Pathway of Oxidative Folding of Secretory Leucocyte Protease Inhibitor: An 8-Disulfide Protein Exhibits a Unique Mechanism of Folding†

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ABSTRACT: Secretory leucocyte protease inhibitor (SLPI) is a 107-amino acid protein with a high density of disulfide pairing (eight). The mechanism of oxidative folding of reduced and denatured SLPI has been investigated here. Despite an exceedingly large number of possible folding intermediates (∼46 million disulfide isomers) and their potential to complicate the refolding process, oxidative folding of SLPI turns out to be surprisingly simple and efficient. Complete oxidative folding and a near-quantitative recovery of the native SLPI can be achieved in a simple buffer solution using air oxidation without any supplementing thiol catalyst or redox agent, a phenomenon that has not yet been observed with other disulfide proteins. Because of the heterogeneity and extensive overlapping of folding intermediates, identification of the predominant intermediate was unfeasible. Nonetheless, studies of reductive unfolding of native SLPI and oxidative folding of a six-disulfide variant of SLPI enable us to propose an underlying mechanism accounting for the unique folding efficiency of SLPI in the absence of a redox agent. Our studies indicate that oxidative folding of SLPI undergoes heterogeneous populations of one-, two-, three-, four-, five-, six-, and seven-disulfide isomers, including two nativelike isomers, SLPI-6A and SLPI-7A, as transient intermediates. Formation of the last two native disulfide bonds leading to the conversion of SLPI-6A → SLPI-7A → N−SLPI is relatively slow and represents the final stage of oxidative folding. Most importantly, free cysteines of SLPI-6A and SLPI-7A also act as a thiol catalyst in promoting the disulfide shuffling of diverse non-native intermediates accumulated along the folding pathway. This explains why a near-quantitative recovery of N-SLPI can be achieved in the absence of any thiol catalyst and redox agent. Properties of SLPI-6A and SLPI-7A were investigated and compared to those of other documented kinetic intermediates of oxidative folding. The correlation between the mechanism of SLPI folding and the three-dimensional structure of SLPI is also elaborated.

The pathway of protein folding can be investigated by tracking the path of disulfide oxidation (formation) of fully reduced disulfide proteins en route to their native structures (*1*, *2*). The process of oxidative folding involves two major chemical events, disulfide formation (oxidation) and disulfide shuffling (isomerization). These two activities follow rather than lead the pathway of folding, which is primarily guided by noncovalent specific interactions. The courses of disulfide formation and shuffling therefore represent a valid signal for tracking the pathway of in vitro protein folding. So far, our knowledge about the mechanism of oxidative folding has been largely derived from the studies of three- and four-disulfide proteins. Two of the best characterized models are bovine pancreatic trypsin inhibitor (BPTI)[1] (*3*−*5*) and bovine ribonuclease A (RNase A) (*6*, *7*). In the case of three-disulfide BPTI, the folding pathway is characterized by a limited number of one- and two-disulfide intermediates that adopt native disulfide bonds and nativelike structures. Of 74 possible disulfide isomers, only five to six one- and two-disulfide intermediates were shown to populate along the folding pathway of BPTI, and all of them were shown to adopt native disulfide bonds (*4*, *5*, *8*). Fully oxidized scrambled isomers of BPTI were not found in the folding pathway of BPTI. In the model of four-disulfide RNase A, the folding intermediates are more heterogeneous and scrambled four-disulfide isomers were shown to be present along the pathway (*2*, *6*, *7*). Subsequent studies of other three- and four-disulfide proteins by different laboratories further reveal a large diversity of disulfide folding pathway (*9*−*24*) exhibited by both folding kinetics and folding pathways.

The folding kinetics varies from protein to protein but is basically adjustable. It is mainly influenced by the pH and redox potential (*1*, *10*). For instance, oxidative folding of reduced hirudin may be completed within a few days or a few seconds, a 5000-fold difference in folding kinetics, depending on the redox agents applied (*10*). The diversity

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[1] Abbreviations: SLPI, secretory leucocyte protease inhibitor; BPTI, bovine pancreatic trypsin inhibitor; EGF, epidermal growth factor; αLA, α-lactalbumin; LCI, leech carboxypeptidase inhibitor; PCI, potato carboxypeptidase inhibitor; RNase A, bovine ribonuclease A; DTT, reduced dithiothreitol; HPLC, high-pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of flight.

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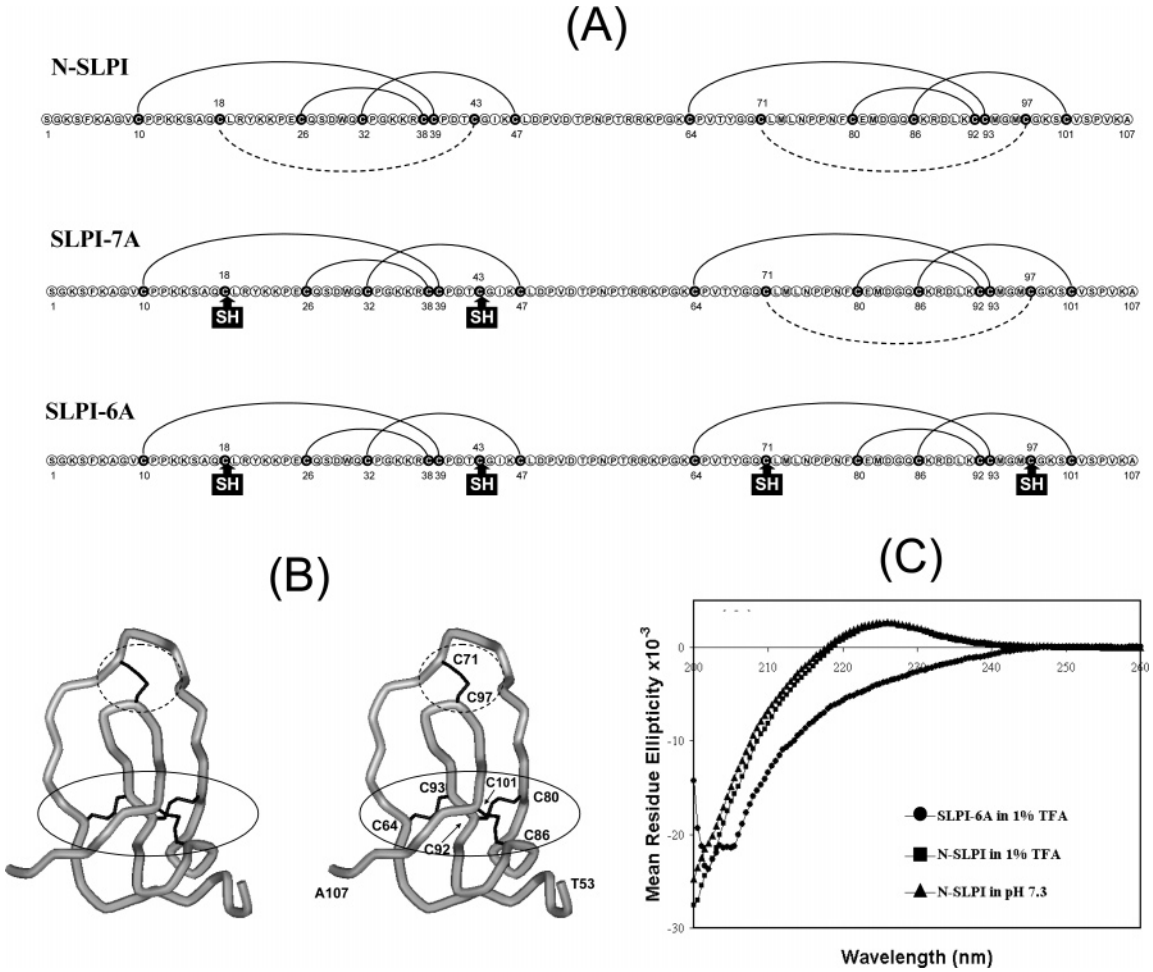

