Refolding and Oxidation of Recombinant Human Stem Cell Factor Produced in Escherichia coli*

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Michael D. Jones, Linda O. Narhi, Wen-Chang Chang‡, and Hsieng S. Lu§
From Amgen Inc., Thousand Oaks, California 91320

Oxidative folding of recombinant human stem cell factor (rhSCF) produced in Escherichia coli was investigated in vitro. Folding of denatured and reduced rhSCF involves at least five intermediate forms, I-1 to I-5, detectable by their differences in hydrophobicity using reverse-phase high performance liquid chromatography. Both I-1 and I-2 contain a native-like disulfide bond, Cys4–Cys89 and Cys43–Cys138, respectively, and I-3 forms a mispaired disulfide, Cys4–Cys89. These forms appear to reach steady state equilibrium and are important folding intermediates. I-1 was found to be the prominent intermediate that directly folds into native rhSCF (N); and the thermodynamically less stable I-2 favors rearrangement into I-1. I-3 may serve as an intermediate for disulfide rearrangement between I-1 and I-2. I-4 and I-5, which are disulfide-linked dimers, are in equilibrium with reduced rhSCF and other intermediates and may not play an important role in rhSCF folding. Both trifluoroacetic acid-trapped I-1 and I-2, after isolation by high performance liquid chromatography, proceed with the remaining oxidative folding process after reconstitution. Iodoacetate-trapped I-1 and I-2 contain low α-helical content and some tertiary structure, while I-3 and reduced rhSCF have little ordered structure. Gel filtration/light-scattering experiments indicate that reduced rhSCF and iodoacetate-trapped I-1, I-2, and I-3 exist as dimeric forms, indicating that rhSCF dimerization precedes formation of disulfide bonds. I-1, I-2, I-3, and the C43,138A analog lacking Cys43–Cys138 bond are not biologically active or exhibit significantly lower activity. The two disulfide bonds in rhSCF seem to be essential for the molecule to maintain an active conformation required for its receptor binding and biological activities.

The mechanistic folding of proteins is a critical area of study in understanding their structural and conformational behaviors in solution. Much experimental work has been done on the thermodynamics and kinetics of folding pathways of proteins (1–4). Disulfide-containing proteins have the advantages that the intra- or interchain disulfide bond is a natural covalent cross-link which, by thermodynamic requirements, stabilizes the cross-linked conformation (1). Oxidative refolding of a disulfide-reduced protein allows one to study the rates of folding and to chemically trap the folding intermediates. Many such folding intermediates are sufficiently long-lived during folding and can be isolated and characterized. In several cases, detailed folding pathways have been defined in terms of occurrence of disulfide-containing intermediates at various stages of folding (4–7). Through the analysis of disulfide intermediates, it is apparent that disulfide formation does not necessarily occur through a simple sequential pathway. As a result, specific issues regarding protein folding pathways have been brought to attention (3, 6–10). These include the significance of non-native disulfides, the kinetic importance of well populated and thermodynamically stable intermediates, and the clarification of the consequence and role of kinetically trapped species that accumulate in the pathway.

Oxidative refolding of recombinant human stem cell factor (rhSCF) is a hematopoietic cytokine that plays an important role in the stimulation, proliferation, differentiation, and functional activation of blood cells (11, 12). The molecules obtained naturally or expressed in recombinant CHO cells are heavily glycosylated (13–15). Recombinant hSCF (rhSCF) can also be produced in genetically engineered Escherichia coli (16). The glycosylated or nonglycosylated molecule exists as a noncovalently linked homodimer in solution (17). Dissociation-reassociation of rhSCF dimer spontaneously occurs at a fairly rapid rate (18). There are two intramolecular disulfide bonds present in the molecule (Cys4–Cys89 and Cys43–Cys138), and the production of active rhSCF from E. coli requires an oxidative folding procedure to recover its biological activity (16). In this study, the refolding pathway of reduced rhSCF is examined by monitoring the distribution of HPLC-separable disulfide-bonded intermediates during refolding and by characterizing physicochemical and biochemical properties of the isolated intermediates. The folding of rhSCF involves intermediates containing native and non-native disulfides, thermodynamically stable and unstable intermediates, as well as disulfide-bonded dimers. These studies allow us better to understand the contribution of native and mispaired disulfide-containing intermediates to the in vitro folding pathway of rhSCF.

