Evidence for the Underlying Cause of Diversity of the Disulfide Folding Pathway†

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Received November 13, 2003; Revised Manuscript Received February 11, 2004

ABSTRACT: The pathways of oxidative folding of disulfide proteins exhibit a high degree of diversity, which is illustrated by the varied extent of (a) the heterogeneity of folding intermediates, (b) the predominance of intermediates containing native disulfide bonds, and (c) the level of accumulation of fully oxidized scrambled isomers as intermediates. BPTI and hirudin exemplify two extreme cases of such divergent folding pathways. We previously proposed that the underlying cause of this diversity is associated with the degree of stability of protein subdomains. Here we present compelling evidence that substantiates this hypothesis by studying the folding pathway of αLA-IIA. αLA-IIA is a partially folded intermediate of α-lactalbumin (αLA). It comprises a structured β-sheet (calcium-binding) domain linked by two native disulfide bonds (Cys^{61}−Cys^{77} and Cys^{73}−Cys^{91}) and a disordered α-helical domain with four free cysteines (Cys^{6}, Cys^{28}, Cys^{111}, and Cys^{120}). Purified αLA-IIA was allowed to refold without and with stabilization of its structured β-sheet domain by calcium. In the absence of calcium, the folding pathway of αLA-IIA resembles that of hirudin, displaying a highly heterogeneous population of folding intermediates, including fully oxidized scrambled species. Upon stabilization of its β-sheet domain by bound calcium, oxidative folding of αLA-IIA undergoes a pathway conspicuously similar to that of BPTI, exhibiting limited species of folding intermediates containing mostly native disulfide bonds.

The process of oxidative folding of a fully reduced and denatured disulfide protein allows construction of the so-called disulfide folding pathway (*1*). It permits tracking and identification of partially oxidized intermediates that lead to the formation of the native structure (*2*). This technique, first used in the study of bovine pancreatic trypsin inhibitor (BPTI)[1] (*3*−*7*) and ribonuclease A (*8*−*12*), has been applied by many different laboratories in elucidating the folding pathway of numerous proteins (*13*−*40*). Despite mounting data, no general consensus regarding the disulfide folding pathway has been reached from the proteins that have been investigated. Even among small proteins comparable in size and with the same number of disulfide bonds, their folding pathways may vary significantly. These differences are mainly manifested in (a) the extent of heterogeneity of folding intermediates, (b) the presence (or absence) of predominant intermediates containing native disulfide bonds, and (c) the level of accumulation of fully oxidized scrambled isomers as folding intermediates. The folding pathways of BPTI (*3*, *6*) and hirudin (*13*, *14*) (both with three disulfides) represent two models at the opposite ends of such diversity. In the case of BPTI, the folding pathway is characterized by the predominance of a limited number of one- and two-disulfide intermediates that adopt native disulfide bonds and native-like structures. Of 74 possible disulfide isomers, only five or six one- and two-disulfide intermediates were shown to populate along the folding pathway of BPTI, and all of them were found to adopt native disulfide bonds (*3*, *4*, *6*). Fully oxidized three-disufide scrambled BPTIs were absent in the folding pathway of BPTI. In contrast, the folding intermediates of hirudin consist of a highly heterogeneous population of one- and two-disulfide species. Well-populated folding intermediates adopting native disulfides are absent in the case of hirudin. Most importantly, scrambled three-disulfide isomers, not observed with BPTI, were shown to serve as folding intermediates of hirudin (*13*, *14*). Among the 14 possible scrambled isomers of hirudin, 11 have been identified, and their disulfide structures were determined (*41*, *42*). Then, there are protein models that feature folding pathways exhibited by both BPTI and hirudin (*16*, *18*, *27*). For instance, the folding pathway of leech carboxypeptidase inhibitor (LCI, four disulfides) comprises heterogeneous populations of one-disulfide, two-disulfide, and four-disulfide (scrambled) intermediates, as well as two predominant three-disulfide kinetic traps containing exclusively native disulfide bonds (*18*).

To understand the underlying cause of these diversities, we have previously conducted a systematic analysis of five different disulfide proteins and revealed a striking correlation between their pathways of reductive unfolding and oxidative folding (*43*). Those with their native disulfide bonds reduced in a collective and simultaneous 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, tick anticoagulant peptide, and potato carboxypeptidase inhibitor) (*13*, *15*, *17*). Those with their disulfide bonds reduced in a sequential fashion during the reductive unfolding display in

† Supported by the endowment of the Robert A. Welch Foundation.

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[1] Abbreviations: αLA, α-lactalbumin; BPTI, bovine pancreatic trypsin inhibitor; HPLC, high-pressure liquid chromatography.

10.1021/bi0360354 CCC: $27.50
Published on Web 03/25/2004

their folding pathway limited species of intermediates adopting mainly native disulfide bonds (e.g., BPTI and EGF). As the stability of an individual disulfide bond is reinforced by its surrounding structure, a sequential reduction of native disulfide bonds is therefore indicative of the presence of subdomains with diverse stability. These results have led to the conclusion that the heterogeneity of folding intermediates of a disulfide protein hinges critically upon the presence (or absence) of localized stable subdomains and can be predicted from the mechanism of its reductive unfolding (*43*).

Here, we present persuasive evidence that supports this hypothesis by studying the folding pathway of αLA-IIA (α-lactalbumin-IIA). The structure of α-lactalbumin (αLA) consists of an α-helical domain that is primarily stabilized by hydrophobic force and a β-sheet domain that is stabilized upon binding to calcium (*44*−*46*). αLA-IIA is a partially folded intermediate of αLA that is capable of carrying on folding to form the native αLA (*19*). αLA-IIA comprises a structured β-sheet (calcium-binding) domain linked by two native disulfide bonds (Cys^{61}−Cys^{77} and Cys^{73}−Cys^{91}) and an unfolded α-helical domain with four reduced free cysteines (Cys^{6}, Cys^{28}, Cys^{111}, and Cys^{120}). We demonstrate here that oxidative folding αLA-IIA may occur via very different pathways, depending on the stabilization of its structured β-sheet domain.