FIGURE 1: Structures of N-SLPI, SLPI-7A, and SLPI-6A. (A) N-SLPI comprises two structurally homologous domains (N- and C-terminal). Each domain is connected by four disulfide bonds. The solid lines connect six disulfide bonds located at the center of the two domains. The dashed lines link two disulfides situated at the enzyme-binding loops. SLPI-7A contains one reduced native disulfide bond ($Cys^{18}-Cys^{43}$) at the N-terminal domain. SLPI-6A lacks both disulfides at the enzyme-binding loops ($Cys^{18}-Cys^{43}$ and $Cys^{71}-Cys^{97}$). (B) 3D structure of the C-terminal domain of SLPI residues 53−107 (*27*). The N-terminal domain is structurally homologous to the C-terminal domain. The three stable disulfide bonds ($Cys^{64}-Cys^{93}$, $Cys^{80}-Cys^{92}$, and $Cys^{86}-Cys^{101}$) at the center of the domain are enclosed by a solid line. The unstable disulfide bond ($Cys^{71}-Cys^{97}$) at the enzyme-binding loop is circled by a dashed line. (C) Far-UV CD spectra of N-SLPI and SLPI-6A. The spectra were analyzed in either 1% aqueous trifluoroacetic acid or Tris-HCl buffer (20 mM, pH 7.3) at 23 °C.

of folding pathway is in general defined by (a) the heterogeneity of disulfide isomers that appear as folding intermediates, (b) the presence or absence of predominant intermediates adopting native disulfide bonds, (c) the presence or absence of kinetic traps, and (d) the presence or absence of fully oxidized non-native isomers as intermediates (these isomers are also termed scrambled isomers and abbreviated as X-isomers). However, the pathway of oxidative folding appears to be an intrinsic property of amino acid sequence and independent of folding kinetics. A systematic study using different combinations of redox agents has shown that the folding pathways of hirudin (*10*) and potato carboxypeptidase inhibitor (*13*) remain essentially unaffected regardless of the variation of its folding kinetics. Similar properties have been observed with several other proteins investigated in our laboratory (*11*, *12*, *14*, *16*).

To further understand the diversity of disulfide folding pathways, investigation of proteins containing more than four disulfide bonds is essential. In this report, we describe the analysis of oxidative folding and reductive unfolding of secretory leucocyte protease inhibitor (SLPI), also known as mucous protease inhibitor (MPI). It is a low-molecular weight, acid stable inhibitor with high affinity and inhibitory activity for trypsin, chymotrypsin, leukocyte elastase, and cathepsin G (*25*, *26*). SLPI consists of 107 amino acids, eight disulfide bonds, and two structurally homologous domains (N- and C-terminal) that are approximately equal in size. Each domain is stabilized by four disulfide bonds with identical bridging patterns (*26*, *27*) (Figure 1).

Unlike three-disulfide proteins which adopt only 74 possible disulfide isomers (six of them are exclusively native), the number of potential disulfide isomers that may exist along the folding pathway of SLPI is astronomical. There are 120 possible one-disulfide isomers, 2 027 024 scrambled eight-disulfide isomers, and 44 179 590 species of two- to seven-disulfide isomers between those groups. If folding pathways of three- and four-disulfide proteins (*9*, *11*, *13*, *16*) signify any hint, at least 15−30% of all possible isomers of SLPI are expected to appear during the folding. Even if all intermediates adopt exclusively native disulfide bonds, similar to that of BPTI (*4*, *5*), there are still 254 possible isomers that may serve as folding intermediates of SLPI. Analysis of such an enormous number of isomers of the same protein would be a daunting if not unfeasible task.

Despite our initial concern and potential obstacle of the complexity of intermediates, oxidative folding of SLPI

happens to be possibly one of the most efficient cases among various proteins that have been investigated. Complete oxidative folding and a near-quantitative recovery of the native SLPI can be achieved in a simple buffer system without any thiol catalyst or redox agent. This phenomenon has not been observed in various three- and four-disulfide proteins studied in our laboratory (*9*−*16*). The mechanism accounting for the efficient folding of SLPI is investigated, and the results are presented in this report.

MATERIALS AND METHODS

Materials. Recombinant SLPI was kindly provided by Novartis AG (Basel, Switzerland). The protein was shown to have a purity of greater than 94%, as judged by the HPLC profiles and MALDI mass analysis. Thermolysin (P-1512), dithiothreitol, and reduced and oxidized glutathione, with purities greater than 99%, were purchased from Sigma.

Oxidative Folding of Reduced and Denatured SLPI. Native SLPI (2 mg/mL) was first reduced and denatured in Tris-HCl buffer (0.1 M, pH 8.4) containing 6 M GdmCl and 30 mM dithiothreitol. The reaction was carried out for 90 min at 23 °C. To initiate folding, the reduced SLPI was passed through a PD-10 column (Sephadex-25-Pharmacia) equilibrated in 0.1 M Tris-HCl buffer (pH 8.4) and diluted immediately with the same Tris-HCl buffer to a final protein concentration of 0.5 mg/mL. The oxidative folding was performed in Tris-HCl buffer (0.1 M, pH 8.4) alone or in the presence of GSH (1 mM) or GSSG (0.5 mM). Folding intermediates of SLPI were trapped in a time course manner by mixing aliquots of the sample with an equal volume of 4% aqueous trifluoroacetic acid and analyzed directly by reverse-phase HPLC using the following conditions. Solvent A was water containing 0.1% trifluoroacetic acid. Solvent B was acetonitrile and water (9:1, v/v) containing 0.086% trifluoroacetic acid. The gradient was from 20 to 60% B (linear) over 50 min. The flow rate was 0.5 mL/min. The column was a Zorbax 300SB C18 column for peptides and proteins (4.6 mm × 5 μm). The column temperature was 23 °C.

Reductive Unfolding of the Native SLPI. The native SLPI (0.5 mg/mL) was dissolved in Tris-HCl buffer (0.1 M, pH 8.4) containing different concentrations of dithiothreitol (0.015−2.0 mM). Reduction was carried out at 23 °C for 10 min or in a time course manner, quenched with an equal volume of 4% aqueous trifluoroacetic acid, and analyzed by HPLC using the conditions described above. The samples were stored at −20 °C.

Determination of the Number of Disulfide Bonds of Folding Intermediates. To quantify the distributions of disulfide species (the fully reduced and one-, two-, three-, four-, five-, six-, seven-, and eight-disulfide isomers) of time course folding samples, the intermediates were directly and collectively derivatized with vinylpyridine (0.13 M) for 35 min at 23 °C in the dark. Derivatized samples were acidified, desalted by gel filtration, and analyzed by MALDI mass spectrometry. The fully reduced SLPI and fully oxidized SLPI exhibit molecular masses of 13 422 and 11 726 Da, respectively. Formation of each additional disulfide bond reduces the molecular mass by 212 Da.

Characterization of Disulfide Structures of Major Unfolding Intermediates of SLPI. Two acid-trapped unfolding intermediates, SLPI-7A and SLPI-6A, were purified from HPLC and freeze-dried. The samples were first derivatized with 50 μL of vinylpyridine (0.13 M) in Tris-HCl buffer (0.1 M, pH 8.4) at 23 °C in the dark, for 35 min. The reaction was quenched with 4% aqueous trifluoroacetic acid. Vinylpyridine-derivatized samples were then analyzed by MALDI mass spectrometry to characterize the composition of their disulfide species. Vinylpyridine-modified SLPI-7A and SLPI-6A were further reduced and denatured with GdmCl (6 M) and DTT (30 mM) and derivatized with iodoacetic acid. Finally, vinylpyridine/iodoacetate double-derivatized SLPI-7A and SLPI-6A (∼10 μg each) were treated with 1.5 μg of thermolysin (Sigma, P-1512) in 30 μL of *N*-ethylmorpholine/acetate buffer (50 mM, pH 6.4). Digestion was carried out at 37 °C for 16 h. Peptides were then isolated by HPLC and analyzed by both mass spectrometry and Edman sequencing to identify peptide fragments containing vinylpyridine-modified cysteine.

