Pathway of Oxidative Folding of Bovine α-Interferon: Predominance of Native Disulfide-Bonded Folding Intermediates†

Curtis C.-J. Lin and Jui-Yoa Chang*

Research Center for Protein Chemistry, Brown Foundation Institute of Molecular Medicine, and Department of Biochemistry and Molecular Biology, University of Texas, Houston, Texas 77030

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ABSTRACT: Bovine α-interferon (BoINF-α) is a single polypeptide protein containing 166 amino acids, two disulfide bonds (Cys^{1}−Cys^{99} and Cys^{29}−Cys^{138}), and five stretches of α-helical structure. The pathway of oxidative folding of BoINF-α has been investigated here. Of the eight possible one- and two-disulfide isomers, only two nativelike one-disulfide isomers, BoINF-α (Cys^{1}−Cys^{99}) and BoINF-α (Cys^{29}−Cys^{138}), predominate as intermediates along the folding pathway. More strikingly, α-helical structures formed almost quantitatively before any detectable formation of a disulfide bond. This is demonstrated by the observation that fully reduced BoINF-α (starting material of oxidative folding) and reduced carboxymethylated BoINF-α both exhibit α-helical structure content indistinguishable form that of native BoINF-α. The folding mechanism of BoINF-α appears to be compatible with the framework model, in which secondary structures fold first, followed by docking (compaction) of preformed secondary structural elements yielding the native structure.

Successful protein folding requires formation of both native secondary and tertiary structures. The interplay and relative folding rate of these two structures have been the subjects of extensive investigations aimed at establishing a general model of protein folding (*1*). Two major models have been put forward to account for the mechanism of protein folding. The framework model (*2*−*4*) proposes that secondary structures (α-helix, *β*-strand, etc.) form first during the early stage of folding, which is followed by docking and packing of preformed secondary structural units yielding the native tertiary structure. The hydrophobic collapse model (*5*−*8*) stipulates that a rapid hydrophobic collapse (interaction) accounts for the major driving force of folding, which is followed by searching and fine-tuning of conformation in a confined volume to reach the native structure. A basic distinction between these two models is the relative kinetics of formation of the secondary structure and the tertiary structure (compaction) of the polypeptide chain during the process of folding. A strong preference of localized conformation would favor the framework model and lead to the rapid formation of secondary structure before the organization of tertiary structure is initiated (*7*).

Results of studies conducted so far, nonetheless, show that extreme mechanisms fitting either model are rare and that the formation of secondary structure and compaction of the protein occur usually in parallel for most proteins during the course of folding. In other words, folding of most proteins follows the middle-of-the-road between the framework and hydrophobic collapse models (*1*). Uversky and Fink (*9*) have analyzed data on the conformational properties of 41 native and partially folded states and concluded a valid correlation between the increase in secondary structure content and the decrease in hydrodynamic volume during folding. Essentially, they found no evidence among analyzed proteins of either compact intermediates lacking secondary structure or unfolded intermediates comprising highly ordered secondary structure. One specific example is demonstrated by the folding of chymotrypsin inhibitor 2 (CI2). As seen in a series of peptide fragments of CI2 with increasing lengths, the secondary structure were observed only after residues that make long-range interaction appear (*10*). These studies have led to the proposal of a new unifying mechanism of folding, the nucleation−condensation model (*11*), which invokes the importance of interplay and the interdependence of the secondary and tertiary structures during protein folding. This model essentially stipulates that secondary structure is inherently unstable, and its stability can be enhanced by protein compaction and tertiary interactions.

However, studies of oxidative folding of disulfide proteins performed in our laboratories have suggested that an extreme mechanism of protein folding compatible with the hydrophobic collapse model does exist and may be more prevalent then previously thought (*1*). One example is demonstrated by the pathway of oxidative folding of hirudin (*12*, *13*). The starting material of hirudin folding (fully reduced hirudin) is essentially structureless. Oxidative folding of reduced hirudin was shown to undergo an initial stage of rapid nonspecific packing to form a mixture of compact scrambled isomers as folding intermediates, which also exhibit largely unordered secondary structure. This is followed by disulfide shuffling and reorganization of scrambled isomers, yielding the native hirudin. The pathways of oxidative folding of potato carboxypeptidase inhibitor (*14*) and cardiotoxin (*15*)

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* To whom correspondence should be addressed: Institute of Molecular Medicine, 1825 Pressler St., Houston, TX 77030. Telephone: (713)500-2458. Fax: (713)500-2447. E-mail: Rowen.Chang@uth.tmc.edu.

