acrylic acid), 0.75 mM for poly-L-glutamine, and 1 mM for poly-L-glutamic acid and poly-L-arginine. Poly-L-glutamine, poly-L-glutamic acid, and poly-L-arginine show only ambient temperature solubility data.

rhTFPI potency by interpolation on the standard curve, which plotted the natural log of the clotting time versus rhTFPI concentration.

Circular Dichroism (CD) Measurement—Far UV CD spectra of rhTFPI samples were obtained with a JASCO-710 spectropolarimeter (Jasco Inc., Easton, MD) using a 0.2-mm path length cylindrical quartz cell.

Results

Solubility Screening Studies—The solubility of rhTFPI in 10 mM sodium phosphate at pH 7 is <0.5 mg/mL. Known amounts of a number of solubilizing solutes were added to this buffer to enhance the aqueous solubility of rhTFPI, and the data are shown in Figure 2. We have divided the tested solutes into three classes: minor, moderate, or good solubilizing agents. Minor solubilizers were defined as compounds that enhanced rhTFPI solubility by several fold to a concentration not exceeding 1 mg/mL; these include NaCl, KCl, mannitol, sorbitol, sucrose, glycine, leucine, glutamic acid, polysorbate 20, polysorbate 80, dextran, and poly-L-glutamine. Moderate solubilizers were defined as those compounds that increase rhTFPI solubility into the range of 1 to 5 mg/mL; these include lysine, arginine, aspartic acid, succinic acid, malic acid, and tartaric acid. Finally, good solubilizers were those that increased rhTFPI solubility to concentrations >5 mg/mL. These include $MgCl_2$, Na_2SO_4, $(NH_4)_2SO_4$, maleic acid, citric acid, poly-L-glutamic acid, poly-L-arginine, dextran sulfate, and poly(acrylic acid). The classification reveals a clear pattern. Neutral compounds, polymers, and monovalent ions have a marginal effect on rhTFPI solubility, whereas multivalent electrolytes and ionic polymers exhibit a more marked effect.

There was a modest temperature effect on rhTFPI solubility. Solubility of rhTFPI at 4 °C was slightly higher than that measured at ambient temperature. This was independent of the type of solvent tested. Thus, rhTFPI has a negative temperature coefficient for solubility.

Effect of pH on Solubility—The solubility of rhTFPI as a function of pH was investigated in the pH range 3−11 buffered by either 20 mM sodium acetate, sodium glutamate, sodium succinate, sodium phosphate, or glycine. Because the pH profile of a protein may depend on the ionic strength,[14] we included 150 mM NaCl in the buffer systems to minimize the difference in ionic strength for these

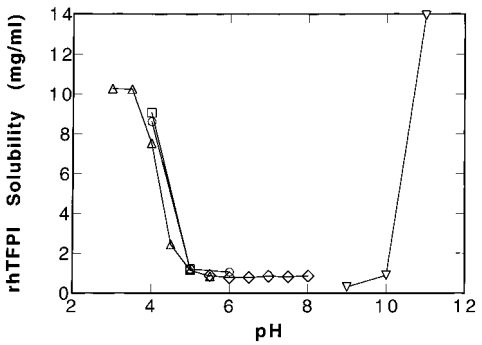

Figure 3—Dependence of rhTFPI solubility on pH in 150 mM NaCl and 20 mM buffer of sodium acetate (triangle), sodium phosphate (diamond), glycine (inverted triangle), sodium L-glutamate (square), and sodium succinate (circle).

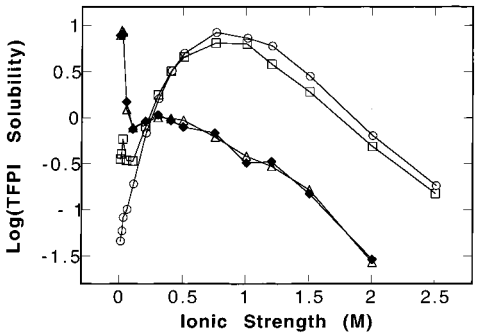

Figure 4—Dependence of logarithm rhTFPI solubility on the ionic strength in sodium chloride solutions buffered with 5 mM sodium acetate at pH 5 (filled diamond) and sodium phosphate at pH 5 (open triangle), pH 6 (open square), and pH 7 (open circle).

buffers. The function appeared as an inverted bell-shaped curve (Figure 3) with a minimum solubility trough between pH 5 and pH 10. The lowest solubility was ~0.5−1 mg/mL centered near pH 7, which is 2−3 pH units away from the isoelectric point of rhTFPI (9.2). Below pH 4.5 and above pH 10.5, rhTFPI solubility rose sharply and eventually exceeded 10 mg/mL.