Amino Acid Sequencing, Mass Spectrometry, and CD Measurement. Amino acid sequences of disulfide-containing peptides were analyzed by automatic Edman degradation using a Perkin-Elmer Procise sequencer (model 494) equipped with an on-line PTH-amino acid analyzer. The molecular masses of disulfide-containing peptides were determined with a MALDI-TOF mass spectrometer (Perkin-Elmer Voyager-DE STR) using 2,5-dihydroxybenzoic acid as a matrix. The molecular masses of analyzed peptides were calibrated by the following standards: bradykinin fragment (residues 1−7) (MH^+ 757.3997), synthetic peptide P14R (MH^+ 1533.8582), ACTH fragment (residues 18−39) (MH^+ 2465.1989), and insulin oxidized B-chain (MH^+ 3494.6513). CD spectra of N-SLPI and SLPI-6A were measured at a protein concentration of 0.5 mg/mL in water containing 1% trifluoroacetic acid in a 200 μL cuvette (1 mm light pass) using a Jasco J-715 spectropolarimeter. A full scan was performed for each sample from 260 to 200 nm. The helicity of each sample was transformed from the mean residue ellipticity at 222 nm.

Inhibitory Activity of SLPI and SLPI-6A. Activities of N-SLPI and SLPI-6A (vinylpyridine modifies) were measured by their ability to inhibit the digestion of a chromogenic substrate (*N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide) by α-chymotrypsin. The reaction was carried out at 23 °C in 80 mM Tris-HCl buffer (pH 7.9) containing 10 mM $CaCl_2$. The digestion was followed at 405 nm for a period of 2 min. The final concentration of the enzyme substrate was 1 mM. The enzyme concentration was 10 nM.

RESULTS

Oxidative Folding of Reduced and Denatured SLPI. The oxidative folding of fully reduced SLPI was investigated systematically under different conditions. The experiments were first carried out in Tris-HCl buffer alone without any thiol catalyst or redox agent. The HPLC chromatograms of the folding intermediates that were trapped in a time course manner by acidification are illustrated in Figure 2A. Under these simple conditions, folding of SLPI was promoted by air oxygen and a near-quantitative recovery of native SLPI was achieved within ∼16 h. Folding intermediates appear as an extended broad peak. Quantification of the predominant fraction is unfeasible due to the complexity of folding intermediates. Analysis of the molecular mass of collective

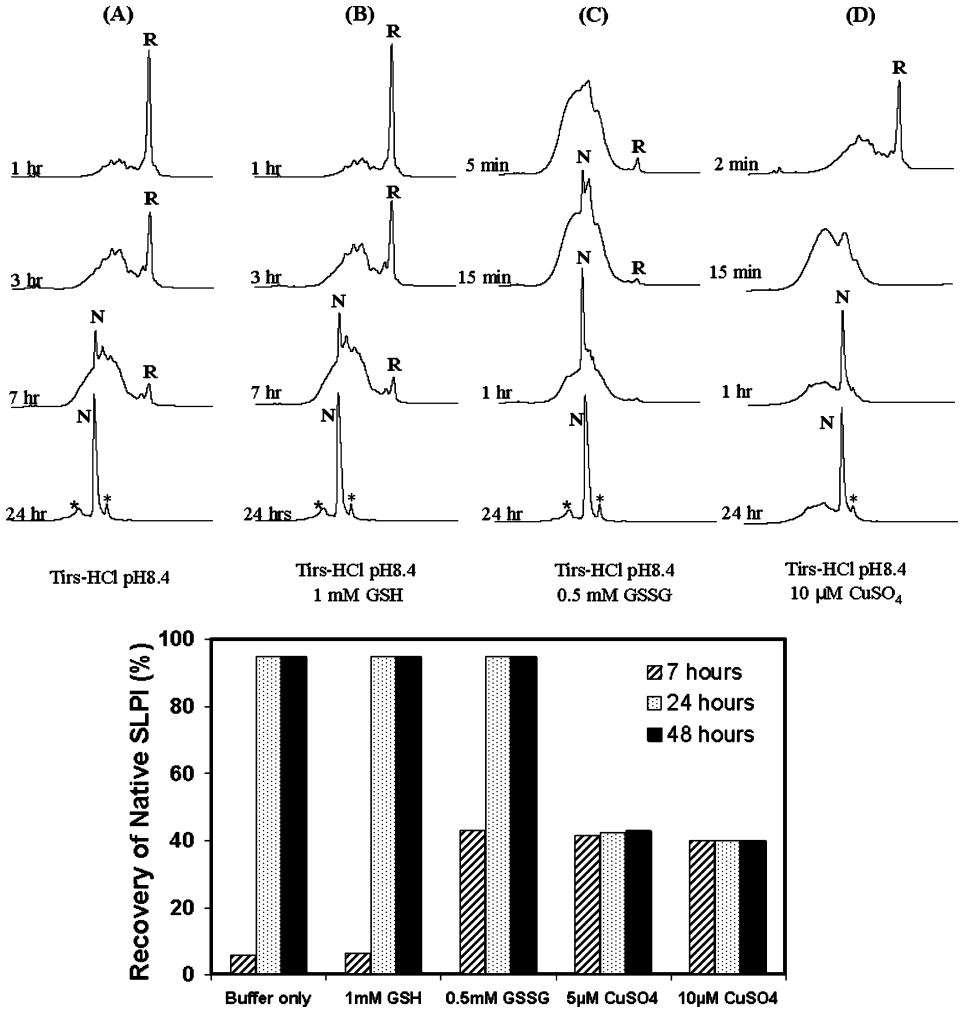

FIGURE 2: Oxidative folding of R-SLPI. HPLC profiles of the intermediates of oxidative folding of SLPI. Folding was performed at 23 °C using the following conditions: (A) Tris-HCl buffer (pH 8.4) alone, (B) Tris-HCl buffer (pH 8.4) containing GSH (1 mM), (C) Tris-HCl buffer (pH 8.4) containing GSSG (0.5 mM), and (D) Tris-HCl buffer (pH 8.4) containing $CuSO_4$ (10 μM). Intermediates of folding were withdrawn at different time points, quenched with an equal volume of 4% aqueous trifluoroacetic acid, and analyzed by reverse-phase HPLC using the conditions described in Materials and Methods. The composition of intermediates is expected to be highly heterogeneous due to the astronomical number of possible isomers. R and N denote the elution positions of the fully reduced (28.7 min) and native SLPI (23.5 min), respectively. The asterisks denote contaminants of the starting material. The bottom panel shows the recovery of N-SLPI obtained under different folding conditions.

intermediates withdrawn at different time points following vinylpyridine modification demonstrates that folding of SLPI passes through sequentially one-, two-, three-, four-, five-, six-, and seven-disulfide intermediates to reach the fully oxidized eight-disulfide SLPI (Figure 3). A seven-disulfide isomer of SLPI appears to act as a transient intermediate during the final stage of folding. This isomer likely corresponds to SLPI-7A identified along the pathway of reductive unfolding of native SLPI (Figures 1A and 4).

The folding of SLPI was then conducted in the same Tris-HCl buffer containing GSH (1 mM). GSH or β-mercaptoethanol (0.2 mM) has been routinely included in the folding buffer to catalyze and facilitate disulfide shuffling of fully oxidized intermediates (scrambled isomers) (*9*−*16*). The results show that inclusion of GSH in the folding buffer affects neither the patterns of folding intermediates nor the recovery of native SLPI (Figure 2B). The inclusion of GSSG, which promotes both disulfide oxidation and disulfide shuffling, significantly accelerates the rate of disulfide formation and the disappearance of fully reduced SLPI. The rate of recovery of native SLPI increases by ~5-fold under these conditions (Figure 2C).

When oxidative folding of SLPI was carried out in the same Tris buffer containing $CuSO_4$ (10 μM) (Figure 2D), the rate of disulfide formation was also accelerated considerably. However, ~60% of the end product comprises heterogeneous eight-disulfide scrambled isomers, unable to convert to the native SLPI due to the absence of thiol catalyst. The inclusion of 5 μM $CuSO_4$ produces a similar outcome (Figure 2, bottom panel). Unlike that of GSSG which may generate GSH, the sole function of $CuSO_4$ is to promote disulfide formation of reduced SLPI. These results thus demonstrate that acceleration of disulfide oxidation (formation) alone has an adverse effect on the recovery of native SLPI.