EXPERIMENTAL PROCEDURES

Materials—E. coli-produced rhSCF was purified according to methods described previously (16). The recombinant molecule contains 165 amino acids plus an N-terminal methionine at position +1. Ultrapure DTTred was purchased from Boehringer-Mannheim, while DTT was from Sigma. These two reagents were verified to be highly pure by reverse-phase HPLC using a C4 column. Expression, isolation and some

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†Amgen’s visiting scientist from the Institute of Biological Chemistry, Academia Sinica and Institute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan.

§To whom correspondence should be addressed: Amgen Inc., M/S 14-2-E, Amgen Center, Thousand Oaks, CA 91320-1789. Tel.: 805-447-3092; Fax: 805-499-7464.

1 The abbreviations used are: hSCF, human stem cell factor; rhSCF, recombinant hSCF; CHO, Chinese hamster ovary; DTT, dithiothreitol; DTTred, oxidized DTT; DTTred, reduced DTT; TFA, trifluoroacetic acid; IAA, iodoacetic acid; RP, reversed phase; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; Cy3(Cm), S-carboxymethylated Cys; GdnHCl, guanidine HCl; FWHM, full width at half emission maximum; N, fully oxidized rhSCF; R, fully reduced rhSCF; and P, SDS-nondissociable but DTT-reducible rhSCF dimer.
characterization of rhSCF C34,138A analog have been described previously (19). Crystallized Iodoacetic acid was purchased from Sigma. HPLC solvents and water were purchased from Burdick and Jackson. Sequencing reagents and solvents were supplied by Applied Biosystems and Hewlett Packard. All other reagents were of the highest quality available.

Denaturation and Reduction of rhSCF—rhSCF (3 mg/ml) was reduced with a 100-fold molar excess of DTT™ in 0.3 mM Tris containing 6 mM GdnHCl (pH 8.5) for 4 h at 25 °C. The sample was quickly buffer-exchanged into refolding buffer using an HR10/10 desalting column (Pharmacia Biotech Inc.) run in a fast protein liquid chromatography system. The refolding buffer is 50 mM Tris-HCl containing 2.5 M urea, 2 mM EDTA, and 60 mM NaCl (pH 8.5). Oxygen in the buffer was completely removed by a continuous nitrogen flushing of the buffer before use. Urea is introduced in the buffer to keep denatured and reduced rhSCF soluble. The concentration of protein was spectrophotometrically determined at 280 nm (absorbance = 0.62 for rhSCF at 1 mg/ml) (17).

Refolding studies were carried out immediately after buffer exchange.

Refolding and Oxidation of Reduced rhSCF with DTT—Fully reduced and denatured rhSCF was diluted to 0.25–1.0 mg/ml in oxygen-free refolding buffer as described. Samples were allowed to refold in the presence of DTT™ at 66.6 mM concentration. At various time intervals, aliquots of folding mixtures were taken, which were then reacted immediately with iodoacetic acid (IAA) or acidified with TFA (see below).

The refolding rhSCF mixtures, after IAA or acid treatment, were injected onto reverse phase columns (Syn-Chro Pak, 300 Å). Solvent A is 0.1% TFA and solvent B is 0.1% TFA in 90% acetonitrile. The column was initially equilibrated with 55% B and eluted by linear gradients from 55 to 65% B over 80 min and from 65 to 95% B over 5 min on an HP-1090 liquid chromatograph equipped with an autosampler and a photodiode array detector. Elution of protein peaks was detected at 215 nm, and protein fractions obtained from HPLC were collected for further analysis.

IAA Modification and Acid Quenching of Intermediates—Aliquots of folding mixture were removed at selected time intervals and unreacted thiols blocked by the addition of 0.1 volume of 1 mM iodoacetic acid in 1.0 mM Tris-HCl (pH 8.0) for 2 min at room temperature. Samples were then immediately examined by HPLC or desalted as described above. TFA-quenched samples were prepared by adding a 0.1 volume of 25% TFA into the folding mixture.

Partial Reduction of rhSCF—Native rhSCF at 1 mg/ml was incubated in the folding buffer (see above) containing 4 mM DTT™ at 25 °C. Aliquots were removed at various times and acidified by the addition of 0.1 volume of 25% TFA for subsequent RP-HPLC analysis.

Preparation and Refolding of Acid-quenched Intermediates—Intermediate forms obtained from acid-quenching of the folding samples or from partial reduction of native rhSCF as described can be prepared by RP-HPLC (see “Results”). The samples obtained were partially dried in a vacuum centrifuge and reconstituted in 10% of 1 M HCl containing 2.5 mM urea (pH 2.0). Folding of each isolated intermediate was resumed by adding 5 volumes of the above-mentioned folding buffer containing DTT™.