EXPERIMENTAL PROCEDURES

Materials. Calcium-depleted bovine αLA (L-6010) was used throughout this study and was obtained from Sigma. The protein was further purified by HPLC and was shown to be more than 97% pure. Thermolysin (P-1512), dithiothreitol, reduced and oxidized glutathione, 2-mercaptoethanol, and GdnCl were also purchased from Sigma with purities of greater than 99%. All buffers used for folding experiments were degassed with a water pump for 30 min and kept in a closed system whenever they were not being used.

Nomenclature of Folding Intermediates of αLA. αLA-IIA represents a two-disulfide species of αLA. αLA-IIIA, αLA-IIIB, and αLA-IIIC are three three-disulfide species of αLA. Scrambled four-disulfide species of αLA are designated by the formula (*47*) X-αLA-species assigned on HPLC, where X stands for scrambled. For instance, X-αLA-b represents species "b" of scrambled αLA. Disulfide structures of αLA-IIA, αLA-IIIA, αLA-IIIB, and αLA-IIIC are shown in Figure 1.

Preparation of αLA-IIA. αLA-IIA can be found along both the folding (oxidative) and unfolding (reductive) pathways of αLA. A higher yield was obtained form the reductive unfolding of αLA (*19*). The native αLA (0.5 mg/mL) was reduced in the Tris-HCl buffer (0.1 M, pH 8.4) containing 10 mM dithiothreitol in the presence of $CaCl_2$ (5 mM). Reduction was carried out at 22 °C for 10 min. The reaction was quenched with an equal volume of 4% aqueous trifluoroacetic acid, and intermediates were separated by HPLC. Under these conditions, the recovery of αLA-IIA amounts to 30% of the total amount of αLA.

Oxidative Folding of αLA-IIA. Folding experiments were carried out at 22 °C in the Tris-HCl buffer (0.1 M, pH 8.4) containing 1 mM GSH and 1 mM GSSG or selected concentrations of GSSG (from 0.05 to 5 mM) in the absence and presence of $CaCl_2$ (5 mM). The protein concentration

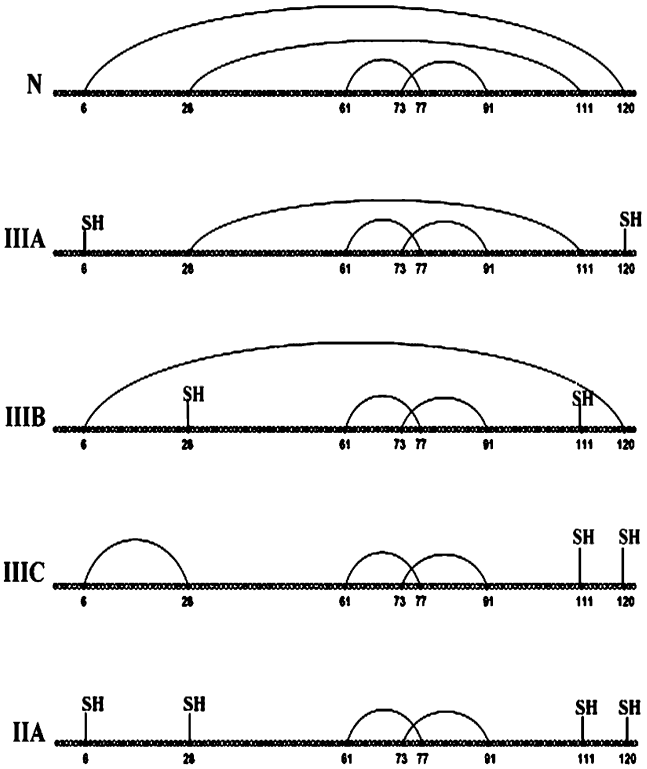

FIGURE 1: Disulfide structures of folding intermediates identified along the folding pathways of αLA-IIA. The four disulfide bonds of native α-LA (N) are as follows: Cys^{6}−Cys^{120}, Cys^{28}−Cys^{111}, Cys^{61}−Cys^{77}, and Cys^{73}−Cys^{91}. IIIA, IIIB, and IIIC are three-disulfide intermediates. Their disulfide structures were derived from the Edman sequencing and MALDI mass analysis of disulfide-containing peptides of thermolysin-digested samples.

was 0.5 mg/mL. Folding was quenched in a time course manner by mixing aliquots of the folding sample with an equal volume of 4% trifluoroacetic acid in water. Trapped folding intermediates were analyzed by HPLC or stored at −20 °C. For stop-and-go folding of αLA-IIIB, experiments were performed at 22 °C in the Tris-HCl buffer (pH 8.4) containing either GSH (1 mM) or GSSG (1 mM) in the presence of $CaCl_2$ (5 mM). Folding intermediates were similarly trapped and analyzed. It is relevant to mention that EDTA has not been used to trap a trace amount of calcium in experiments that are designated calcium free. The potential calcium contaminant, if it exists at all, will be at most at the low nanomole level, which is 1000-fold lower than the concentration of αLA-IIA (0.5 mg/mL, ∼36 μmol). The rationale is that these two major folding experiments (Figure 2A,B) are designed to be performed under strictly identical conditions except for the difference in the calcium concentration.

Analysis of the Folding Intermediates by Reversed Phase HPLC. Analysis and isolation of folding intermediates of αLA-IIA were achieved by the reversed phase HPLC using the following conditions. Solvent A for HPLC was water containing 0.1% trifluoroacetic acid. Solvent B was an acetonitrile/water mixture (9:1, v/v) containing 0.086% trifluoroacetic acid. The gradient was from 22 to 37% solvent B over the course of 15 min and from 37 to 56% solvent B from 15 to 60 min. The flow rate was 0.5 mL/min. The column was a Zorbax 300SB C-18 model for peptides and proteins (4.6 mm, 5 μm). The column temperature was 23 °C.