Effect of Ionic Strength on Solubility—We investigated the dependence of rhTFPI solubility on ionic strength in NaCl solutions buffered by sodium phosphate at pH 5, 6, and 7 and by sodium acetate at pH 5. Figure 4 shows the logarithm of rhTFPI solubility as a function of the ionic strength at the three pH conditions. The ionic strength, I, was defined as

$$I = 1/2 \, \Sigma \, C_i \, Z_i^2 \qquad (2)$$

where C_i is the concentration of the i^{th} ion and Z_i is the charge on the i^{th} ion. At pH 6 and 7, rhTFPI solubility shows typical "salting-in" and "salting-out" biphasic curves. The solubility initially increased with ionic strength to ~1 M and then decreased at subsequent, higher ionic strengths. The entire solubility curve at pH 6 was lower than that at pH 7. At pH 5, buffered by either phosphate or acetate, a triphasic solubility curve was observed. At this pH, the rhTFPI solubility curve in the normal salting-in and salting-out regions was lower than the curve at pH 6. In contrast to the behavior at pH 6 and 7, a large part of the salting-in phase was diminished. Instead, a third phase at low ionic strength conditions was observed in which rhTFPI solubility increased as the ionic strength decreased.

The salting-out part of the solubility curves at pH 5 (0.5−2 M ionic strength), 6, and 7 (1−2.5 M ionic strength) exhibited a linear dependence of the logarithm of the solubility value on ionic strength. This part of the solubility

Table 1—Salting-Out Parameters for rhTFPI in Sodium Chloride Solutions Buffered by Either Acetate or Phosphate to pH 5, 6, and 7[a]

pH (buffer species)	β	K_s (M^{-1})
5 (sodium acetate)	0.51	0.95
5 (sodium phosphate)	0.56	0.99
6 (sodium phosphate)	1.90	1.09
7 (sodium phosphate)	2.07	1.12

[a] The salting-out parameters were calculated by linear fitting salting-out part of the solubility curves from 0.5 to 2 M ionic strength for pH 5 and from 1 to 2.5 M ionic strength for pH 6 and 7.

curve was analyzed by the following empirical equation:[8]

$$\log S = \beta - K_s I \quad (3)$$

where S is protein solubility, β is a constant, K_s is the salting-out constant, and I is the ionic strength. The two salting-out parameters, β and K_s, were calculated (Table 1). Results in Table 1 show the parameter β (solubility extrapolates to zero salt concentration without considering the salting-in effect) depended on pH, whereas the salting-out constant K_s did not and was averaged to be 1.04 M^{-1}. These results are consistent with similar data for other proteins.[15]

Resolubilization of Insolubility Precipitates—Protein precipitates are usually formed from one of two distinct mechanisms. (1) exceeding the solubility limit of the protein in the medium leads to an "insolubility precipitate"; and (2) a partial or complete unfolding of the protein resulting in aggregation that eventually gives rise to an "instability precipitate". The latter is so-called because this type of precipitate is usually seen as a result of prolonged storage of the protein. Experiments were designed to understand these processes by examining the resolubilization behavior of the two types of precipitates in selected media.

A double-dialysis approach was employed for investigation of the insolubility precipitate. In the first step of this experiment, insolubility precipitates were generated within dialysis bags. A bulk solution of rhTFPI was dialyzed against phosphate-buffered saline (PBS) because of its rather limited solubility in this medium. One bag containing the precipitate was removed, and the amount of soluble protein was measured to obtain a control solubility value for each experiment. In a subsequent step, the remaining dialysis bags containing precipitate were transferred into resolubilizing media such as sodium citrate, arginine, or urea, and further dialyzed against those media. After completion of this second dialysis step, the amount of soluble protein in the bag was measured. We compared the amount of rhTFPI resolubilized by a given medium with the control solubility in PBS obtained from the first step to evaluate the effect of the resolubilization condition. We also measured the in vitro bioactivity of the resolubilized rhTFPI to assess its conformational state.