Protease Digestion and Peptide Mapping—IAA-alkylated intermediates isolated from HPLC were dried and reconstituted in 50 mM Tris buffer containing 2.5 mM urea, 2 mM EDTA, and 60 mM NaCl (pH 8.5) for endoproteinase Glu-C digestion. Peptide mapping was performed as described previously (20). Samples prepared for Asp-N peptide mapping were reconstituted using the same buffer described above but without addition of 2 mM EDTA, and detailed procedures were also as reported previously (16).

Sequence Analysis and Mass Spectrometry—Sequence analysis of peptides was performed on a Waters PicoTag mass spectrometer. Mass spectrometric analysis of peptides was performed on a Sciex API III electrospray mass spectrometer as described elsewhere (20).

Circular Dichroism (CD) and Fluorescence Spectroscopic Analyses—CD spectra were obtained using a Jasco J-720 spectropolarimeter and cuvettes with a 0.02 cm (for far UV CD) or a 1 cm (for near UV CD) path length. Protease samples were dialyzed through sterile, 0.2-μm membrane, and concentrations determined by their absorbance at 280 nm. All proteins were analyzed in 50 mM Tris-HCl containing 2.5 mM urea, 2 mM EDTA, and 60 mM NaCl (pH 8.5). The α-helical content of proteins was estimated according to Greenfield and Fasman (21). The thermal stability was measured by the temperature-induced change in ellipticity at 222 nm, using a J-70000 thermal control unit, and a rectangular cuvette with a path length of 0.1 cm. The proteins were heated at 20 °C/h, and the spectra from 240 to 200 nm were collected every 2 ± 0.5 °C. The ellipticity at 222 nm alone was simultaneously monitored every 0.5 °C. The curve of ellipticity at 222 nm versus temperature was fitted by a computer program which fits the base lines before and after heating individually, and then fits the transition region compensating for the base lines.

The fluorescence spectra were determined at ambient temperature on an SLM Aminco 500C spectrofluorimeter, using a cuvette with a path length of 0.5 cm and protein concentrations of 0.22 mg/ml. The solutions were excited at 280 nm, and the fluorescence spectra from 280 to 420 nm were recorded, using slit widths of 5 nm. rhSCF Biological Assay and SDS-PAGE—[3H]Thymidine incorporation by the human megakaryoblastic leukemia cell line UT-7 was monitored in cellular proliferation assays as described previously (22). Nonreducing SDS-PAGE was carried out using 10% polyacrylamide gels (Novex, San Diego, CA) was performed using a Laemmli (23) gel system.

Size-exclusion Chromatography and Light-scattering Detection—Gel-filtration was performed in Superdex 75 column (Pharmacia) preequilibrated with 0.1 mM phosphate buffer (pH 7.0) containing 0.5 M NaCl. Samples were analyzed by an HP-1050 Ti system (Hewlett Packard) equipped with an autosampler, a diode array detector, and a ChromStaPC-based software package for data acquisition and analysis using conditions as described elsewhere (17). Molecular weights were determined using two detectors in series: an on-line laser light-scattering detector (Mini-Dawn, Wyatt Technologies) and a refractive index detector (Hewlett Packard HP1047A). Chromatograms were collected and analyzed on the Hewlett Packard ChromStaPC. Molecular weights were determined using the ratio of light-scattering and refractive index signals, using procedures similar to those described previously (24, 25), after calibration using ribonuclease, ovalbumin, and bovine serum albumin monomer/dimer. The molecular weight of samples determined by this procedure is independent of their respective elution times.

RESULTS

Refolding of Reduced and Denatured rhSCF—In its native state, folded and disulfide-bonded rhSCF forms a noncovalently linked dimer in solution (17). In RP-HPLC, native rhSCF has been dissociated into monomers under acidic elution conditions and behaved as a sharp symmetrical peak. Under conditions using a shallow acetonitrile gradient elution in TFA, the reduced rhSCF form was well separated from the oxidized form and eluted approximately 10 min later. As shown in Fig. 1A, this HPLC procedure allows the separation of various folding intermediates for studying oxidative folding of denatured and reduced rhSCF.