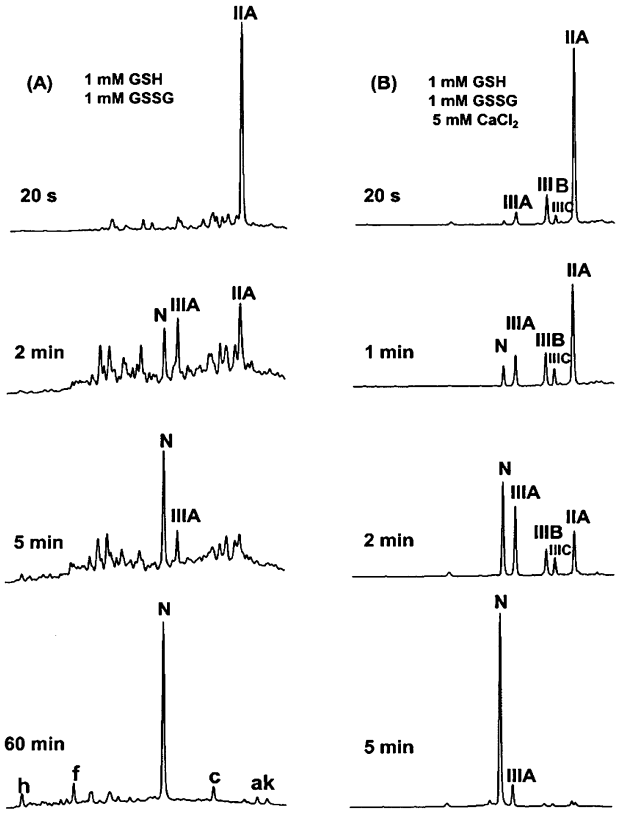

FIGURE 2: Intermediates of oxidative folding of αLA-IIA. Folding experiments were carried out at 22 °C in the Tris-HCl buffer (pH 8.4, 0.1 M) containing (A) GSH and GSSG (1 mM each) or (B) GSH and GSSG (1 mM each) and $CaCl_2$ (5 mM). The protein concentration was 0.5 mg/mL. Intermediates of folding were trapped at different time points by acidification (4% trifluoroacetic acid) and analyzed by HPLC using the conditions described in Experimental Procedures. N denotes the elution position of native αLA. IIIA, IIIB, and IIIC are three major three-disulfide folding intermediates of αLA-IIA. Their disulfide structures are presented in Figure 1. a, c, f, h, and k (see the 60 min samples) indicate the elution positions of five major four-disulfide scrambled intermediates. Their disulfide structures are documented in ref *47*.

Analysis of Disulfide Structures of α*LA-IIA,* α*LA-IIIA,* α*LA-IIIB, and* α*LA-IIIC.* HPLC-fractionated intermediates (20 μg) were isolated, freeze-dried, and derivatized with 50 μL of vinylpyridine (0.13 M) in the Tris-HCl buffer (0.1 M, pH 7.5) at 23 °C for 45 min. Vinylpyridine-derivatized samples were further purified by HPLC, freeze-dried, and treated with 2 μg of thermolysin (Sigma, P-1512) in 65 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 amino acid sequencing and mass spectrometry to identify the disulfide-containing peptides.

Amino Acid Sequencing and Mass Spectrometry. The amino acid sequences of disulfide-containing peptides were analyzed by automatic Edman degradation using a Perkin-Elmer Procise sequencer (model 494) equipped with an online PTH amino acid analyzer. The molecular masses of disulfide-containing peptides were determined with a MALDI-TOF mass spectrometer (Perkin-Elmer Voyager-DE STR).

RESULTS

Disulfide Structures of α*LA-IIA,* α*LA-IIIA,* α*LA-IIIB, and* α*LA-IIIC.* To help readers, the disulfide structures of major folding intermediates of αLA will be described first. These intermediates all contain two native disulfide bonds (Cys^{61}−Cys^{77} and Cys^{73}−Cys^{91}) that are required to stabilize the β-sheet domain, but differ in their structures at the α-helical domain of αLA. αLA-IIA and αLA-IIIA are two major intermediates also identified along the pathway of oxidative folding and reductive unfolding of αLA (*19*) when the reactions of αLA were performed in the presence of calcium. αLA-IIIB and αLA-IIIC are two additional intermediates identified in the pathway of oxidative folding initiated with αLA-IIA in the presence of calcium.

Fractions of αLA-IIA, αLA-IIIA, αLA-IIIB, and αLA-IIIC were treated with vinylpyridine, were further purified by HPLC, and were all shown to contain a single species. Analysis by MALDI mass spectrometry revealed molecular masses of 14604 Da for αLA-IIA and 14 391 Da for αLA-IIIA, αLA-IIIB, and αLA-IIIC. These molecular masses are 424 and 212 Da higher, respectively, than that of native αLA (14 179 Da), for which 4 and 2 mol of conjugated vinylpyridine account (molecular mass of 105 Da). The results thus confirm that αLA-IIA is a two-disulfide species and αLA-IIIA, αLA-IIIB, and αLA-IIIC are all three-disulfide species. Vinylpyridine-treated intermediates were digested with thermolysin. Thermolytic peptides were isolated by HPLC and analyzed by Edman sequencing and MALDI mass spectrometry to identify the structures of cysteine- and disulfide-containing peptides. These data lead to the conclusion of disulfide structures of αLA-IIA, αLA-IIIA, αLA-IIIB, and αLA-IIIC as illustrated in Figure 1 (sequencing and mass spectrometry data available upon request).

Folding Pathway(s) of α*LA-IIA in the Absence of Calcium.* The pathway of oxidative folding of αLA-IIA in the absence of calcium is illustrated by their folding intermediates shown in Figure 2 (left panel). Without calcium, a majority (>85%) of αLA-IIA needs to unravel the already structured β-sheet domain and revert to a more unfolded state to carry on the folding to reach the native structure. This indirect route is obviously based on the heterogeneity of folding intermediates detected along its folding pathway. Without unfolding of the existing β-sheet domain, there are only six possible three-disulfide isomers that may serve as the folding intermediates of αLA-IIA. With the unraveling of the two native disulfide bonds in the β-sheet domain and their subsequent involvement in the formation and shuffling of disulfide bonds, a total of 734 possible two-, three-, and four-disulfide intermediates exist. Here, at least 40 fractions of intermediates were identified, and there is no evidence of the appearance of well-populated species (Figure 2A). Analysis by MALDI mass spectrometry following the reaction with vinylpyridine indicates that these heterogeneous intermediates comprise populations of two-, three-, and four-disulfide scrambled isomers. The presence of four-disulfide scrambled species becomes apparent at the late stage of folding. After 60 min of folding, the remaining intermediates are almost exclusively four-disulfide scrambled isomers (Figure 2A). This clearly demonstrates that an extensive disruption of the existing β-sheet domain is a major route for the folding of αLA-IIA in the absence of calcium. Another important characteristic is that this pattern of heterogeneity of folding intermediates is independent of the composition of the redox agent that is employed (either 1 mM GSH, 1 mM GSSH, or 1 mM GSH and 1 mM GSSG).