The resolubilization results are shown in Table 2. The control sample (dialyzed against PBS only) showed that 0.69 mg of the initial 10 mg of rhTFPI present per mL in the bag remained soluble after the first dialysis step; that is, >90% of the protein precipitated. In the second dialysis for resolubilization, 0.15 M arginine was able to convert approximately one-fourth to one-third of the precipitated amount into soluble protein, and 0.15 M sodium citrate converted almost the entire precipitate into soluble protein. Urea proved to be another good resolubilization agent, with 2 M urea converting half and 8 M urea converting all of the precipitated rhTFPI into soluble protein. A slightly higher recovery in 8 M urea than the starting concentration was probably caused by concentration due to osmotic pressure produced during dialysis.

Table 2—Resolubilization of rhTFPI Solubility Precipitate[a]

resolubilization buffer	soluble rhTFPI (mg/mL)	specific bioactivity
control (PBS)	0.69	1.10
PBS + 2 M urea	5.52	0.82
PBS + 8 M urea	12.2	1.04
PBS + 2 M urea + 0.1 M DTT	0.01	0.10
PBS + 8 M urea + 0.1 M DTT	11.4	0.11
0.15 M L-arginine	3.01	0.92
0.15 M sodium citrate	9.44	1.01

[a] The concentration of soluble rhTFPI was measured by the RP-HPLC method regardless of peak shift for samples containing DTT. Specific bioactivity was estimated from the PT assay results versus the protein concentration measured by the RP-HPLC.

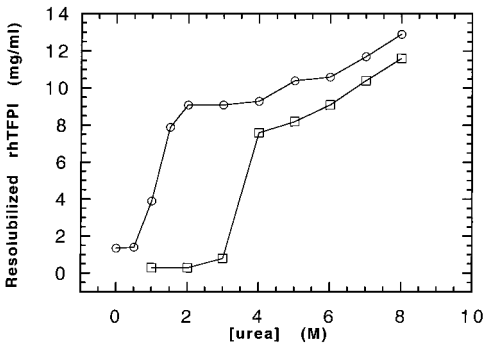

Figure 5—Resolubilization of rhTFPI "insolubility precipitate" in urea solvents in the absence (circle) and presence (square) of 0.1 M DTT.

The addition of 0.1 M dithiothreitol (DTT) to the two different concentrations of urea produced dissimilar effects. In 2 M urea containing 0.1 M DTT, almost no soluble rhTFPI was detected after the second dialysis step, showing that the presence of DTT had an adverse effect on resolubilization in the more dilute urea solution. In contrast, 0.1 M DTT in 8 M urea had little effect on the resolubilization of rhTFPI. Regardless of its effect on resolubilization, DTT evidently produced conformational changes in the rhTFPI molecule. The bioactivity data in Table 2 indicate that resolubilized rhTFPI remained fully active (within the ±20% variation of the assay) except when DTT was present in the resolubilization medium.

To understand the role of DTT in the resolubilization studies more clearly, the urea/DTT resolubilization experiment was repeated using a broader urea concentration range (Figure 5). When the amount of rhTFPI resolubilized in the second dialysis step was plotted as a function of the urea concentration in the dialysis medium, an initial low phase was followed by a transition phase and a high recovery phase regardless of the presence of DTT. However, the transition occurred at a much lower urea concentration (~1.5 M) in the absence of DTT. In 1.5 M urea, rhTFPI was still biologically active, as determined by the PT assay. Measurements by CD or fluorescence showed that the molecule retained its native conformation (data not shown). With DTT present in the resolubilization medium, the transition was shifted to a higher urea concentration (~3.5 M). Because disulfide bonds have been shown to play a critical role in folding and conformational stability of Kunitz-domain-containing proteins,[16] it is thought that reduction of these bonds by DTT may have resulted in the exposure of hydrophobic regions to the aqueous medium, leading subsequently to rhTFPI denaturation. Thus, stronger denaturing conditions were needed to resolubilize the precipitated protein when DTT was present in the medium.