Fig. 1 shows RP-HPLC analysis of IAA-treated sample aliquots taken at different time intervals. Folding after TFA acidification also exhibited a similar HPLC profile containing the same relative distribution of intermediate forms. RP-HPLC can resolve several IAA-trapped intermediates in addition to the native (N) and fully reduced (R) forms in rhSCF folding mixtures. In the initial stages of the reaction (~2 h), R and N species were readily identified along with five intermediate forms, designated as I-1, I-2, I-3, I-4, and I-5. I-4 and I-5 seemed to form very early (~20 min) and decreased to a very low level later. Intermediate I-1, I-2, and I-3 still remain to some extent throughout the whole folding process. At later stages in the folding (~20 h) a particular form P that elutes more hydrophilically than R also appeared. P was identified to be an SDS-nondissociable but DTTreducible rhSCF dimer as described in a companion study (26).

The folding kinetics were investigated further by plotting folding times versus rate of generation for each intermediate estimated from HPLC peak integration. Fig. 1B shows the kinetic profiles for the five intermediate forms as well as R and N, while Fig. 1C depicts generation rates for intermediates within the first 20 min of folding. Initially R disappeared rapidly with concomitant formation of various intermediate forms and the finally folded form N. The data clearly show that the I-1 concentration increased rapidly and persisted for a longer period of time during folding. After 48 h of folding, 72.9% of the total protein has completely folded into N, with 12.1% remaining in I-1 and 12.9% in P. Only 1.3% of the
protein remained fully reduced.

The initial first order folding rates for conversion of R into each of the intermediates and the time required to reach maximal concentration derived from the results shown in Fig. 1 are listed in Table I. Initial rates of I-1 to I-5 are similar. However, the time required for I-1 to reach its higher maximal concentration is much longer than those for I-2 and I-3. I-4 and I-5 reached maximal concentration by two min after which their rates of formation decreased. As a result, their initial rate constants could not be determined. In separate folding experiments, decreasing rhSCF concentration (i.e. 0.43 mg/ml) has dramatically reduced the initial folding rate and increased the times for intermediates to reach their maximal concentrations (Table I). For example, the initial folding rate has decreased 5 to 10-fold for I-1, I-2, and I-3. In the folding at lower rhSCF concentrations (0.2–0.5 mg/ml), the disulfide linked dimer, I-4, and I-5, have diminished.

Different rhSCF intermediates were subjected to nonreducing SDS-PAGE. As listed in Table II, R migrates as an extended, larger molecule than the native (18 and 15 kDa, respectively). IAA-trapped I-1 and I-3 also have expanded molecular sizes between N and R. Both I-4 and I-5 migrated as a dimer having a molecular mass of 38 kDa. Upon reduction, their molecular masses reduced to 18 kDa.

### Table I

| Folding products | Initial rate | Time reaching maximal concentration | Disulfide formed |
|------------------|--------------|------------------------------------|------------------|
| A. Folding of denatured and reduced rhSCF: | | | |
| 1 mg/ml | 0.43 mg/ml | 1 mg/ml | 0.43 mg/ml |
| I-1 | 1.24 | 0.24 | 120 | 240 | Cys4–Cys89 |
| I-2 | 1.07 | 0.12 | 120 | 240 | Cys43–Cys138 |
| I-3 | 0.94 | 0.10 | 5 | 240 | Cys43–Cys89 |
| I-4 | ND | ND | 2 | ND | Cys43–Cys43 |
| I-5 | ND | ND | 2 | ND | Cys89–Cys89 |
| B. Folding of reduced and denatured Cys(Cm) I-2 and C43,138A analog at 0.43 mg/ml: | | | |
| Cys(Cm) I-2 | ND | 0.281 | ND | Cys43–Cys138 |
| C43,138A analog | ND | 0.298 | ND | Cys89–Cys89 |

a Cys(Cm) I-2 was prepared by HPLC isolation from the IAA-trapped folding mixture.
b ND, not determined.
Structural Characterization of IAA-trapped Intermediates—

For structural characterization, the above-mentioned IAA-modified R and intermediates were isolated by RP-HPLC as described (Fig. 1A) and subjected to peptide mapping after endoproteinase Glu-C digestion. Fig. 2 compares peptide maps derived from the digestion of IAA-trapped I-1, I-2, I-3, I-4, I-5, as well as Cys(Cm)-rhSCF. The obtained peptides were characterized by sequence analysis and electrospray mass spectrometry. R after IAA modification had a profile identical to that of Cys(Cm)-rhSCF (chromatogram 1). I-1 contains a single Cys4–Cys89 disulfide bond with Cys43 and Cys138 remaining unpaired, and I-2 has a Cys43–Cys138 disulfide bond with unpaired Cys4 and Cys89 (chromatograms 3 and 4, respectively). I-3 forms a non-native Cys43–Cys89 disulfide bond and leaves Cys4 and Cys138 unpaired (chromatogram 2). Both I-4 and I-5 also form an incorrect disulfide bond, i.e. Cys43–Cys43 and Cys89–Cys89, respectively, to become disulfide-linked dimers (chromatograms 6 and 7). I-4 and I-5 have three free cysteines as seen in their respective peptide maps. The disulfide structures of form N was confirmed to be identical to rhSCF standard by direct comparison of peptide maps obtained from Asp-N endoproteinase digestion (data not shown, also see Ref. 16).