Folding Pathway(s) of αLA-IIA in the Presence of Calcium. In the presence of calcium (0.5−20 mM $CaCl_2$) and upon stabilization of the existing β-sheet domain, the folding pathway of αLA-IIA is distinctly different. Under these conditions, the two native disulfide bonds in the β-sheet domain of αLA-IIA (Cys^{61}−Cys^{77} and Cys^{73}−Cys^{91}) remain intact throughout the folding. The pathway by which αLA-IIA reaches the native structure thus involves only direct arrangement of two disulfide bonds within the α-helical domain. There are six potential three-disulfide intermediates that may lead αLA-IIA to the formation of native αLA. Three of them were identified here, and they are designated αLA-IIIA, αLA-IIIB, and αLA-IIIC (Figure 2B). αLA-IIIA and αLA-IIIB are predominant species, and each includes an additional native disulfide bond, Cys^{28}−Cys^{111} and Cys^{6}−Cys^{120}, respectively (Figure 1). The third disulfide bond of αLA-IIIC is non-native (Cys^{6}−Cys^{28}).

αLA-IIIA was shown to form the fourth native disulfide bond (Cys^{6}−Cys^{120}) and be directly converted to the native αLA regardless of the composition of the redox agents that were employed. αLA-IIIB, despite also acquiring a third native disulfide bond (Cys^{6}−Cys^{120}), is not converted directly to the native αLA via formation of the Cys^{28}−Cys^{111} disulfide bond. A systematic analysis revealed that αLA-IIIB and αLA-IIIC appear to exist in equilibrium and need to be reshuffled and converted to αLA-IIIA to reach the native structure. This was shown by performing stop-and-go folding initiated with purified αLA-IIIB in buffer solutions containing either GSH or GSSG. In the presence of GSH (1 mM) alone, in which the rate of disulfide formation is slow and the rate of equilibrium among disulfide isomers is promoted, αLA-IIIB quickly equilibrates with αLA-IIIC. Both αLA-IIIB and αLA-IIIC are then rapidly converted to and accumulate as αLA-IIIA (Figure 3A, 5 min sample). In the presence of GSSG (1 mM) when the kinetics of disulfide formation are accelerated, a decrease in the levels of αLA-IIIB and αLA-IIIC is accompanied by an increase in the level of αLA-IIIA, which then rapidly forms the native αLA (Figure 3B, 5 min sample). Therefore, the folding kinetics of αLA-IIA may differ substantially depending on the relative concentration of GSH and GSSG applied in the folding buffer. To further assess the effect of GSSG, a folding experiment with αLA-IIA was performed in the buffer containing $CaCl_2$ (5 mM) and varying concentrations of GSSG (from 0.05 to 5 mM). The results showed that the concentration of αLA-IIIA accumulated along the folding pathway of αLA-IIA was inversely proportional to the concentration of GSSG applied (Figure 4). In a similar manner, the rate of recovery of the native αLA was positively relative to the concentration of GSSG employed in the folding buffer. When folding of αLA-IIA was carried out in the presence of a high concentration of GSSG (5 mM), the most prevalent intermediate was αLA-IIIB (Figure 4D). In this proposed pathway, αLA-IIIA represents the most stable intermediate of αLA-IIA folding and acts as a direct precursor of native αLA.

Another significant finding of this study is the effect of the extent of the stability of the structured β-sheet domain on the folding kinetics of αLA-IIA. It shows that the more stable the structured β-sheet domain, the faster folding of the unstructured α-helical domain. This is primarily because stabilization of β-sheet domain averts the indirect pathway

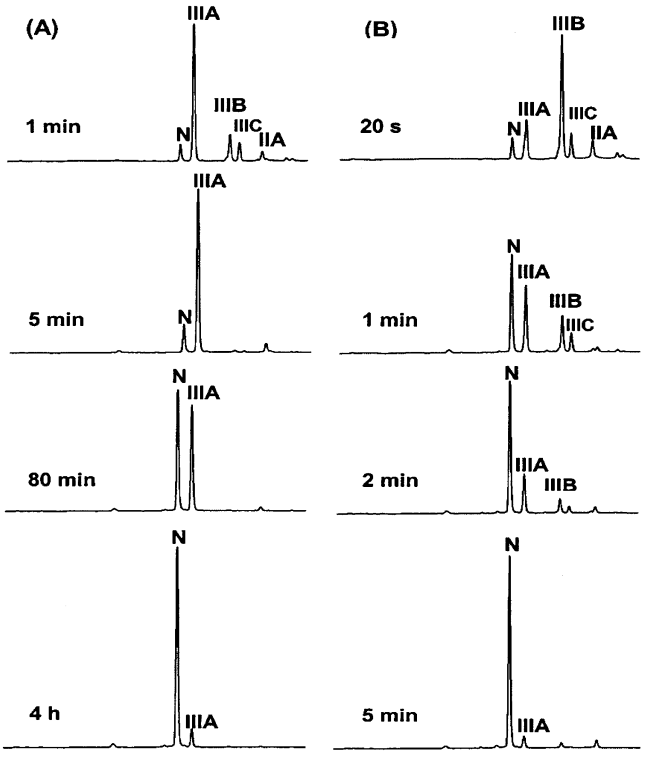

FIGURE 3: Stop-and-go folding initiated with purified αLA-IIIB. Folding experiments were carried out at 22 °C in the Tris-HCl buffer (pH 8.4) containing (A) GSH (1 mM) and $CaCl_2$ (5 mM) and (B) GSSG (1 mM) and $CaCl_2$ (5 mM). The protein concentration was 0.5 mg/mL. Intermediates of folding were trapped at different time points by acidification (4% trifluoroacetic acid) and analyzed by HPLC using the conditions described in Experimental Procedures. N denotes the elution position of native αLA. IIIA and IIIC are two major three-disulfide folding intermediates of IIIB. When folding of αLA-IIIB was performed in the presence of GSH (A), rapid conversion of IIIB to IIIA and accumulation of IIIA along the pathway occur.

of folding. It is also plausible that stabilization of the β-sheet domain facilitates the formation of a native-like conformation in the α-helical domain that in turn allows for rapid oxidation of correct cysteines to form αLA-IIIA and αLA-IIIB. This is demonstrated here by performing folding experiments with αLA-IIA at increasing concentrations of $CaCl_2$, in the presence of a mixture of GSH and GSSG. We observe that the increasing stability of the existing β-sheet domain by calcium binding increases the folding kinetics of αLA-IIA. For instance, when folding of αLA-IIA was performed in the buffer containing GSH and GSSG (1 mM each), the rate of generation of native αLA increases by 6-fold after inclusion of 5 mM $CaCl_2$ (compare panels A and B of Figure 2) The rate was improved by an additional 1.5-fold as the concentration of $CaCl_2$ increased from 5 to 20 mM.