To test this hypothesis further, we analyzed rhTFPI in 8 M urea with and without DTT by CD and a RP-HPLC procedure. As shown in Figure 6, the far UV CD spectrum

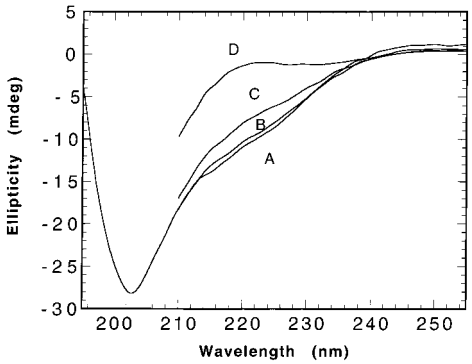

Figure 6—Far UV CD spectra of four rhTFPI samples. These four samples are (A) 0.48 mg/mL rhTFPI in PBS; (B) 0.94 mg/mL rhTFPI in 2 M urea and PBS; (C) 1.24 mg/mL rhTFPI in 8 M urea and PBS; (D) and 1.27 mg/mL rhTFPI in 0.1 M DTT, 8 M urea, and PBS (D). The CD signals were normalized to the 0.48 mg/mL rhTFPI sample for comparison.

Table 3—Percent Remaining Soluble Protein in rhTFPI Samples after Incubation at 40 °C for 0, 3, and 13 Days and after Resolubilization[a]

samples	soluble protein in stability samples with no resolubilization, %	total soluble protein in resolubilized stability samples by the following solvents, %		
		0.1 M citrate	2 M urea	8 M urea
t = 0	100	–	100	–
t = 3 days	73	–	73	110
t = 13 days	44	51	54	96

[a] Resolubilization by 0.1 M sodium citrate (only for $t = 13$ days stability sample), 2 M urea (all three stability samples), and 8 M urea (only for $t = 3$ and 13 days stability samples). All values were normalized to the $t = 0$ stability sample.

of rhTFPI in 8 M urea resembles the spectra in both 2 M urea and PBS, but with a slight loss of conformation, whereas addition of 0.1 M DTT in 8 M urea results in a significant loss of secondary structure. Similarly, RP-HPLC chromatograms showed that the rhTFPI peak eluted at 13 min in both a control sample without urea and an 8 M urea sample. With the addition of 0.1 M DTT in the 8 M urea solution, the rhTFPI peak shifted to 17.5 min, indicating increased hydrophobicity of the protein under this condition. These observations support the conclusion that reduction of disulfide bonds by DTT unfolds the protein, thus exposing hydrophobic regions at the surface.

Resolubilization of Instability Precipitates—Instability precipitates of rhTFPI were generated by incubating a 0.4 mg/mL rhTFPI solution in 10 mM sodium phosphate at pH 7, 150 mM NaCl, and 0.005% polysorbate 80 at 40 °C for various periods of time. Under these conditions, the major degradation pathway of the protein has been determined to be protein aggregation that eventually results in formation of a visible precipitate over time. Stability samples at $t = 0$, 3, and 13 days were withdrawn and were analyzed for remaining soluble protein after removal of the visible precipitate by filtration. Samples containing precipitate (i.e., without filtration) were also dialyzed against 0.1 M sodium citrate, 2 M urea, and 8 M urea in attempts to resolubilize the precipitated protein.

Table 3 displays the remaining soluble protein in stability samples and the total amount of soluble rhTFPI in the stability samples after resolubilization. Upon incubation at 40 °C for 3 and 13 days, the amount of soluble protein decreased to 73 and 44% of the initial value, respectively. When the stability samples containing the soluble and precipitated protein were dialyzed into citrate and urea resolubilizing media, only small fractions of the precipitated protein could be resolubilized using 0.1 M sodium citrate or 2 M urea solutions (7−10% resolubilized from the 13-day stability sample containing 56% rhTFPI as a precipitate). On the other hand, resolubilization by an 8 M urea solution afforded a quantitative recovery of the instability precipitate. These data indicate that the precipitated protein in stability samples was not resolubilized by native or near-native media (such as 0.1 M sodium citrate or 2 M urea) but needed strong denaturing conditions for resolubilization.