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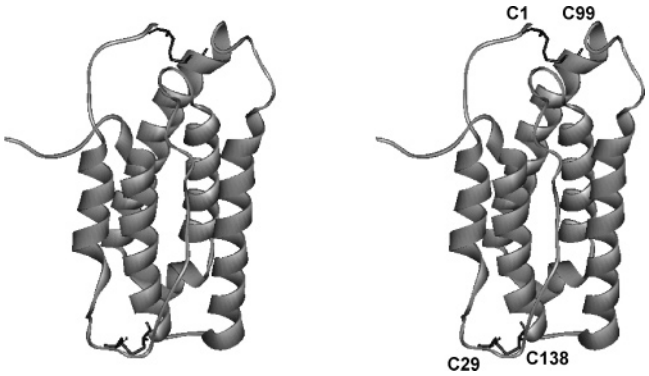

FIGURE 1: Structure of BoINF-α. The native BoINF-α comprises five stretches of α-helical structure and is stabilized by two disulfide bonds (Cys^1-Cys^{99} and $Cys^{29}-Cys^{138}$). The structure is based on the homology modeling of human interferon α-2 (*16*).

are similarly close to the mechanism of the hydrophobic collapse model.

In this report, we investigate the pathway of oxidative folding of bovine α-interferon (BoINF-α)[1] and demonstrate a folding model that is in agreement with the framework model. BoINF-α comprises 166 amino acids and five segments of α-helices (*16*−*18*) that are intraconnected by two native disulfide bonds (Cys^1-Cys^{99} and $Cys^{29}-Cys^{138}$) (Figure 1) (*19*). A two-disulfide protein represents the simplest model for studying the pathway of oxidative folding. There are eight disulfide isomers (six one-disulfide isomers and two two-disulfide isomers) that may serve as folding intermediates. Among them, only two one-disulfide intermediates contain native disulfide bonds. Despite the simplicity, the mechanism of oxidative folding of BoINF-α is shown to be consistent with the case of framework model, in which secondary structures fold first, followed by packing and docking of preformed secondary structures yielding the native protein. The folding mechanism of BoINF-α is characterized by (a) the exclusive predominance of native disulfide-bonded folding intermediates and (b) the rapid formation of α-helical structure during the early stage of folding.

EXPERIMENTAL PROCEDURES

Materials. Recombinant BoINF-α was kindly provided by Novartis AG (Basel, Switzerland). The protein was shown to have a purity of >97%, as judged by the HPLC profiles and MALDI mass analysis. Trypsin (T-1426), dithiothreitol, β-mercaptoethanol, and GdnCl, all with purities of >99%, were purchased from Sigma.

Oxidative Folding of the Reduced and Denatured BoINF-α. The native BoINF-α (2 mg/mL) was first reduced and denatured in Tris-HCl buffer (0.1 M, pH 8.4) containing 6 M GdnCl and 30 mM dithiothreitol. The reaction was carried out for 90 min at 23 °C. To initiate folding, the reduced BoINF-α was passed through a PD-10 column (Sephadex-25, Pharmacia) which was equilibrated in 0.1 M Tris-HCl buffer (pH 8.4). The desalted sample was diluted immediately with the same Tris-HCl buffer to a final protein concentration of 0.5 mg/mL. 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 BoINF-α were quenched by mixing aliquots of the reaction 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 linear from 40 to 60% B over 60 min. The flow rate was 0.5 mL/min. The column was a VYDAC C18 column for peptides and proteins (catalog no. 218TP104), 4.6 mm × 5 μm. The column temperature was 23 °C.

Stop−*Go Folding Experiments of Isolated Folding Intermediates.* Folding intermediates of BoINF-α-IA and -IB were isolated via HPLC, freeze-dried, and allowed to resume the folding by being reconstituted in 0.1 M Tris-HCl buffer (pH 8.4) with a final protein concentration of 0.5 mg/mL. The process of folding was quenched by acidification and analyzed by HPLC as described above.