Reductive Unfolding of Native SLPI. Reductive unfolding of N-SLPI was performed in the buffer without addition of denaturant. The protein was reduced either with varying concentrations of DTT (from 0.015 to 2 mM) for a fixed time point (10 min) (Figure 4A) or with a fixed concentration of DTT in a time course manner (Figure 4B,C). The reduction converts N-SLPI via SLPI-7A to form SLPI-6A as a stable kinetic trap (Figure 4A). Quantitative conversion of N-SLPI to SLPI-6A can be achieved at 22 °C within 10 min using 2 mM DTT as the reducing agent. The kinetics

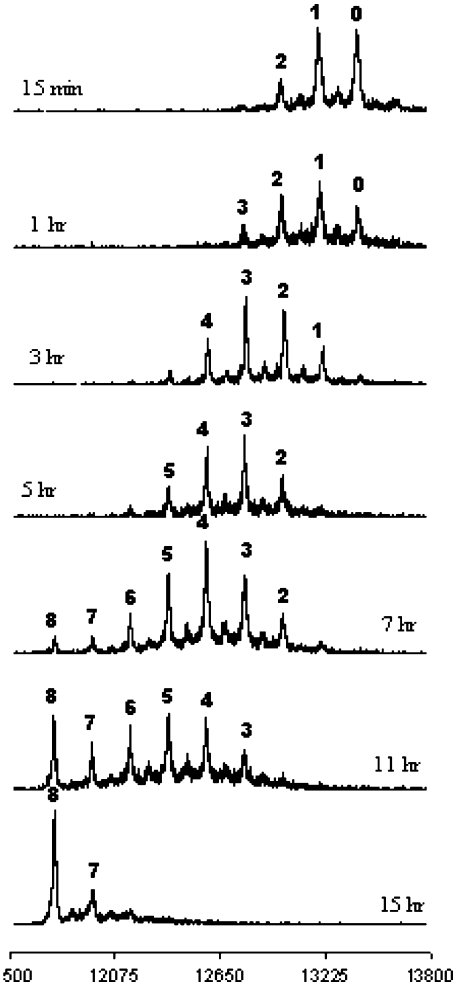

FIGURE 3: Composition of disulfide species identified along the folding pathway of SLPI. Folding of SLPI was carried out at 23 °C in Tris-HCl buffer (pH 8.4). The folding intermediates were trapped by reaction with vinylpyridine and analyzed by MALDI-MS. The folding intermediates of SLPI comprise various concentrations of isomers adopting zero to eight disulfide pairings. As a result of the reaction with vinylpyridine, these nine populations of disulfide isomers can be distinguished by their relative molecular masses and identified by MALDI mass spectrometry. Each additional pair of vinylpyridine modifications increases the molecular mass by 212 Da.

of N → 7A → 6A conversion is extremely fast. In the presence of 0.5 mM DTT (Figure 4B), the measured rate constant ($k_{N\rightarrow 6A}$) is 6.5×10^{-2} s^{-1}. Further reduction of SLPI-6A to generate fully reduced R-SLPI proceeds very slowly, requires much a higher concentration of DTT, and occurs in an all-or-none fashion without accumulation of any significant intermediate. In the presence of 25 mM dithiothreitol (Figure 4C), N-SLPI to SLPI-6A conversion exhibits a rate constant ($k_{N\rightarrow 6A}$) of $>1.76 \times 10^{-1}$ s^{-1}. In contrast, the rate constant of SLPI-6A to R-SLPI conversion ($k_{6A\rightarrow R}$) is shown to be 1.82×10^{-4} s^{-1}, ~1000-fold slower.

SLPI-6A and -7A were treated with vinylpyridine and analyzed by MALDI mass spectrometry. The results confirm that SLPI-6A and SLPI-7A are six-disulfide and seven-disulfide isomers of SLPI, respectively.

Disulfide Structures of Major Unfolding Intermediates SLPI-6A and SLPI-7A. Vinylpyridine-trapped SLPI-6A and -7A were further reduced and carboxymethylated with iodoacetic acid and digested with thermolysin. Peptides were separated by HPLC and analyzed by MALDI mass spectrometry and Edman sequencing to identify peptide-containing vinylpyridine-modified Cys. The results (available upon request) reveal that SLPI-7A contains a reduced Cys^{18}—Cys^{43} disulfide bond at the N-domain. SLPI-6A comprises reduced Cys^{18}—Cys^{43} and Cys^{71}—Cys^{97} disulfide bonds at the N- and C-domains. Both selectively reduced disulfide bonds are located at the enzyme-binding loops of SLPI (Figure 1A,B).

Oxidative Folding of SLPI-6A and Formation of the Last Two Native Disulfide Bonds. SLPI-6A was purified from HPLC and reconstituted in Tris-HCl buffer to permit reoxidation of the two reduced disulfide bonds. The folding intermediates were similarly trapped by acidification and analyzed by HPLC. The results show that SLPI-6A refolds directly via SLPI-7A as an intermediate to yield N-SLPI (Figure 5). There is no evidence based on HPLC analysis that significant unfolding and reshuffling of the remaining six native disulfide bonds of SLPI are required for the SLPI-6A → SLPI-7A → N-SLPI conversion pathway. However, the kinetics may vary significantly depending on the conditions that are applied. When folding was performed in the buffer using air oxidation, $k_{6A\rightarrow 7A}$ is greater than $k_{7A\rightarrow N}$. Consequently, SLPI-7A accumulates as a predominant intermediate (Figure 5A). This is consistent with the accumulation of a seven-disulfide intermediate during the oxidative folding starting with fully reduced SLPI (Figure 3). When folding of SLPI-6A was carried out in the buffer containing $CuSO_4$ (10 μM), the rate of recovery of N-SLPI increased by ~3-fold (Figure 5B), but $k_{6A\rightarrow 7A}$ remains greater than $k_{7A\rightarrow N}$. In the presence of GSSG, which promotes disulfide formation, the kinetics is reversed. Under these conditions, $k_{7A\rightarrow N}$ appears to be greater than $k_{6A\rightarrow 7A}$ and the concentration of SLPI-7A is diminished considerably during the refolding, especially at higher concentrations of GSSG (Figure 5C). The underlying mechanism that explains why GSSG is more effective in promoting the oxidation of the Cys^{18}—Cys^{43} disulfide bond than that of the Cys^{71}—Cys^{97} disulfide bond is not immediately clear to us. Presumably, reduced free Cys^{18} and Cys^{43} may be structurally more accessible to GSSG.

Oxidative Folding of Vinylpyridine-Modified SLPI-6A, a Six-Disulfide Protein. Vinylpyridine-modified SLPI-6A (4VP-SLPI-6A) was reduced and denatured in Tris-HCl buffer (pH 8.4) containing 30 mM DTT and 6 M GdmCl. Fully reduced 4VP-SLPI-6A was then allowed to refold in the same buffer in the absence and presence of thiol catalyst (1 mM GSH). Folding was quenched by acidification and analyzed by HPLC (Figure 6). Again, the folding intermediates appear as a spread-out peak, indicating a highly diverse population of folding intermediates. In the absence of thiol catalyst, folding of 4VP-SLPI-6A cannot be completed (Figure 6A). Approximately 60% of the protein was trapped as fully oxidized scrambled isomers, incapable of reaching the native 4VP-SLPI-6A. In the presence of GSH, completion of the folding was achieved within approximately 16 h (Figure 6B). The patterns of folding intermediates for these two experiments are nearly identical during the early stage of folding (up to 7 h).