Discussion

Adequate solubility of a protein in aqueous media is a critical requirement for development of commercial manufacturing processes and stable formulations for clinical application. Determination of protein solubility often requires preparation of a saturated solution in the given medium. Common techniques include dialysis, ultrafiltration, dissolution of a dried protein powder, or precipitation with poly(ethylene glycol).[10,17,18] We have used a dialysis method to study rhTFPI solubility. The dialysis approach is simple and allows rapid screening of a large number of potential solvents. If the protein concentration in the starting material is sufficiently high and precipitation is observed during dialysis, the soluble fraction of the protein measured within the dialysis membrane represents its saturated solubility in the selected medium. If protein solubility in a particular solvent exceeds the protein concentration in the starting material, further concentration of the dialyzed solution by ultrafiltration may be necessary to obtain the true maximum solubility. In this paper, we used the dialysis approach not only for obtaining the solubility value for a protein but also for performing further studies to elucidate solubility mechanisms.

Attractive Ionic Interaction Limits rhTFPI Solubility—Proteins with poor aqueous solubility are often considered to be "hydrophobic".[9] We have reason to believe that the insolubility of rhTFPI's may originate from attractive electrostatic interaction between the termini of the protein. The rhTFPI molecule consists of a single polypeptide chain of 277 amino acid residues. It has a total of 11 aspartate, 25 glutamate, 17 arginine, and 25 lysine residues. (The histidine residue is not included because the pK_a of its side chain is close to neutral pH, making its protonation state difficult to predict.) These ionizable residues in the protein are randomly distributed within the polypeptide structure, including the three Kunitz domains, except at the two termini where they are present in clusters (Figure 1). The N-terminus contains 3 aspartate and 5 glutamate residues that are located between residues 2 to 16. Similarly, 10 lysine, 4 arginine, and 2 glutamate residues are located between residues 240 to 275 at the C-terminus. At neutral pH, the N-terminus (containing 8 negatively charged residues) behaves as an anionic polymer and the C-terminus (containing 14 positively charged residues) behaves as a cationic polymer. Such uneven charge distribution may result in a large dipole moment on the protein molecule. Thus, electrostatic attractive forces between the two termini and dipole−dipole interaction between two protein molecules may cause both intra- or intermolecular interactions, resulting in reduction of protein solubility.[19]

The hypothesis that insolubility of rhTFPI is attributable to the electrostatic interaction of its two charged termini is supported by the following facts: (1) The rhTFPI pH−solubility curve shows an unusually broad solubility minimum trough around neutral pH (Figure 3). This trough is probably caused by the strong ionic interaction of positively charged lysine and arginine residues with negatively charged aspartate and glutamate residues. In the trough

pH region, these residues are all strongly charged. (2) The C-terminal truncation analogues of rhTFPI exhibit a much greater aqueous solubility than the full-length rhTFPI. For example, an analogue containing the N-terminus and the first two Kunitz domains (analogue 1−160) and missing the positively charged C-terminus, has an aqueous solubility >15 mg/mL (Gustafson, M. E., personal communication). This solubility is almost 2 orders of magnitude higher than the submilligram solubility of the full-length protein. (3) Both cationic (e.g., poly-L-arginine) and anionic polymers (e.g., dextran sulfate, poly-L-glutamate, and poly(acrylic acid)) have remarkable solubilizing effects on rhTFPI. Presumably, ionic polymers can bind to the termini clustered with oppositely charged residues. Such binding shields charges on one terminus, preventing it from interacting with the other terminus. (4) Compounds likely to affect hydrophobic properties of proteins have no effect on rhTFPI solubility. For instance, hydrophobic and neutral amino acids have little effect on rhTFPI solubility (Figure 2). In addition, polysorbate surfactants and poly(ethylene glycol) also did not enhance the protein solubility. Therefore, it is unlikely that hydrophobic interactions play a critical role in rhTFPI insolubility. (5) The solubility of rhTFPI shows a negative temperature coefficient. Because most of the tested conditions are within the salting-in region, where the electrostatic interaction dominates the solubility, the negative temperature effect is probably due to the decreased dielectric constant instead of the increase in the hydrophobic interaction at high temperatures. Decrease in dielectric constant strengthens the electrostatic interaction between the protein termini, thus reducing the solubility. Taken together, these observations strongly suggest that poor aqueous solubility of rhTFPI is a result of ionic interaction of the two charged termini.