Folding of Acid-quenched Intermediates and Partial Reduction of Native rhSCF—Following HPLC isolation of TFA-trapped intermediates, folding of individual intermediates was subsequently investigated. Fig. 3, A and B, shows the folding of I-1 and I-2 by HPLC analysis. Folding of I-1 appears to generate only N. In contrast, I-2 folds into I-1 and I-3, which then convert to N. A small amount of R and an uncharacterized form X near I-1 were detected as well.

Folding of rhSCF C43,138A Analog and Alkylated I-2—Both rhSCF C43,138A analog and alkylated I-2 were isolated, denatured, reduced, and refolded. Fig. 4A and Table I shows that the initial rates for formation of the Cys4–Cys89 disulfide for reduced rhSCF C43,138A analog and wild type rhSCF are similar. Their overall folding rates over 20 h are also similar. This observation appears to indicate that I-1 may directly fold into N in reduced I-2. The initial oxidation rate for Cys43–Cys138 from the reduced form of I-2 was also similar to that of the formation of the same disulfide in the wild type protein. However, the overall folding rate for formation of I-2 in the wild type rhSCF is significantly slower.

Physicochemical Analyses—Fig. 5A shows the far UV CD spectra of various folding intermediates. Both N and rhSCF standard in 2.5 M urea have approximately identical spectra.
Both samples contain about 45% $\alpha$-helix, consistent with results observed in the absence of urea (17). The reduced rhSCF has a spectrum reflecting primarily a random-coiled structure that contains only 3–5% $\alpha$-helix. Secondary structures of I-1 and I-2 are similar, and both contain approximately 18% $\alpha$-helix. I-3 appears to have very little structure, containing only trace amounts of $\alpha$-helix ($\approx$5%).

Fig. 5B shows the near UV CD spectra. N also has a spectrum identical in shape to that of standard rhSCF, with a slight decrease in magnitude. The spectrum of R indicates that the reduced protein is structurally disordered. I-1 and I-2 have identical spectra that are also similar in shape to that of N, but with signals reduced at levels between N and R. Both of the single disulfide intermediates appear to partially display a native-like tertiary structure.

The changes in ellipticity at 222 nm versus temperature for various rhSCF forms are depicted in Fig. 5C. Increase of temperature causes loss of $\alpha$-helical structure as a result of thermal denaturation. Table II lists the transition midpoint of thermal denaturation of various rhSCF intermediates and analog. SCF standard and fully refolded rhSCF (N) are very stable molecules, with identical transition temperature of 60°C. I-1 and C43,138A analog with a temperature of 48°C are of intermediate stability. I-2 with a temperature of 36°C is the least stable, consistent with increased flexibility as indicated by its fluorescence spectrum (described below).

The fluorescence spectra of rhSCF species are shown in Fig. 5D. The spectra for both refolded rhSCF and rhSCF standard consist of a single peak at 322 nm, consistent with the single Trp (at position 44) being in a hydrophobic environment. The FWHM is about 50 nm. As is usually the case for Trp-containing proteins, there is no Tyr signal, indicating energy transfer is occurring between Tyr and Trp. R has a single peak at 350 nm with a wider FWHM of 56 nm, typical of a Trp spectrum in solution; and its Tyr fluorescence is not apparent. I-1 has a spectrum slightly red shifted to 325 nm, indicating that the Trp is more exposed to the solvent than N. However, Trp in I-1 appears to be in a native-like environment. I-2 has a fluorescence maximum at 333 nm with a FWHM of 57 nm, indicating that the Trp in this molecule is slightly more exposed to solvent than I-1. Like R, the spectrum of I-3 has a maximum at 350 nm, apparently due to Trp being fully exposed. I-3 also contains a clear shoulder representing the presence of substantial Tyr fluorescence, indicating that the Tyr is no longer transferring energy to the Trp. This suggests that the distance between Tyr and Trp has increased. C43,138A analog lacking the Cys43–Cys138 disulfide bond, exhibits both CD and fluorescence spectra identical to those for the rhSCF standard. Its thermostability, however, is similar to I-1 (see above).