DISCUSSION

The Pathway of Oxidative Folding of Disulfide Proteins Exhibits a Wide Spectrum of Diversity. The diversity of the disulfide folding pathway demonstrated by various small proteins has been an intriguing and debated subject. Although the pathway of oxidative folding is sensitive to folding conditions, including pH, redox agents, and the method of trapping intermediates (*2*, *6*, *14*, *48*), these parameters by themselves cannot adequately explain the vast difference observed between BPTI (*3*, *6*) and hirudin (*13*, *14*), two

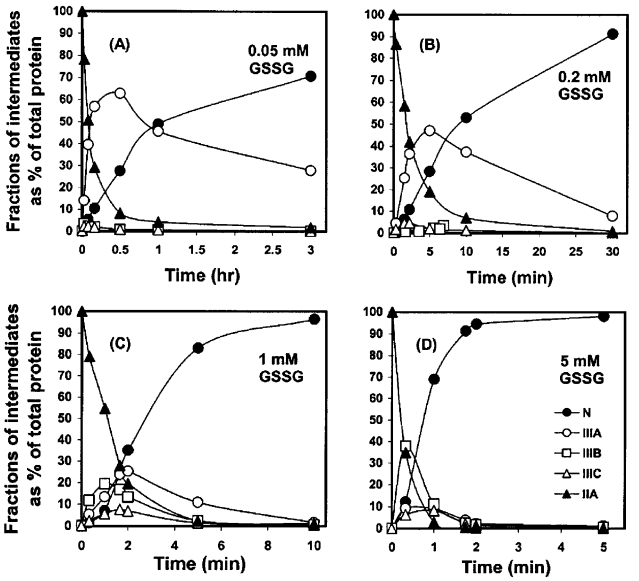

FIGURE 4: Quantitative analysis of three-disulfide folding intermediates along the pathway of oxidative folding of αLA-IIA. Folding was carried out at 22 °C in the Tris-HCl buffer (pH 8.4) containing $CaCl_2$ (5 mM) and the following concentrations of GSSG: (A) 0.05, (B) 0.2, (C) 1, and (D) 5 mM. Time course recoveries of native αLA (●), αLA-IIA (▲), αLA-IIIA (○), αLA-IIIB (□), and αLA-IIIC (△) were calculated as fractions of the total protein.

distinct models with extreme folding characteristics. Indeed, folding pathways of BPTI and hirudin have virtually nothing in common, despite their similar size (~60 amino acids), comparable conformational stability (*49*, *50*), and identical numbers of disulfide bonds (three). The native BPTI is generated through an orderly formation of native-like subdomains. All major intermediates in the folding of BPTI contain only native disulfide bonds. Therefore, the same interactions that stabilize the native BPTI also actively participate in guiding unfolded BPTI in reaching the native structure. In contrast, folding of hirudin appears to adopt a mechanism of trial and error. The folding proceeds through an initial stage of nonspecific disulfide formation (packing) to form the scrambled isomers, which is followed by disulfide reshuffling (consolidation) of the heterogeneous scrambled population to reach the native structure (*14*). Unlike that of BPTI, interactions that stabilize the native hirudin take effect only at the late stage of hirudin folding. Hirudin is not unique in displaying such properties. The pathways of oxidative folding of potato carboxypeptidase inhibitor (*17*), tick anticoagulant peptide (*15*), and α-amylase inhibitor (*21*) closely resemble that of hirudin. These discrepancies clearly imply that one of the structural determinants accounting for the diversity of the disulfide folding pathway is stable subdomains, which are present in BPTI (*11*, *51*−*53*) but absent in hirudin. We reveal here that a protein (αLA-IIA) may fold either like hirudin or like BPTI, depending on whether its structured domain is being stabilized.

The Folding Pathway(s) of αLA-IIA in the Absence of Calcium Resembles That of Hirudin. In the absence of calcium and without stabilization of the structured β-sheet domain, oxidative folding of αLA-IIA proceeds via primarily an indirect pathway, in which ~85% of αLA-IIA needs to unfold its structured β-sheet domain through scrambling of its two native disulfide bonds (Cys^{61}−Cys^{77} and Cys^{73}−Cys^{91}) to carry on the folding. As a result, the folding intermediates are highly heterogeneous that precludes the presence of predominant species. At least 40 fractions of mainly two-, three-, and four-disulfide isomers were observed as folding intermediates, and many of these fractions may likely contain more than one disulfide species. The presence of four-disulfide isomers is evident at the late stage of folding (see the 60 min sample of Figure 2A; fractions marked alphabetically in lowercase). Among them, disulfide structures of seven four-disulfide isomers of αLA have been characterized (*47*). The mechanism of oxidative folding of αLA-IIA thus is almost indistinguishable from that of hirudin (*13*, *14*), potato carboxypeptidase inhibitor (*17*), tick anticoagulant peptide (*15*), and *Amaranthus* α-amylase inhibitor (*21*). Their folding pathways all share the following characteristics: (a) an exceedingly high degree of heterogeneity of folding intermediates, (b) the absence of predominant folding intermediates containing native disulfide bonds, and (c) the presence of fully oxidized scrambled isomers as essential folding intermediates.