Unbalanced Charge Distribution on the Two Termini Shifts the Solubility Minimum Away from the p*I*—Many proteins show an inverted bell-shaped pH−solubility curve with a solubility minimum at or close to the isoelectric point.[20,21] The net zero charge on the protein surface at its p*I* decreases the repulsive forces between protein molecules and makes the protein prone to precipitation. Solubility rises as net charge increases at lower pH due to the protonation of negatively charged residues, and at higher pH, because of deprotonation of positively charged residues. The p*I* of rhTFPI is 9.2 and its solubility minimum is centered around pH 7. The considerable deviation of the solubility minimum from its p*I* may be attributed to the nature of the charge distribution on the polypeptide chain. As already mentioned, the N-terminus contains 8 negatively charged residues and the C-terminus contains 14 positively charged residues. Because the number of positively charged residues on the C-terminus is greater than the number of negatively charged residues on the N-terminus, the interaction between the two termini may not reach a maximum at its p*I* but at neutral pH where all the anionic and cationic residues are fully charged. Therefore, the minimum solubility of rhTFPI is shifted to the neutral pH region.

Salting-In, Salting-Out, and the Third Solubility Phase at Low Ionic Strength—When salt is gradually added to a protein solution, the solubility of the protein usually exhibits two macroscopic processes. First, protein solubility increases, reaching a maximum when the salt concentration produces low to moderate ionic strength conditions. Then, protein solubility decreases as the salt concentration further increases the ionic strength of the solution. These two macroscopic processes are known as "salting-in" and "salting-out" effects. The salting-in effect can be readily explained using the Debye−Hückel screening through nonspecific electrostatic interactions between a charged protein macro-ion and surrounding small ions.[20,22] This theory assumes protein molecules are approximately spherical macro-ions with uniformly distributed charges. Interaction with small counterions stabilizes protein macro-ions and decreases their activity coefficients, resulting in an increase in solubility. At low ionic strengths where the salting-in effect predominates, protein solubility is usually a function of the ionic strength and independent of the ion type.

At higher concentrations of salt, the solute molecules compete with protein molecules for water molecules to attain hydration. An increase in salt concentration promotes hydrophobic interactions between protein molecules, decreasing protein solubility. In contrast to the salting-in effect, protein salting-out depends strongly on the ion type. The effectiveness of a salt for this phenomenon follows the lyotropic or Hofmeister series.[23]

At pH 5, in addition to the expected salting-in and salting-out, a new phase was observed for rhTFPI at low ionic strength conditions (5 to 50 mM). Addition of salt resulted in an effect opposite to the salting-in effect, as protein solubility decreased with increase in salt concentration. The origin of this phase is not clear to the authors at the present time. However, we speculate that increased electrostatic repulsive forces between protein molecules at low ionic strength may increase its solubility. At pH 5, positively charged residues, such as arginine and lysine, are fully charged, whereas negatively charged residues, such as glutamate and aspartate, are only partially charged. Under low ionic strength conditions, an overwhelming population of positively charged residues on rhTFPI may yield a repulsive force strong enough to prevent it from precipitating. The addition of salt to such a solution would increase the dielectric constant of the solution, resulting in a weakening of the electrostatic interactions, thus decreasing solubility.

In addition to the third phase observed at the low ionic strength conditions at pH 5, we also noticed that rhTFPI solubility became somewhat pH independent around an ionic strength of 0.25 M under the tested pH range. Further work is needed to explore this effect.