These data indicate that fully oxidized six-disulfide (scrambled) 4VP-SLPI-6A must represent an essential population of folding intermediates. In the absence of GSH (Figure 6A), free cysteines of partially oxidized intermediates

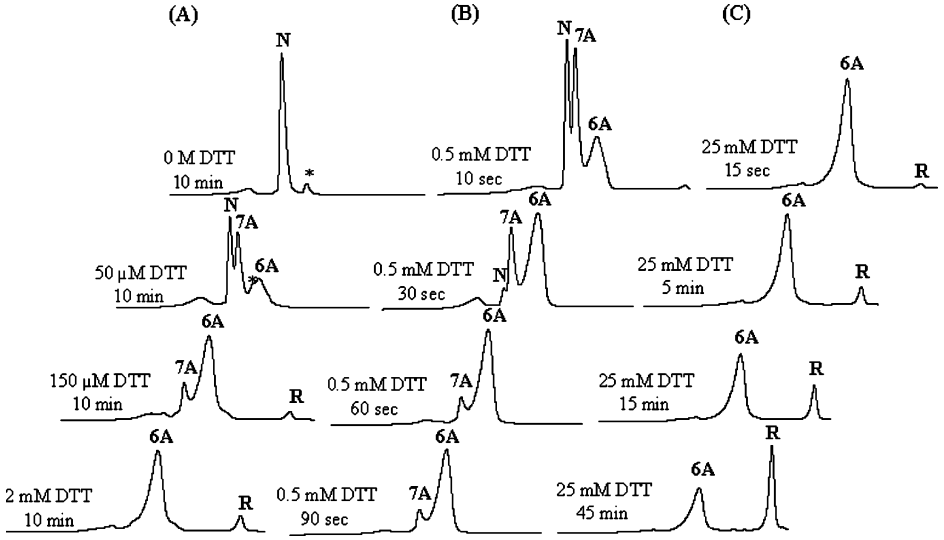

FIGURE 4: Reductive unfolding of N-SLPI. (A) Native SLPI was reduced with different concentrations of DTT for a fixed time point (10 min). (B) Native SLPI was reduced with a fixed concentration of DTT (0.5 mM) for different time points. (C) Native SLPI was reduced with a fixed concentration of DTT (25 mM) for different time points. All reactions were carried out at 23 °C. Reactions were quenched with an equal volume of 4% aqueous trifluoroacetic acid and analyzed by HPLC. N and R denote the elution positions of the native and fully reduced SLPI, respectively. Asterisks denote impurities of the starting material. 7A and 6A denote two major unfolding intermediates.

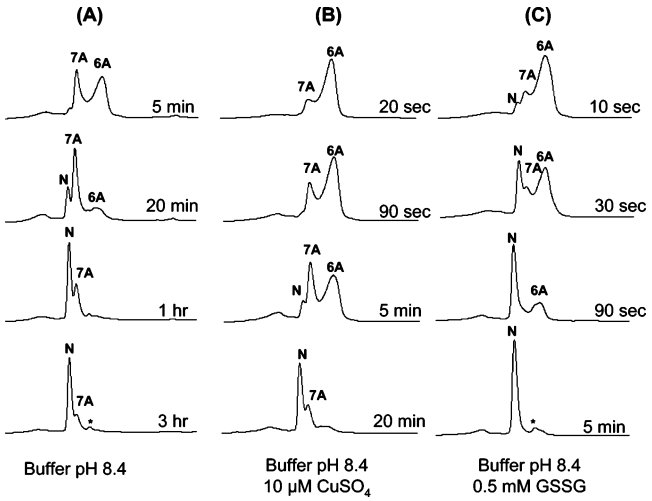

FIGURE 5: Oxidative folding of SLPI-6A. (A) Purified SLPI-6A was allowed to refold in Tris-HCl buffer (pH 8.4) for extended periods of time. (B) Purified SLPI-6A was refolded in Tris-HCl buffer (pH 8.4) containing 10 μM $CuSO_4$. (C) Purified SLPI-6A was refolded in Tris-HCl buffer (pH 8.4) containing 0.5 mM GSSG. All reactions were carried out at 23 °C. Reactions were quenched at different time points with an equal volume of 4% aqueous trifluoroacetic acid and analyzed by HPLC. N denotes the native SLPI. 7A denotes the refolding intermediate.

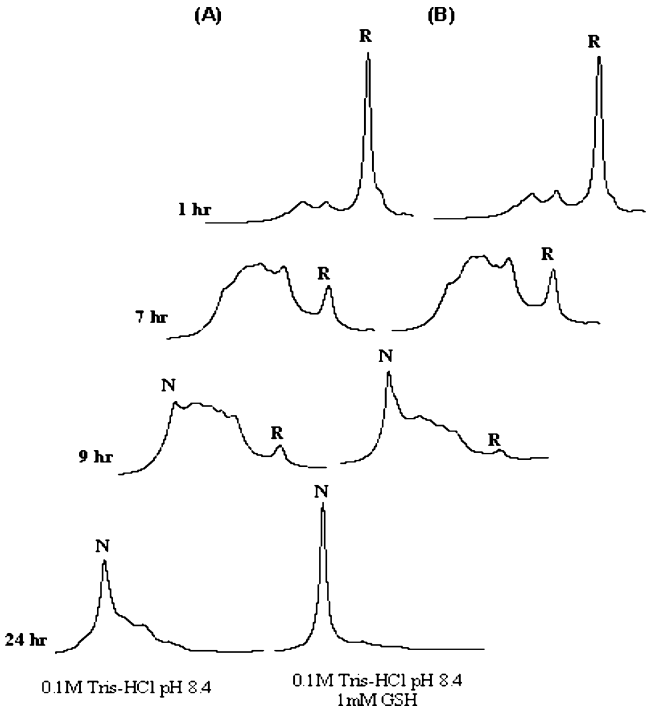

FIGURE 6: Oxidative folding of 4VP-SLPI-6A. 4VP-SLPI-6A, a six-disulfide protein, was fully reduced and denatured and allowed to refold at 23 °C using the following conditions: (A) Tris-HCl buffer (pH 8.4) alone and (B) Tris-HCl buffer (pH 8.4) containing GSH (1 mM). Intermediates of folding were quenched with an equal volume of 4% aqueous trifluoroacetic acid and analyzed by reverse-phase HPLC using the conditions described in Materials and Methods. R and N denote the elution positions of the fully reduced (25.5 min) and native 4VP-SLPI-6A (20.3 min), respectively.

(one- to five-disulfide species) act as transient thiol catalysts and assist conversion of scrambled 4VP-SLPI-6A to the native 4VP-SLPI-6A. As the folding and oxidation progress, free cysteines are depleted gradually, and scrambled 4VP-SLPI-6A thus becomes stuck and unable to convert to the native 4VP-SLPI-6A. Addition of GSH as a thiol catalyst (Figure 6B) lifts this trap and facilitates the process by which scrambled 4VP-SLPI-6A undergoes disulfide shuffling and reaches the native 4VP-SLPI-6A.

Conformational Property and Inhibitory Activity of SLPI-6A. The three-dimensional (3D) structure of N-SLPI indicates that it has no hydrophobic core and very little internally hydrogen bonded secondary structure (*27*). The CD spectrum of N-SLPI is consistent with its 3D structure, which reveals no detectable α-helical or β-sheet structures (Figure 1C). SLPI-6A exhibits a CD spectrum that is distinguishable from that of N-SLPI but also reveals no secondary structure.

The enzyme inhibitory activity of N-SLPI and 4VP-SLPI-6A was determined by their ability to inhibit α-chymotrypsin digestion of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. The digestion was followed at 405 nm for a period of 2 min. Reduction of the two native disulfide bonds of SLPI at the enzyme-binding site was shown to drastically impair its

inhibitory activity. Under the conditions described here, the inhibitory activity of N-SLPI is significant and concentration-dependent, whereas SLPI-6A exhibits less than 2−3% of the inhibitory activity of N-SLPI.

DISCUSSION

Mechanism Accounting for the Efficient Oxidative Folding of SLPI in the Absence of a Redox Agent. For an eight-disulfide protein, there are more than 46 million disulfide isomers that may serve as intermediates of oxidative folding of SLPI. Despite potential complications from such a vast number of possible intermediates, oxidative folding of SLPI is surprisingly efficient and straightforward. Quantitative recovery of the native SLPI can be accomplished by allowing the folding of reduced SLPI in the buffer without including any thiol catalyst or redox agent. This phenomenon is extraordinary and has not yet been observed with other disulfide proteins. The simplicity of SLPI folding is even more remarkable if one considers that SLPI has no hydrophobic core and very little internally hydrogen bonded secondary structure (*27*), two major structural elements known to drive protein folding (*28*).