The Folding Pathway(s) of αLA-IIA in the Presence of Calcium Bears a Resemblance to That of BPTI. In the presence of calcium, oxidative folding of αLA-IIA exclusively undergoes the direct pathway without disruption of its structured β-sheet domain. Among all six possible three-disulfide isomers that may serve as folding intermediates of αLA-IIA, only two would comprise solely native disulfide bonds. These two isomers (αLA-IIIA and αLA-IIIB) (Figures 1 and 2B) are shown to be the predominant intermediates of αLA-IIA folding. Moreover, αLA-IIIB is unable to connect the fourth native disulfide bond (Cys^{28}−Cys^{111}) and fold to form the native αLA directly. It needs to be rearranged and converted to αLA-IIIA to attain the native αLA. The stability of αLA-IIIA and its direct conversion to the native αLA account for the rate-limiting step of αLA-IIA folding. The folding pathway of αLA-IIA in the presence of calcium exhibits a striking resemblance to that of BPTI (*3*−*6*), a three-disulfide protein. Their folding intermediates are displayed by the presence of a limited number of isomers containing mainly native disulfide bonds and the absence of fully oxidized scrambled isomers. The similarities are further illustrated in Figure 5. Here, only the predominant folding pathway of BPTI (*6*) is compared with that of αLA-IIA. A major stable native-like one-disulfide intermediate of BPTI, BPTI(Cys^{30}−Cys^{51}) (akin to αLA-IIA), also reaches the native BPTI (Cys^{30}−Cys^{51}, Cys^{14}−Cys^{38}, and Cys^{5}−Cys^{55}) via two native-like two-disulfide intermediates, BPTI-(Cys^{30}−Cys^{51}/Cys^{14}−Cys^{38}) and BPTI(Cys^{30}−Cys^{51}/Cys^{5}−Cys^{55}) (akin to αLA-IIIB and αLA-IIIA). Like αLA-IIIB, BPTI(Cys^{30}−Cys^{51}/Cys^{14}−Cys^{38}) is unable to attain the native BPTI directly. It has to be rearranged to the state of BPTI(Cys^{30}−Cys^{51}/Cys^{5}−Cys^{55}) which then rapidly forms the third native disulfide bond (Cys^{14}−Cys^{38}) to reach the native structure of BPTI (*3*, *6*). Thus, αLA-IIIA and BPTI-(Cys^{30}−Cys^{51}/Cys^{5}−Cys^{55}) both act as the immediate precursors of native αLA and native BPTI, respectively (Figure 5). Nonetheless, there is one noticeable difference with respect to their folding kinetics that needs to be addressed. This difference was observed and compared when folding experiments with BPTI and αLA-IIA were conducted in the presence of similar concentrations of GSSG (0.15−0.2 mM) (*6*). In the case of BPTI, conversion of BPTI(Cys^{30}−Cys^{51}/

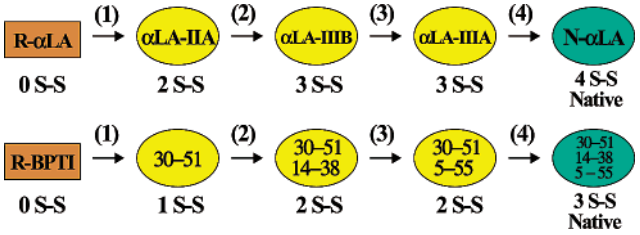

FIGURE 5: Resemblance of the major pathways of oxidative folding of αLA-IIA and BPTI. All six folding intermediates depicted here contain exclusively native disulfide bonds. Folding of reduced αLA was performed in the presence of calcium. The disulfide structures of folding intermediates of αLA-IIA are given in Figure 1. The numbering of BPTI represents the pairing of disulfide bonds. R stands for the fully reduced protein. In step 1, fully reduced and denatured αLA and BPTI fold to form predominant intermediates αLA-IIA and BPTI(30−51), respectively. In steps 2 and 3, both αLA-IIA and BPTI(30−51) carry on the folding via two predominant intermediates to reach the native structure. αLA-IIA advances via αLA-IIIB, which is reshuffled to form αLA-IIIA. BPTI(30−51) proceeds to form BPTI(30−51,14−38), which then rearranges to form BPTI(30−51,5−55). In step 4, both αLA-IIIA and BPTI-(30−51,5−55) act as direct precursors of their respective native structures. They attain the native structure by forming the last native disulfide bond (Cys^{6}−Cys^{120}) in the case of αLA and the Cys^{14}−Cys^{38} disulfide bond in the case of BPTI. It is necessary to point out that only the predominant folding pathway of BPTI is depicted here for comparison. Another native-like two-disulfide intermediate, BPTI(5−55,14−38), also plays a role similar to that of BPTI(30−51,14−38) during BPTI folding (*3*, *6*). In addition, two minor two-disulfide intermediates containing the non-native disulfide bond were also observed along folding pathway of BPTI (*3*, *4*).

Cys^{14}−Cys^{38}) to the direct precursor accounts for the rate-limiting step of BPTI folding. The very low concentration of BPTI(Cys^{30}−Cys^{51}/Cys^{5}−Cys^{55}) found in the folding pathway of BPTI is consistent with its rapid conversion to native BPTI (*6*), whereas in the case of αLA-IIA, consumption of the direct precursor (αLA-IIIA) and its conversion to the native αLA represent the rate-limiting step of αLA-IIA folding. This is demonstrated by the significant accumulation of αLA-IIIA along the folding pathway (see Figure 4B).