Hydrodynamic properties of several intermediates and analog were analyzed by size exclusion chromatography in conjunction with light-scattering detection. As expected, the determined molecular weights for rhSCF standard, N and intermediates are all around 33,000–35,000, indicative of being a dimer in solution (Table II). Fig. 6A illustrates a typical chromatographic separation profile for I-2 detected by UV absorption, refractive index, and light scattering. Fig. 6B shows the calibration plot for the molecular weight determination of
various species. N was determined to have a molecular weight close to that of the rhSCF standard. IAA-trapped I-1, I-3, and I-2 are also dimeric molecules. With the exception of standard rhSCF and N, every isolated intermediate and R contain small amounts of high molecular weight aggregates (only detectable by light scattering; see Fig. 6A for I-2), indicating that intermediates and R are less stable in solution (in an order of N < I-1 < I-2, I-3 < R).

Biological Activity—Table III summarizes the data obtained from the cell proliferation and radioreceptor binding assays. N, as expected, elicits full mitogenic activity on UT-7 assay but is about half active in radioreceptor assay. The lower activity in radioreceptor assay may be due to loss of activity during sample handling in HPLC step as rhSCF standard proceeded through an HPLC step also yielded a reduced affinity to the receptor. In contrast to N, iodoacetate-modified R, I-2, and I-3 show no or very low rhSCF biological activity and receptor binding affinity. Iodoacetate-modified I-1 and the C43,138A analog (see Ref. 19 also) retain only some residual biological activity in the UT-7 assay, but display a significant amount of binding to the kit receptor (15).

**DISCUSSION**

In vitro folding of rhSCF proceeds via a number of intermediate forms containing native and non-native disulfide bonds. There are five partially oxidized intermediates together with reduced rhSCF. At the completion, the folding mixture contained a major folded and oxidized form N along with small fractions of I-1 and P. I-1, I-2, and I-3 appear to reach steady state equilibrium during folding (Fig. 1), implying that they are important intermediates in the rhSCF folding pathway.
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refolding studies of the isolated intermediates and analog (Figs. 3 and 4), it appears that I-1 is an essential intermediate directly folding into N, while the majority of I-2 needs to reconvert to I-1 via I-3 by S-S rearrangement. By estimation of equilibrium concentrations detected by HPLC (Table I), I-1 is the most stable intermediate while I-2 and I-3 are less stable.

Several observations indicated that the conformation of I-1 is mostly native-like. For example, I-1 is the only intermediate still retaining residual biological activity (5%) (Table III). Upon SV-8 proteolytic digestion, an appreciable amount of I-1 remains as large, undigested fragments, whereas other intermediates can be easily digested (Fig. 2). By fluorescence spectroscopic analysis, Trp44 in I-1 or I-2 appears to be in a hydrophobic environment similar to that found for rhSCF standard (17). By partial reduction of native rhSCF, I-2 is the major species accumulated; and therefore stability of Cys43-Cys138 bond is higher than Cys4-Cys89 bond (see Ref. 26 for details). Taken together with the single Trp being in a hydrophobic core structure, it is highly possible that Cys43-Cys38 is stabilized by being in this environment. This hydrophobic environment should also stabilize I-1 by sequestering the free Cys43 and Cys138 away from the aqueous surrounding needed for the formation of Cys43-Cys138 bond. As a result, I-1 appears to be stable and remains as the major intermediate form.

Our data indicated that the disulfide-mispaired I-3 may serve as an intermediate for the conversion between I-1 and I-2. This is supported by refolding of isolated I-2 which converts to I-1 through I-3 by disulfide rearrangement (Figs. 3B and 4). Formation of Cys4-Cys89 disulfide bond does not necessarily require the rearrangement since rhSCF C43,138A analog, a homolog of I-1, forms Cys4-Cys89 bond at the same rate as that in the wild type molecule. However, during folding of the reduced rhSCF, disulfide rearrangement between I-1 and I-2 via I-3 actually decreased the folding rate for I-2 (Fig. 4B). The folding of isolated I-2 produced I-1 and I-3 before N, indicating that the formation of N from I-2 is preferentially directed to I-1 via disulfide rearrangement (Fig. 3B). Since a small fraction of N was detected in addition to I-1 and I-3 at the early folding stage of isolated I-2, direct folding of I-2 into N may also occur at a slow rate.