Successful oxidative folding of disulfide proteins generally requires the presence of redox agents (e.g., a mixture of GSSG and GSH, Cys-Cys and Cys, or oxidized and reduced DTT) to promote the activity of disulfide formation and disulfide shuffling. Moreover, the majority of disulfide proteins are capable of refolding quantitatively in the presence of either GSSG or GSH alone, which is similar to the conditions described in the legend of Figure 2. In the presence of GSSG alone (Figure 2C), a fast disulfide formation and a slow disulfide shuffling (catalyzed by GSH generated from GSSG) usually occurs. In the presence of only GSH (Figure 2B), slow disulfide formation is promoted by air oxygen and an efficient disulfide shuffling is catalyzed by GSH. However, when oxidative folding was carried out in the buffer without any redox agent, most reduced proteins are unable to complete the folding. The end products of folding typically consist of a mixture of native (N) and fully oxidized scrambled (X) isomers (*9*−*14*). This is because intermediates containing non-native disulfide bonds usually exist along the folding pathway. A significant portion of these non-native intermediates advances to the state of fully oxidized X-isomers. During the early stage of folding, free Cys residues of partially oxidized intermediates may act at a thiol catalyst to promote X-isomer → N-protein conversion. As the folding progresses, more intermediates are oxidized and less free Cys is available; therefore, X-isomers become trapped as end products. Thus, the finding that oxidative folding of reduced SLPI can be accomplished in the alkaline buffer using air oxidation alone is both unique and intriguing.

One plausible explanation is that the folding pathway of SLPI comprises stepwise and orderly formation of intermediates adopting exclusively native disulfide bonds and native-like structures. Under these circumstances, the presence of air oxygen alone in the buffer will be sufficient to catalyze quantitative oxidation of all native disulfide bonds of SLPI. However, this is unlikely to occur for the following reasons. (a) Folding intermediates of SLPI appear to be highly heterogeneous as shown in Figures 2 and 3. (b) Oxidative folding of a six-disulfide variant of SLPI (4VP-SLPI-6A) demonstrates that a substantial number of non-native scrambled isomers exist along the folding pathway (Figure 6). (c) If folding intermediates of SLPI comprise exclusively native disulfide bonds, then inclusion of $CuSO_4$ (which promotes disulfide formation) is unlikely to cause the accumulation of 60% X-SLPI as the end product of folding (Figure 2D). Additionally, the efficiency of SLPI folding may be facilitated by the interaction of homologous N- and C-terminal domains. However, docking reactions of domains and association of subunits have also been shown to represent slow steps on the pathway to the functional protein structure (*35*).

A two-stage mechanism proposed here can account for the simplicity of SLPI folding in the buffer, however. In the first stage of folding, reduced SLPI passes through heterogeneous populations of intermediates to reach the six-disulfide and seven-disulfide intermediates that comprise both non-native isomers and two nativelike isomers, SLPI-6A and SLPI-7A. In the second stage of folding, SLPI-6A converts slowly and sequentially via SLPI-7A to form eight-disulfide native SLPI. At the same time, free cysteines of the lingering SLPI-7A (and SLPI-6A) also function as a thiol catalyst to facilitate disulfide shuffling of any intermediates that still contain non-native disulfide bonds. In this scenario, completion of non-native disulfide → native disulfide transformation via disulfide shuffling must precede the slow and final act of SLPI-6A → SLPI-7A → N-SLPI conversion. This explains why all reduced SLPI is able to reach the native SLPI in the absence of a thiol catalyst.

This proposed two-stage folding mechanism of SLPI is supported by the collective evidence observed in this study. (a) SLPI-7A and SLPI-6A are predominant intermediates identified along the pathway of SLPI reductive unfolding. Their presence in pathways of both reductive unfolding and oxidative folding is consistent with properties of many documented nativelike folding intermediates (Figure 7). (b) A lingering seven-disulfide intermediate can be identified at the final stage of folding (Figure 3). (c) Oxidative folding of a six-disulfide variant of SLPI (4VP-SLPI-6A) clearly implies that during the first stage of SLPI folding, intermediates adopting non-native disulfide bonds are prevalent (Figure 6). (d) An optimized and slow process of SLPI-6A → SLPI-7A → N-SLPI conversion is essential for the quantitative folding of SLPI in the absence of a redox agent. In case this process is accelerated, X-SLPI will accumulate and become trapped as an end product (Figure 2D).

Mechanisms of these two stages of SLPI folding, their relevance, and comparison to the folding mechanism of other disulfide proteins are further elaborated in the following two sections.

The First Stage of SLPI Folding Resembles That of Hirudin, As Elucidated by the Mechanism of Oxidative Folding of 4VP-SLPI-6A. 4VP-SLPI-6A is a six-disulfide variant of native SLPI, with four free cysteines covalently modified with vinylpyridine. We have performed oxidative folding of fully reduced 4VP-SLPI-6A in an effort to understand the initial stage of oxidative folding of SLPI (Figure 6). The results demonstrate that the mechanism of oxidative folding of 4VP-SLPI-6A is fundamentally indistinguishable from that of many three-disulfide and four-disulfide proteins, including hirudin (*9*, *10*), TAP (*11*, *29*), PCI (*13*), LCI (*14*, *15*), CTX-III (*30*), and apo-α-lactalbumin

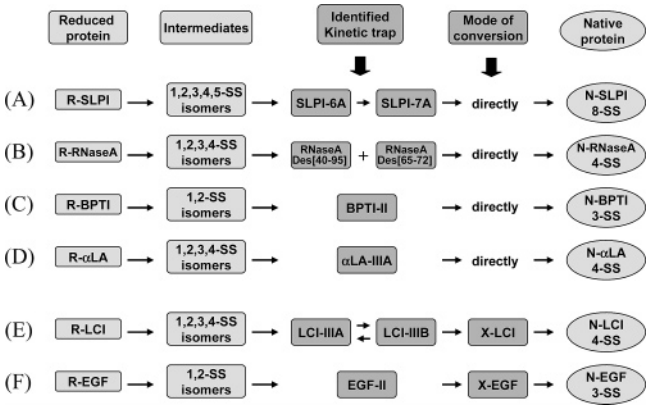

FIGURE 7: Flowcharts of disulfide folding pathways and kinetic traps of six different proteins. All kinetic traps (intermediates) listed here were shown to exist along the pathways of both oxidative folding and reductive unfolding; all contain exclusively native disulfide bonds, and aside from SLPI-6A, all lack only one native disulfide bond. (A) SLPI has two major kinetic traps, SLPI-6A and SLPI-7A, as demonstrated in this study. The SLPI-6A → SLPI-7A → N-SLPI flow is direct and sequential. (B) RNase A also has two kinetic intermediates, Des[40−95] and Des[65−72]. These two isomers do not equilibrate. They convert to the native RNase A directly, but independently, by forming their fourth native disulfide bonds, Cys^{40}−Cys^{95} and Cys^{65}−Cys^{72}, respectively (*6*, *7*). (C) BPTI-II (also known as N^{SH}_{SH}) contains two native disulfide bonds of BPTI. BPTI-II converts to N-BPTI directly by forming the third native disulfide bond, Cys^{5}−Cys^{55} (*3*, *5*). (D) αLA-IIIA converts to N-αLA directly by forming the fourth native disulfide bond, Cys^{6}−Cys^{120} (*16*). (E) LCI has two major kinetic traps, LCI-IIIA and LCI-IIIB, which exist in equilibrium. They are unable to form the last native disulfide bond directly. For LCI-IIIA and LCI-IIIB to reach the native structure, they will have to unfold already acquired nativelike structures and pass through heterogeneous four-disulfide X-LCI as intermediates (*14*, *15*, *33*). (F) EGF-II is the major kinetic trap of EGF folding. Like that of LCI-IIIA and LCI-IIIB, EGF-II needs to unfold and pass through three-disulfide X-EGF to reach N-EGF (*12*).

(*16*). When oxidative folding of these proteins was carried out in the buffer alone without supplementing thiol catalyst or redox agents (conditions identical to that described in the legend of Figure 6A), the end products of their folding invariably comprise a mixture of native and scrambled isomers with a ratio of 50:50 (±20%). In addition, they all share the following characteristics of oxidative folding: (a) a highly heterogeneous population of folding intermediates, (b) the absence of predominant intermediates containing exclusively native disulfide bonds, and (c) the presence of fully oxidized scrambled isomers as essential folding intermediates.