Characterization of Disulfide Structures of Major Folding Intermediates of BoINF-α. Two acid-trapped folding intermediates, BoINF-α-IA and -IB, were purified via HPLC and freeze-dried. The samples were first derivatized with 0.1 M iodoacetamide in Tris-HCl buffer (0.1 M, pH 8.4) at 23 °C in the dark, for 30 min. The reaction was quenched with 4% aqueous trifluoroacetic acid. Carboxymethylated samples were then analyzed by MALDI mass spectrometry to characterize the number of disulfide bonds. Modified BoINF-α-IA and -IB (~10 μg each) were further treated with 1 μg of trypsin (Sigma, T-1426) in 50 μL of ammonium bicarbonate (0.1 M, pH 7.9). Digestion was carried out at 37 °C for 8 h. Peptides were then isolated by HPLC. To identify peptide fragments containing disulfide bond and carboxymethylated cysteine, peptides were analyzed with a MALDI-TOF mass spectrometer (Perkin-Elmer Voyager-DE STR) using 2,5-dihydroxybenzoic acid as the 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). 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.

Measurement of the Secondary Structure Content of Disulfide Isomers of BoINF-α. Disulfide isomers of BoINF-α, including the native (N), fully reduced (R), reduced carboxymethylated (R-CM), and two predominant folding intermediates (IA and IB), were analyzed by CD. They were all isolated from HPLC, freeze-dried, and then dissolved in 0.5% aqueous TFA (0.1 mg/mL) prior to CD measurements. Immediately after the CD measurement, the samples were also analyzed by HPLC to ensure their identity. HPLC conditions are as described above. The CD spectra were analyzed using a JASCO J-715 circular dichroism spectrophotometer. Ten scans were accumulated for each sample. The *X*-scale was scanning wavelength from 200 to 260 nm. The *Y*-scale was measured in mean residue ellipticity, $[\theta]_{MRW}$, and ranged from −20 to 20. Mean residue ellipticity, $[\theta]_{MRW}$, was calculated for each data point using the formula $[\theta]_{MRW} = \theta_{obs}/(10Cnl)$, where *C* is the concentration in molar,

[1] Abbreviations: BoINF-α, bovine α-interferon; DTT, reduced dithiothreitol; HPLC, high-pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of flight.