The generation of intermolecular disulfide bonds found in I-4 and I-5 is interesting. They are relatively unstable as they only transiently exist at an early stage of folding. In the refolding of I-4 and I-5, it appears that both intermediates refold back into R which then fold into N via I-1, I-2, and I-3. Since dimerization of either I-4 or I-5 uses the same cysteine residue (Cys43 or Cys89, respectively), their structures may be different from any of the isolated intermediates as well as product P found at the end of folding (see subsequent paper, ref. 26). These observations suggest that I-4 and I-5 may not be as important as other intermediates in rhSCF folding. In summary, properties of all characterized intermediates appear to indicate that I-1, I-2, and I-3 are on-pathway folding intermediates important in the folding of rhSCF, while I-4 and I-5 may be off-pathway intermediates.

In comparison with native rhSCF, each trapped intermediate and R appears to be unstable in solution as they form a large amount of high molecular weight aggregates detectable in gel permeation chromatography by light-scattering detection. This observation implies that rhSCF disulfide bonds and/or the fully folded conformation play a role in the stability of rhSCF molecule. Characterization of partially oxidized intermediates seems to indicate that each of the two rhSCF disulfide bonds may contribute equally in maintaining proper tertiary structural folding of rhSCF.

All rhSCF folding species exist as noncovalently linked dimers (Table III). We observe that the completely reduced rhSCF (R) is also dimeric. However, the completely denatured and reduced R requires 1–2 h to become dimeric in oxygen-free folding conditions, and R prepared from partial reduction experiments in the absence of 6 M GdnHCl is present in dimeric form as well.2 Refolding rate of this reduced rhSCF preparation is similar to that for the reduced rhSCF prepared in 6 M GdnHCl. Therefore, the formation of dimer may occur at an earlier folding time preceding oxidation of disulfide bonds and may not affect the overall folding.

To elicit its full biological function, rhSCF has to be fully oxidized and folded. None of the isolated intermediates and analog show significant biological activity, indicating that they do not have native rhSCF conformations. Cys4-Cys38 and Cys43,Cys138 are essential to maintain a proper structural folding of rhSCF for binding to its receptor, c-kit, and exerting full biological functions. Although the completely folded and purified C43,138A analog has a gross conformation indistinguishable from the wild type rhSCF and displays significant receptor binding activity, this molecule is biologically less active (Table III).

In summary, a putative in vitro folding pathway was postulated: I-1 is identified as an important and productive intermediate for formation of fully oxidized and folded rhSCF. I-2 seems to favor rearrangement through I-3 to I-1 and N. I-2 may also directly fold into N at a much slower rate. Our preliminary data indicated that the folding of rhSCF can be affected by protein disulfide isomerase.2 It would be interesting to further study the effect of the isomerase or chaperone proteins (2, 22) in the catalysis of rhSCF folding. I-4 and I-5, which are disulfide-linked dimers, are in equilibrium with other intermediates including R. These off-pathway intermediates are kinetically identifiable and play less important roles in the in vitro folding of rhSCF. The observation that R, I-1, I-2, and I-3 exist as dimeric molecules appears to indicate that dimer formation together with a small extent of secondary and tertiary structural folding occur at a very earlier folding stage.

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REFERENCES

1. Creighton, T. E. (1990) Biochem. J. 270, 1–16

2. Creighton, T. E., Bagley, C. J., Cooper, L., Darby, N. J., Friedman, R. B., Kemmnik, J., and Sheikht, A. (1993) Mol. Biol. 232, 1176–1196

3. Chang, J.-Y. (1993) J. Biol. Chem. 268, 4043–4049

4. Mui, P. W., Konishi, Y., and Schera, H. A. (1985) Biochemistry 24, 4481–4489

5. Creighton, T. E., and Goldenberg, D. P. (1984) J. Biol. Chem. 259, 497–526

6. Weissman, J. S., and Kim, P. S. (1991) Science 253, 1386–1393

7. Lu, H. S., Clugston, C. L., Narhi, L. O., Merewether, L. A., Pearl, W. R., and Boone, T. C. (1992) J. Biol. Chem. 267, 8770–8777