In fact, the pathway of oxidative folding of 4VP-SLPI-6A may have been able to be predicted on the basis of the pathway of its reductive unfolding (Figure 4). We have previously shown that for most disulfide proteins, there is a striking correlation between the pathways of their reductive unfolding and oxidative folding (*31*). Those with their disulfide bonds reduced (by dithiothreitol) in an all-or-none manner were shown to exhibit both a high degree of heterogeneity of folding intermediates and the accumulation of scrambled isomers along the pathway of oxidative folding (e.g., hirudin, TAP, and PCI) (*9*−*11*, *13*, *32*). Those with their disulfide bonds reduced in a sequential fashion during the reductive unfolding display in their folding pathway limited species of intermediates adopting mainly native disulfide bonds (e.g., BPTI and EGF) (*5*, *12*, *31*). Reductive unfolding of the six disulfide bonds of SLPI-6A was shown to occur in a collective and simultaneous manner without detectable intermediates (Figure 4C), an all-or-none mechanism similar to that of hirudin, PCI, and TAP. It is therefore not surprising that oxidative folding of 4VP-SLPI-6A follows the hirudin-like pathway (*9*, *10*).

The Second Stage of SLPI Folding Involves the Formation of the Last Two Native Disulfide Bonds, Leading to a Sequential SLPI-6A → SLPI-7A → N-SLPI Conversion. The presence of SLPI-7A (and SLPI-6A) as a transient intermediate of SLPI folding is primarily inferred from the molecular mass analysis of folding intermediates (Figure 3) and their existence as kinetic intermediates along the pathway of reductive unfolding (Figure 4). The presence of SLPI-6A and SLPI-7A along the pathways of both oxidative folding and reductive unfolding is consistent with the roles of many nativelike intermediates (kinetic traps) that have been documented in different proteins. However, the properties of SLPI-6A and SLPI-7A differ somehow from the properties of these documented kinetic intermediates. These differences are summarized in the legend of Figure 7.

The most remarkable diversity is illustrated by comparison of the two kinetic intermediates between SLPI and leech carboxypeptidase inhibitor (LCI) (*14*). Indeed, the folding pathways of SLPI and LCI are surprisingly similar except for the properties of their kinetic intermediates (*14*, *15*). Unlike SLPI-6A and SLPI-7A which convert to the native SLPI directly and sequentially, LCI-IIIA and LCI-IIIB exist in a state of equilibrium (*15*, *33*). More importantly, LCI-IIIA and LCI-IIIB (each contains three native disulfide bonds) are unable to form their respective fourth native disulfide bonds directly. For LCI-IIIA and LCI-IIIB to reach N-LCI, they will have to unfold already acquired nativelike structures and pass through scrambled four-disulfide X-LCI to form the native structure (*15*, *34*). Because of these differences, when oxidative folding of LCI was conducted in the buffer alone, the end products of folding comprise a mixture of X-LCI and N-LCI (*14*), similar to that observed in the case of hirudin (*9*), tick anticoagulant peptide (TAP) (*11*), and potato carboxypeptidase inhibitor (PCI) (*13*). Another striking example is the kinetic trap found in the oxidative folding of epidermal growth factor (EGF). When folding of reduced EGF was performed in the buffer alone, a nativelike two-disulfide kinetic trap (EGF-II) accumulates rapidly (*12*). But for EGF-II to form the third native disulfide bond (Cys^{6}−Cys^{20}), it will also have to reorganize and pass through three-disulfide X-EGF (*12*), a route similar to that of LCI-IIIA and LCI-IIIB. Perhaps the kinetic intermediate that bears the closest resemblance to SLPI-6A and SLPI-7A is the one found in the folding pathway of α-lactalbumin (αLA) (*16*). In the presence of calcium, αLA-IIIA converts to the native αLA directly by forming the fourth native disulfide bond (Cys^{6}−Cys^{120}). All these variations further highlight the diversity of the mechanism of oxidative folding.

Finally, the reversible and stepwise N-SLPI ⇔ SLPI-7A ⇔ SLPI-6A process clearly indicates that the Cys^{18}−Cys^{43} disulfide bond is less stable than the Cys^{71}−Cys^{97} disulfide bond despite the structural homology between the N- and C-terminal domains.

Interpretation of the Two-Stage Mechanism of SLPI Folding and Unfolding Based on the 3D Structure of SLPI. The two-stage mechanism of SLPI folding and unfolding

can be explained by the 3D structure of SLPI (*27*). The unique mechanism of SLPI folding and unfolding also has significant implications on the structural organization and stability of SLPI. The native SLPI consists of two structurally homologous domains (N- and C-terminal domains) with approximately equal size (*26*). The four disulfide bonds in both domains also share an identical bridging pattern (Figure 1A). According to the structure of the C-terminal domain (Figure 1B) (*27*), three of the four native disulfide bonds ($Cys^{64}-Cys^{93}$, $Cys^{80}-Cys^{92}$, and $Cys^{86}-Cys^{101}$) are spatially close and knot four peptide strands by a covalent network in the center of the C-terminal domain. A fourth disulfide bond ($Cys^{71}-Cys^{97}$) connects the proteinase-binding loop with the adjacent hairpin loop and is distant from the center of the C-terminal domain (*27*). The N-terminal domain of SLPI adopts a similar fold, with three disulfide bridges ($Cys^{10}-Cys^{39}$, $Cys^{26}-Cys^{38}$, and $Cys^{32}-Cys^{47}$) clustering at the center and a fourth disulfide ($Cys^{18}-Cys^{43}$) located at the edge of the enzyme-binding loop.

On the basis of this structure of SLPI, it is understandable that unfolding and refolding of SLPI at the center of two domains may occur in a cooperative manner. It is therefore not surprising to observe that (a) reductive unfolding of the six native disulfide bonds at the center of both domains occurs in an all-or-none fashion (SLPI-6A → R-SLPI) and (b) oxidative folding of 4VP-SLPI-6A exhibits a hirudin-like pathway. These properties are almost predictable (*31*) and consistent with the unfolding and refolding behaviors of numerous disulfide proteins investigated previously in our laboratory (*9*−*14*).

What is really surprising is the fragility of the two native disulfide bonds ($Cys^{18}-Cys^{43}$ and $Cys^{71}-Cys^{97}$) at the enzyme-binding loops of SLPI. Indeed, the covalent stability of the $Cys^{18}-Cys^{43}$ and $Cys^{71}-Cys^{97}$ bonds is even lower than those of most non-native disulfide bonds of scrambled proteins, as judged from their relative ability to withstand the reduction by DTT. In Tris-HCl buffer (pH 8.4) containing 0.5 mM DTT, for instance, the rate constant of reduction of 13 collective non-native disulfide bonds of scrambled hirudins was shown to be 1.32×10^{-3} s^{-1} (*32*). The rate constant for the reduction collective non-native disulfide bonds of scrambled TAP was 5.8×10^{-4} s^{-1} (*32*). Under identical conditions (Figure 4B), the rate constant of reduction of native $Cys^{18}-Cys^{43}$ and $Cys^{71}-Cys^{97}$ disulfide bonds is $\sim 6.5 \times 10^{-2}$ s^{-1}, an astounding 50- and 112-fold greater than those of non-native disulfide bonds of scrambled hirudin and TAP, respectively.

These data thus suggest that formation and stability of $Cys^{18}-Cys^{43}$ and $Cys^{71}-Cys^{97}$ bonds are not enforced and reinforced by any meaningful noncovalent interactions of adjacent structures. Their rapid cleavage during reductive unfolding and slow formation during oxidative folding are all consistent with this notion. On the other hand, formation of these two disulfide bonds is required for the enzyme inhibitory activity of SLPI. It is unclear why two native disulfide bonds essential for the function SLPI are at the same time so unstable. Nonetheless, the sluggish oxidation of $Cys^{18}-Cys^{43}$ and $Cys^{71}-Cys^{97}$ bonds represents an underlying cause accounting for the unique mechanism of SLPI folding.