Relative Stability of the α-Helical Domain and β-Sheet Domain of αLA. αLA consists of a hydrophobic α-helical domain and a calcium-binding β-sheet domain. We have shown here that stabilization of the β-sheet domain may direct the folding pathways of the α-helical domain. An important question is whether stabilization of the α-helical domain will have the same effect on the pathway of oxidative folding of the β-sheet domain. Performing this folding experiment will require (a) a two-disulfide intermediate that comprises two native disulfide bonds within the α-helical domain as a starting material and (b) a method for manipulation of the stability of the α-helical domain; both are currently unavailable. We have indeed addressed to some extent this issue during the study of oxidative folding of fully reduced αLA (*19*). In these studies, the presence of calcium also favors the rapid accumulation of αLA-IIA and αLA-IIIA as intermediates. Although the conformational stability of the α-helical domain of αLA is well-documented (*56*, *57*), its relative stability with respect to the β-sheet domain, even in the absence of bound calcium, depends critically on the denaturation conditions that are employed. This has been demonstrated in a study of the conformational stability of αLA using the method of disulfide scrambling (*58*). For instance, an organic solvent selectively facilitates the denaturation of the hydrophobic α-helical domain of αLA. Acetonitrile-denatured αLA (either calcium-depleted or calcium-bound) comprises a predominant scrambled isomer (X-αLA-b) containing two intact native disulfide bonds within the β-sheet domain and two scrambled non-native disulfide bonds at the α-helical domain (*58*). Urea favors denaturation of αLA at the α-helical domain as well. On the other hand, thermal denaturation disrupts mainly the β-sheet domain of αLA, generating a predominant denatured X-αLA-c isomer (*58*) that exhibits structural characteristics consistent with the well-documented molten globule state (*59*, *60*). X-αLA-c retains a substantial content of α-helical structure (*61*) and is stabilized by two native disulfide bonds within the α-helical domain and two non-native disulfide bonds at the β-sheet domain. Comparative folding experiments (*47*) have shown that both X-αLA-b and X-αLA-c fold to form the native αLA without accumulation of any significant intermediate along the pathway. The same study also reveals that X-αLA-b is kinetically ∼65% more competent than X-αLA-c in the conversion to the native αLA, implying that folding of the α-helical domain is more efficient than that of the β-sheet domain in the absence of bound calcium.

The Folding Mechanism of αLA-IIA Provides an Important Model in Understanding the Diversity of Disulfide Folding Pathways. In conclusion, the distinct folding mechanism of αLA-IIA observed in the absence and presence of calcium underlies the importance of the stability of domain structure in dictating the pathways of protein folding. Data obtained from these studies provide persuasive evidence that supports the proposed underlying cause (*43*) for the diversity of disulfide folding pathways. It is understandable that stabilization of subdomain structure (*11*, *51*−*55*) may greatly restrict the conformation allowed within the remaining part of a protein and thus drastically reduce the number of potential intermediates presented along the pathway of protein folding. The case of αLA-IIA folding represents a useful model to account for the large diversity of folding pathways observed among BPTI, hirudin, and various disulfide-containing proteins.

ACKNOWLEDGMENT

I acknowledge the technical assistance of Mrs. Li Li.

REFERENCES

1. Creighton, T. E. (1978) Experimental studies of protein folding and unfolding, *Prog. Biophys. Mol. Biol. 33*, 231−297.
2. Creighton, T. E. (1986) Disulfide bonds as probes of protein folding pathways, *Methods Enzymol. 131*, 83−106.
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. Creighton, T. E., and Goldenberg, D. P. (1984) Kinetic role of a metastable native-like two-disulfide species in the folding transition of bovine pancreatic trypsin inhibitor, *J. Mol. Biol. 179*, 497−526.
6. Weissman, J. S., and Kim, P. S. (1991) Re-examination of the folding of BPTI: predominance of native intermediates, *Science 253*, 1386−1393.
7. 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.