8. Creighton, T. E. (1991) Science 256, 111–112

9. Zhang, J.-X., and Goldenberg, D. P. (1993) Biochemistry 32, 14075–14081

10. Kim, P. S., and Baldwin, R. L. (1990) Annu. Rev. Biochem. 59, 631–660

11. Zsebo, K. M., Wyzych, J., McNiece, I. K., Lu, H. S., Smith, K. A., Karkare, S. B., Sachdev, R. K., Yuschenkoff, V. B., Birckett, N. C., Williams, L. R., Satyagal, V. N., Tung, W., Bosselman, R. A., Mendiz, E. A., and Langley, K. E. (1990) Cell 63, 195–201

12. Martin, F. H., Suggs, S. V., Langley, K. E., Lu, H. S., Ting, J., Okino, K. H., Morris, C. F., McNiece, I. K., Jackson, W. F., Mendiz, E. A., Birckett, N. C., Smith, P. A., Johnson, M. J., Parker, V. P., Flores, J. C., Patel, A. C., Fisher, E. F., Erjavec, H. O., Herrera, C. J., Wyzych, J., Sachdev, R. K., Pope, J. A., Leslie, I., Wen, D., Lin, C. W., Capples, R. L., and Zsebo, K. M. (1990) Cell 63, 203–211

13. Lu, H. S., Clugston, C. L., Wyzych, J., Fausset, P. R., Sulston, S., Mendiz, E. A., Zsebo, K. M., and Langley, K. E. (1991) J. Biol. Chem. 266, 8102–8107

14. Lu, H. S., Clugston, C. L., Wyzych, J., Parker, V., Lee, T. D., Swiderski, K., Balterza, R. F., Patel, A. C., Chang, D. C., Brankow, D. W., Liu, X.-D., Ogden, S. G., Karkare, S. B., Hu, S. S., Zsebo, K. M., and Langley, K. E. (1992) Arch. Biochem. Biophys. 298, 150–158

2 M. D. Jones, L. O. Narhi, W.-C. Chang, and H. S. Lu, unpublished data.
15. Langley, K. E., Mendiaz, E. A., Liu, N., Narhi, L. O., Zeni, L., Perseghian, C., Clogston, C. L., Leslie, I., Pope, J. A., Lu, H. S., Zsebo, K. M., and Boone, T. C. (1994) Arch. Biochem. Biophys. 311, 55–61
16. Langley, K. E., Wypych, J., Mendiaz, E. A., Clogston, C. L., Parker, V. P., Farrar, D. H., Brotherson, M. O., Satyagal, V. N., Leslie, I., Birkett, N. C., Smith, K. A., Balteria, R. J., Lyons, D. E., Hogan, J. M., Crandall, C., Boone, T. C., Pope, J. A., Karkare, S. B., Zsebo, K. M., Sachdev, R. K., and Lu, H. S. (1992) Arch. Biochem. Biophys. 295, 21–28
17. Arakawa, T., Yphantis, D. A., Lary, J. M., Narhi, L. O., Lu, H. S., Prestrelski, S. J., Clogston, C. L., Zsebo, K. M., Mendiaz, E. A., Wypych, J., and Langley, K. E. (1991) J. Biol. Chem. 266, 18942–18948
18. Lu, H. S., Chang, W. C., Mendiaz, E. A., Mann, M. B., Langley, K. E., and Hsu, Y.-R. (1995) Biochem. J. 305, 563–568
19. Langley, K. E., Mendiaz, E. A., Liu, N., Narhi, L. O., Zeni, L., Perseghian, C., Clogston, C. L., Leslie, I., Pope, J. A., Lu, H. S., Zsebo, K. M., and Boone, T. C. (1994) Arch. Biochem. Biophys. 311, 55–61
20. Jones, M. D., Merewether, L. A., Clogston, C. L., and Lu, H. S. (1994) Anal. Biochem. 216, 135–146
21. Greenfield, M., and Fasman, G. D. (1969) Biochemistry 8, 4108–4116
22. Smith, K. A., and Zsebo, K. M. (1992) in Current Protocols in Immunology (Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M., and Strober, W., eds.) pp 6.17.1–6.17.11, John Wiley & Sons, New York
23. Laemmli, U. K. (1970) Nature 227, 680–685
24. Takagi, T. (1990) J. Chromatogr. 280, 409–416
25. Kato, A., Kameyama, K., and Takagi, T. (1992) Biochim. Biophys. Acta 1159, 22–28
26. Lu, H. S., Jones, M. D., Shieh, J.-H., Mendiaz, E., Feng, D., Watler, P., Narhi, L. O., and Langley, K. E. (1996) J. Biol. Chem. 271, 11309–11316
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