REFERENCES

1. Creighton, T. E. (1986) Disulfide bonds as probes of protein folding pathways, *Methods Enzymol. 131*, 83−106.
2. Scheraga, H. A., Wedemeyer, W. J., and Welker, E. (2001) Bovine pancreatic ribonuclease A: Oxidative and conformational folding studies, *Methods Enzymol. 341*, 189−221.
3. Creighton, T. E. (1992) The disulfide folding pathway of BPTI, *Science 256*, 111−114.
4. Goldenberg, D. P. (1992) Native and non-native intermediates in the BPTI folding pathway, *Trends Biochem. Sci. 17*, 257−261.
5. Weissman, J. S., and Kim, P. S. (1991) Re-examination of the folding of BPTI: Predominance of native intermediates, *Science 253*, 1386−1393.
6. Rothwarf, D., Li, Y.-J., and Scheraga, H. A. (1998) Regeneration of Bovine Pancreatic Ribonuclease A: Identification of Two Native-like Three-Disulfide Intermediates Involved in Separate Pathways, *Biochemistry 37*, 3760−3766.
7. Welker, E., Narayan, M., Wedemeyer, W. J., and Scheraga, H. A. (2001) Structural determinants of oxidative folding in proteins, *Proc. Natl. Acad. Sci. U.S.A. 98*, 2312−2316.
8. Dadlez, M., and Kim, P. S. (1996) Rapid formation of the native 14−38 disulfide bond in the early stages of BPTI folding, *Biochemistry 35*, 16153−16164.
9. Chatrenet, B., and Chang, J.-Y. (1993) The disulfide folding pathway of hirudin elucidated by stop/go folding experiments, *J. Biol. Chem. 268*, 20988−20996.
10. Chang, J.-Y. (1994) Controlling the speed of hirudin folding, *Biochem. J. 300*, 643−650.
11. Chang, J.-Y. (1996) The disulfide folding pathway of tick anticoagulant peptide, a kunitz-type inhibitor structurally homologous to BPTI, *Biochemistry 35*, 11702−11709.
12. Chang, J.-Y., Li, L., and Lai, P. H. (2001) A major kinetic trap for the oxidative folding of human epidermal growth factor, *J. Biol. Chem. 276*, 4845−4852.
13. Chang, J.-Y., Cannals, F., Schindler, P., Querol, E., and Aviles, F. X. (1994) The disulfide folding pathway of potato carboxypeptidase inhibitor, *J. Biol. Chem. 269*, 22087−22094.
14. Salamanca, S., Li, L., Vendrell, J., Aviles, F. X., and Chang, J.-Y. (2003) Major kinetic traps for the oxidative folding of leech carboxypeptidase inhibitor, *Biochemistry 42*, 6754−6761.
15. Arolas, J. L., Bronsoms, S., Lorenzo, J., Aviles, F. X., Chang, J.-Y., and Ventura, S. (2004) Role of kinetic intermediates in the folding of carboxypeptidase inhibitor, *J. Biol. Chem. 279*, 37261−37270.
16. Chang, J.-Y., and Li, L. (2002) Pathway of oxidative folding of α-lactalbumin: A model for illustrating the diversity of disulfide folding pathway, *Biochemistry 41*, 8405−8413.
17. Ewbank, J. J., and Creighton, T. E. (1993) Pathway of disulfide-coupled unfolding and refolding of bovine α-lactalbumin, *Biochemistry 32*, 3677−3693.
18. Cemazar, M., Zahariev, S., Lopez, J. J., Carugo, O., Jones, J. A., Hore, P. J., and Pongor, S. (2003) Oxidative folding intermediates with nonnative disulfide bridges between adjacent cysteine residues, *Proc. Natl. Acad. Sci. U.S.A. 100*, 5754−5759.
19. Hua, Q. X., Jia, W., Frank, B. H., Phillips, N. F., and Weiss, M. A. (2002) A Protein Caught in a Kinetic Trap: Structures and Stabilities of Insulin Disulfide Isomers, *Biochemistry 41*, 14700−14715.
20. Hua, Q. X., Narhi, L., Jia, W., Arakawa, T., Rosenfeld, R., Hawkins, N., Miller, J. A., and Weiss, M. A. (1996) Native and Non-native Structure in a Protein-folding Intermediate: Spectroscopic Studies of Partially Reduced IGF-I and an Engineered Alanine Model, *J. Mol. Biol. 259*, 297−313.
21. Yang, Y., Wu, J., and Watson, J. T. (1999) Probing the folding pathways of long R(3) insulin-like growth factor-I (LR(3)IGF-I) and IGF-I via capture and identification of disulfide intermediates by cyanylation methodology and mass spectrometry, *J. Biol. Chem. 274*, 37598−37604.
22. Pattanaik, P., Sooryanarayana, A. P. R., and Visweswariah, S. S. (1998) Refolding of native and recombinant chicken riboflavin carrier (or binding) protein: Evidence for the formation of non-native intermediates during the generation of active protein, *Eur. J. Biochem. 258*, 411−418.
23. Qiao, Z. S., Min, C. Y., Hua, Q. X., Weiss, M. A., and Feng, Y. M. (2003) In vitro refolding of human proinsulin. Kinetic intermediates, putative disulfide-forming pathway folding initiation site, and potential role of C-peptide in folding process, *J. Biol. Chem. 278*, 17800−17809.

24. Rosenfeld, R. D., Miller, J. A., Narhi, L. O., Hawkins, N., Katta, V., Lauren, S., Weiss, M. A., and Arakawa, T. (1997) Putative folding pathway of insulin-like growth factor-I, *Arch. Biochem. Biophys. 342*, 298−305.
25. Seemuller, U., Arnhold, M., Fritz, H., Wiedenmann, K., Machleidt, W., Heinzel, R., Appelhans, H., Gassen, H. G., and Lottspeich, F. (1986) The acid-stable proteinase inhibitor of human mucous secretions (HUSI-I, antileukoprotease). Complete amino acid sequence as revealed by protein and cDNA sequencing and structural homology to whey proteins and Red Sea turtle proteinase inhibitor, *FEBS Lett. 199*, 43−48.
26. Thompson, R. C., and Ohlsson, K. (1986) Isolation, properties, and complete amino acid sequence of human secretory leukocyte protease inhibitor, a potent inhibitor of leukocyte elastase, *Proc. Natl. Acad. Sci. U.S.A. 83*, 6692−6696.
27. Gruetter, M., Fendrich, G., Huber, R., and Bode, W. (1988) The 2.5 Å X-ray crystal structure of the acid stable proteinase inhibitor from human mucous secretions analyzed in its complex with bovine α-chymotrypsin, *EMBO J. 7*, 345−352.
28. Dill, K. A. (1990) Dominant force in protein folding, *Biochemistry 29*, 7133−7155.
29. Chang, J.-Y., and Ballatore, A. (2000) Structure and heterogeneity of the one- and two-disulfide folding intermediates of tick anticoagulant peptide, *J. Protein Chem. 19*, 299−310.
30. Chang, J.-Y., Lu, B.-Y., Lin, C., and Chin, Y. (2005) Fully oxidized scrambled isomers are essential and predominant folding intermediates of cardiotoxin-III, *FEBS Lett*. (in press).
31. Chang, J.-Y., Li, L., and Bulychev, A. (2000) The underlying mechanism for the diversity of disulfide folding pathway, *J. Biol. Chem. 275*, 8287−8289.
32. Chang, J.-Y. (1997) A two-stage mechanism for the reductive unfolding of disulfide-containing proteins, *J. Biol. Chem. 272*, 69−75.
33. Arola, J. L., Popowicz, G. M., Bronsoms, S., Aviles, F. X., Huber, R., Holak, T. A., and Ventura, S. (2005) Study of a major intermediate in the oxidative folding of leech carboxypeptidase inhibitor: Contribution of the fourth disulfide bond, *J. Mol. Biol. 352*, 961−975.
34. Arolas, J. L., D'Silva, L., Popowicz, G. M., Aviles, F. X., Holak, T. A., and Ventura, S. (2005) NMR structural characterization and computational predictions of the major intermediate in oxidative folding of leech carboxypeptidase inhibitor, *Structure 13*, 1193−1202.
35. Seckler, R., and Jaenicke, R. (1992) Protein folding and protein refolding, *FASEB J. 6*, 2545−2552.

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