The salting-in effect explains quite well the solubility effectiveness of the salts we examined in Figure 2. Compared with the relatively poor solubilizing salts NaCl and KCl, the good solubilizing salts $MgCl_2$, Na_2SO_4, $(NH_4)_2SO_4$, and $MgSO_4$ all affect the ionic strength to a much greater extent and are, therefore, believed to be more effective in solubilizing rhTFPI. In contrast, the polyelectrolytes showing good solubilizing effect on rhTFPI act by binding to the basic C-terminus or the acidic N-terminus but not by merely increasing the ionic strength. For example, 0.75 mM poly-L-arginine (MW 11 800 daltons) and 1 mM poly-L-glutamate (MW 1000 daltons) both solubilized rhTFPI to the same extent (Figure 2) despite having widely different effects on the solution ionic strength because of the approximately 10-fold variance in the number of charges they are carrying.

Insolubility Precipitates and Instability Precipitates—As shown in Table 2, rhTFPI insolubility precipitate can be redissolved in media containing sodium citrate, arginine, or low concentrations of urea. These media do not denature the protein and are good solubilizers for native rhTFPI. This result indicates that the interactions responsible for formation of insolubility precipitates are weak and probably nativelike. Therefore, insolubility precipitates are most likely formed from native rhTFPI molecules. The following reaction scheme is indicated:

$$N \leftrightarrow \text{Insolubility Precipitates}$$

In contrast, instability precipitates formed as a result of a thermal treatment or on addition of DTT require media with high concentrations (4 M) of urea for resolubilization (Figure 5). The interactions between precipitated protein molecules are strong in these cases and require denaturing conditions to break them down, suggesting that instability precipitates must be formed from denatured (or partially denatured) protein molecules. The reaction scheme can be described as follows:

$$N \leftrightarrow U \rightarrow \text{Instability Precipitates}$$

Although it is possible that instability precipitates may be converted from insolubility precipitates as shown in the following mechanism, we do not believe this mechanism fits our data on rhTFPI:

$$N \leftrightarrow \text{Insolubility Precipitates} \rightarrow \text{Instability Precipitates}$$

The small temperature coefficient for rhTFPI solubility is unlikely to limit its solubility at 40 °C to create an insolubility precipitate. Further, addition of 2 M urea failed to recover any instability precipitates for the $t = 3$ days stability sample (Table 3), demonstrating an absence of the insolubility precipitates. Finally, the slow kinetics of rhTFPI degradation at 40 °C also ruled out the possibility of a quick conversion from insolubility precipitates to instability precipitates.

The kinetics of formation of insolubility precipitates differ from those for instability precipitates. The amorphous precipitation caused by protein insolubility is often a much faster event than the aggregation and precipitation resulting from protein instability. In rhTFPI, the insolubility precipitate is formed immediately upon buffer exchange, and the turbidity of the solution develops within minutes. On the other hand, rhTFPI instability precipitates are formed over much longer periods of time (weeks to months at high temperatures). For example, loss of soluble rhTFPI (increase of precipitated protein) at 40 °C was estimated to have a half-life of ~12 days, based on the data in Table 3. The kinetics of formation of an insolubility precipitate often contains a lag phase (nucleation) and a rapid growth phase (polymerization),[10] whereas instability precipitate formation may be governed by its unimolecular unfolding as the rate-limiting step that is then followed by a rapid polymerization process.[24]

Conclusions

In conclusion, our studies to determine the solubility of rhTFPI under various solvent conditions showed that rhTFPI solubility is predominantly affected by the charge distribution on the protein molecule itself as well as on the solvent ions present in the surrounding medium. Considering these interactions, the limited solubility of the protein under physiological conditions could be explained and solutes were identified to enhance rhTFPI solubility in aqueous media. Detailed examinations of protein−salt interactions have revealed a third solubilizing phase for rhTFPI at pH 5 under low ionic strength condition that is different from the normal salting-in and salting-out phases of the protein. Finally, by comparing the dissolving power of nondenaturing and denaturing solvent conditions, we have shown that rhTFPI precipitates formed due to the limited protein solubility are different from those resulting from protein instability. Whereas insolubility precipitates can be resolubilized by nondenaturing solvent conditions, such as citrate, arginine, and low concentrations of urea, strong denaturing conditions, such as >4 M concentrations of urea, are required for resolubilization of instability precipitates. These observations are expected to be useful in designing purification processes and formulations needing high aqueous solubility of rhTFPI for further development of the molecule as a therapeutic agent.

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