8. Konishi, Y., Ooi, T., and Scheraga, H. A. (1981) Regeneration of ribonuclease A from the reduced protein, isolation and identification of intermediates, and equilibrium treatment, *Biochemistry 20*, 3945−3955.
9. Rothwarf, D. M., Li, Y. J., and Scheraga, H. A. (1998) Regeneration of Bovine Pancreatic Ribonuclease A: Detailed Kinetic Analysis of Two Independent Folding Pathways, *Biochemistry 37*, 3767−3776.
10. Scheraga, H. A., Wedemeyer, W. J., and Welker, E. (2001) Bovine pancreatic ribonuclease A: oxidative and conformational folding studies, *Methods Enzymol. 341*, 189−221.
11. van Mierlo, C. P., Darby, N. J., and Creighton, T. E. (1992) The partially folded conformation of the Cys-30 Cys-51 intermediate in the disulfide folding pathway of bovine pancreatic trypsin inhibitor, *Proc. Natl. Acad. Sci. U.S.A. 89*, 6775−6779.
12. Welker, E., Narayan, M., Volles, M. J., and Scheraga, H. A. (1999) Two new structured intermediates in the oxidative folding of RNase A, *FEBS Lett. 460*, 477−479.
13. 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.
14. Chang, J.-Y. (1994) Controlling the speed of hirudin folding, *Biochem. J. 300*, 643−650.
15. Chang, J.-Y. (1996) The disulfide folding pathway of tick anticoagulant peptide, a kunitz-type inhibitor structurally homologous to BPTI, *Biochemistry 35*, 11702−11709.
16. 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.
17. 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.
18. 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.
19. 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.
20. Ewbank, J. J., and Creighton, T. E. (1993) Pathway of disulfide-coupled unfolding and refolding of bovine α-lactalbumin, *Biochemistry 32*, 3677−3693.
21. 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.
22. Daly, N. L., Clark, R. J., and Craik, D. J. (2003) Disulfide folding pathways of cystine knot proteins, *J. Biol. Chem. 278*, 6314−6322.
23. 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.
24. 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.
25. Liang, S., Shu, Q., Wang, X., and Zong, X. (1999) Oxidative folding of reduced and denatured huwentoxin-I, *J. Protein Chem. 18*, 619−625.
26. Lu, H. S., Clogston, C. L., Narhi, L. O., Merewether, L. A., Pearl, W. R., and Boone, T. C. (1992) Folding and oxidation of recombinant human granulocyte colony stimulating factor produced in *Escherichia coli*. Characterization of the disulfide-reduced intermediates and cysteine−serine analogs, *J. Biol. Chem. 267*, 8770−8777.
27. Wu, J., Yang, Y., and Watson, J. T. (1998) Trapping of intermediates during the refolding of recombinant human epidermal growth factor (hEGF) by cyanylation, and subsequent structural elucidation by mass spectrometry, *Protein Sci. 7*, 1017−1028.
28. 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.
29. Miller, J. A., Narhi, L. O., Hua, Q. X., Rosenfeld, R., Arakawa, T., Rohde, M., Prestrelski, S., Lauren, S., Stoney, K. S., Tsai, L., and Weiss, M. A. (1993) Oxidative refolding of insulin-like growth factor 1 yields two products of similar thermodynamic stability: a bifurcating protein-folding pathway, *Biochemistry 32*, 5203−5213.
30. Milner, S. J., Carver, J. A., Ballard, F. J., and Francis, G. L. (1999) Probing the disulfide folding pathway of insulin-like growth factor-I, *Biotechnol. Bioeng. 62*, 693−703.
31. 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.
32. Pegoraro, S., Fiori, S., Cramer, J., Rudolph-Bohner, S., and Moroder, L. (1999) The disulfide-coupled folding pathway of apamin as derived from diselenide-quenched analogs and intermediates, *Protein Sci. 8*, 1605−1613.
33. Qiao, Z. S., Guo, Z. Y., and Feng, Y. M. (2001) Putative disulfide-forming pathway of porcine insulin precursor during its refolding in vitro, *Biochemistry 40*, 2662−2668.
34. 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.
35. 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.
36. Ruoppolo, M., Talamo, F., Pucci, P., Moutiez, M., Quemeneur, E., Menez, A., and Marino, G. (2001) Slow folding of three-fingered toxins is associated with the accumulation of native disulfide-bonded intermediates, *Biochemistry 40*, 15257−15266.
37. Singh, R. R., and Appu Rao, A. G. (2002) Reductive unfolding and oxidative refolding of a Bowman-Birk inhibitor from horsegram seeds (*Dolichos biflorus*): evidence for "hyperreactive" disulfide bonds and rate-limiting nature of disulfide isomerization in folding, *Biochim. Biophys. Acta 1597*, 280−291.
38. Wei, C., Tang, B., Zhang, Y., and Yang, K. (1999) Oxidative refolding of recombinant prochymosin, *Biochem. J. 340*, 345−351.
39. Wilkins, J. A., Cone, J., Randhawa, Z. I., Wood, D., Warren, M. K., and Witkowska, H. E. (1993) A study of intermediates involved in the folding pathway for recombinant human macrophage colony-stimulating factor (M-CSF): evidence for two distinct folding pathways, *Protein Sci. 2*, 244−254.
40. Zhang, Y. H., Yan, X., Maier, C. S., Schimerlik, M. I., and Deinzer, M. L. (2002) Conformational analysis of intermediates involved in the in vitro folding pathways of recombinant human macrophage colony stimulating factor β by sulfhydryl group trapping and hydrogen/deuterium pulsed labeling, *Biochemistry 41*, 15495−15504.
41. Chang, J.-Y. (1995) The properties of scrambled hirudins, *J. Biol. Chem. 270*, 25661−25666.
42. Chang, J.-Y., Schindler, P., and Chatrenet, B. (1995) The disulfide structures of scrambled hirudins, *J. Biol. Chem. 270*, 11992−11997.
43. Chang, J.-Y., Li, L., and Bulychev, A. (2000) The underlying mechanism for the diversity of disulfide folding pathways, *J. Biol. Chem. 275*, 8287−8289.
44. Rao, K. R., and Brew, K. (1989) Calcium regulates folding and disulfide-bond formation in α-lactalbumin, *Biochem. Biophys. Res. Commun. 163*, 1390−1396.
45. Ewbank, J. J., and Creighton, T. E. (1993) Structural characterization of the disulfide folding intermediates of bovine α-lactalbumin, *Biochemistry 32*, 3694−3707.
46. Permyakov, E. A., and Berliner, L. J. (2000) α-Lactalbumin: Structure and function, *FEBS Lett. 473*, 269−274.
47. Chang, J.-Y. (2002) The folding pathway of α-lactalbumin elucidated by the technique of disulfide scrambling, *J. Biol. Chem. 277*, 120−126.
48. Creighton, T. E. (1984) Disulfide bonds formation in proteins, *Methods Enzymol. 107*, 305−329.
49. Chang, J.-Y., and Ballatore, A. (2000) The structure of denatured bovine pancreatic trypsin inhibitor (BPTI), *FEBS Lett. 473*, 183−187.
50. Bulychev, A., and Chang, J.-Y. (1999) Unfolding of hirudin characterized by the composition of denatured scrambled isomers, *J. Protein Chem. 18*, 771−778.

51. Creighton, T. E., Darby, N. J., and Kemmink, J. (1996) The roles of partly folded intermediates in protein folding, *FASEB J. 10*, 110−118.
52. Staley, J. P., and Kim, P. S. (1990) Role of a subdomain in the folding of bovine pancreatic trypsin inhibitor, *Nature 344*, 685−688.
53. Staley, J. P., and Kim, P. S. (1994) Formation of a native-like subdomain in a partially folded intermediate of bovine pancreatic trypsin inhibitor, *Protein Sci. 3*, 1822−1832.
54. Carty, R. P., Pincus, M. R., and Scheraga, H. A. (2002) Interactions that Favor the Native over the Non-Native Disulfide Bond among Residues 58−72 in the Oxidative Folding of Bovine Pancreatic Ribonuclease A, *Biochemistry 41*, 14815−14819.
55. Narhi, L. O., Hua, Q. X., Arakawa, T., Fox, G. M., Tsai, L., Rosenfeld, R., Holst, P., Miller, J. A., and Weiss, M. A. (1993) Role of native disulfide bonds in the structure and activity of insulin-like growth factor 1: genetic models of protein-folding intermediates, *Biochemistry 32*, 5214−5221.
56. Wu, L. C., Peng, Z. Y., and Kim, P. S. (1995) Bipartite structure of the α-lactalbumin molten globule, *Nat. Struct. Biol. 2*, 281−286.
57. Peng, Z. Y., Wu, L. C., and Kim, P. S. (1995) Local structural preferences in the α-lactalbumin molten globule, *Biochemistry 34*, 3248−3252.
58. Chang, J.-Y., and Li, L. (2002) The structure of denatured α-lactalbumin elucidated by the technique of disulfide scrambling, *J. Biol. Chem. 276*, 9705−9712.
59. Kuwajima, K. (1989) The molten globule state as a clue for understanding the folding and cooperativity of globular-protein structure, *Proteins: Struct., Funct., Genet. 6*, 87−103.
60. Ptitsyn, O. B. (1995) Molten globule and protein folding, *Adv. Protein Chem. 47*, 83−229.
61. Chang, J.-Y., Bulychev, A., and Li, L. (2000) A stabilized molten globule protein, *FEBS Lett. 487*, 298−300.

BI0360354