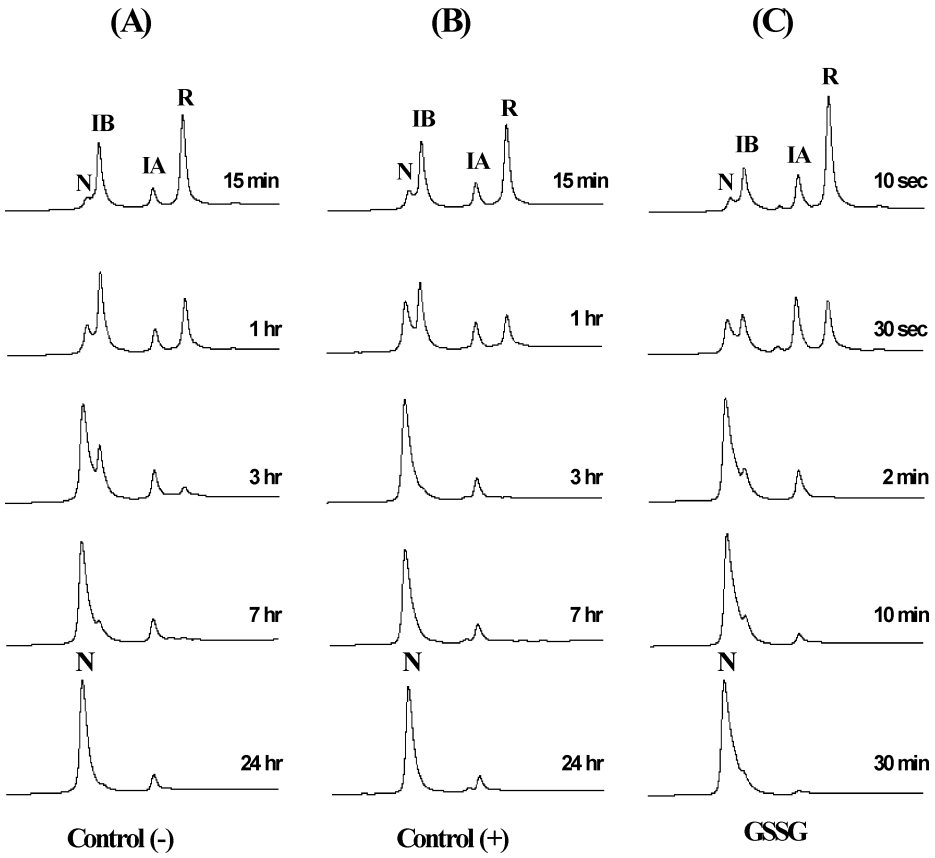

FIGURE 2: HPLC profiles of the process of oxidative folding of reduced denatured BoINF-α. Folding was performed at 22 °C using the following conditions: (A) Tris-HCl buffer (0.1 M, pH 8.4) alone, (B) Tris-HCl buffer (0.1 M, pH 8.4) containing GSH (1 mM), or (C) Tris-HCl buffer (0.1 M, pH 8.4) containing GSSG (0.5 mM). Intermediates of folding were withdrawn at different time points, quenched with an equal volume of 4% aqueous TFA, and analyzed by reverse-phase HPLC using the conditions described in Experimental Procedures. R and N indicate the elution positions of the fully reduced and native BoINF-α, respectively. IA and IB denote two major folding intermediates (their disulfide structures are given in Figure 3).

n is the number of residues, and *l* is the path length of the cell.

RESULTS

Oxidative Folding of Reduced and Denatured BoINF-α. The oxidative folding of fully reduced BoINF-α was investigated under various conditions. The experiment was first carried out in the Tris-HCl buffer alone without any thiol catalyst or redox agent. The HPLC chromatograms of the time course-trapped folding intermediates are illustrated in Figure 2A. Under this simple condition, folding of reduced BoINF-α was promoted by air oxygen, and ~93% of the native BoINF-α was recovered after folding for 24 h. Folding intermediates were shown to consist of two major fractions, designated IA and IB. Analysis of molecular masses following modification with iodoacetamide reveals that both IA and IB are one-disulfide isomers of BoINF-α.

The folding of BoINF-α 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 facilitate and catalyze disulfide shuffling of intermediates containing non-native disulfide bonds (*13*, *20*). The results show that inclusion of GSH in the folding buffer affects neither the patterns of folding intermediates nor the recovery of native BoINF-α (Figure 2B), although the kinetics of folding increases by ~2-fold. Like that performed in buffer alone (Figure 2A), ~5−7% of BoINF-α remained as IA after folding for 24 h. The inclusion of GSSG, which promotes disulfide oxidation, significantly increases the rate of disulfide formation and the disappearance of fully reduced BoINF-α. The rate of recovery of native BoINF-α increases by ~120-fold (Figure 2C) compared to that carried out in buffer alone (Figure 2A).

Disulfide Structures of the Folding Intermediates of BoINF-α. IA and IB were isolated from HPLC, freeze-dried, modified with iodoacetamide, and digested with trypsin. Fragmented tryptic peptides were then isolated by HPLC (Figure 3A) and analyzed by Edman amino acid sequencing and MALDI mass spectrometry. The data reveal that IA contains the $Cys^{29}-Cys^{138}$ disulfide bond and IB contains the $Cys^{1}-Cys^{99}$ disulfide bond (Figure 3B); both are native disulfide connectivity of BoINF-α.

Stop−Go Folding of the Two Predominant Folding Intermediates of BoINF-α. The two major folding intermediates, IA and IB, were isolated from HPLC, freeze-dried, and allowed to continue the oxidative folding by reconstitution in Tris-HCl buffer. Folding intermediates were trapped by sample acidification and analyzed by HPLC (Figure 4). A few conclusions can be drawn from these experiments. (a) IA and IB do not equilibrate between each other, indicating that structural elements of intermediates connected by $Cys^{1}-Cys^{99}$ (IB) and $Cys^{29}-Cys^{138}$ (IA) disulfide bonds exhibit a significant degree of stability. (b) The rate of IA → N conversion is ~4-fold faster than that of IB → N conversion,

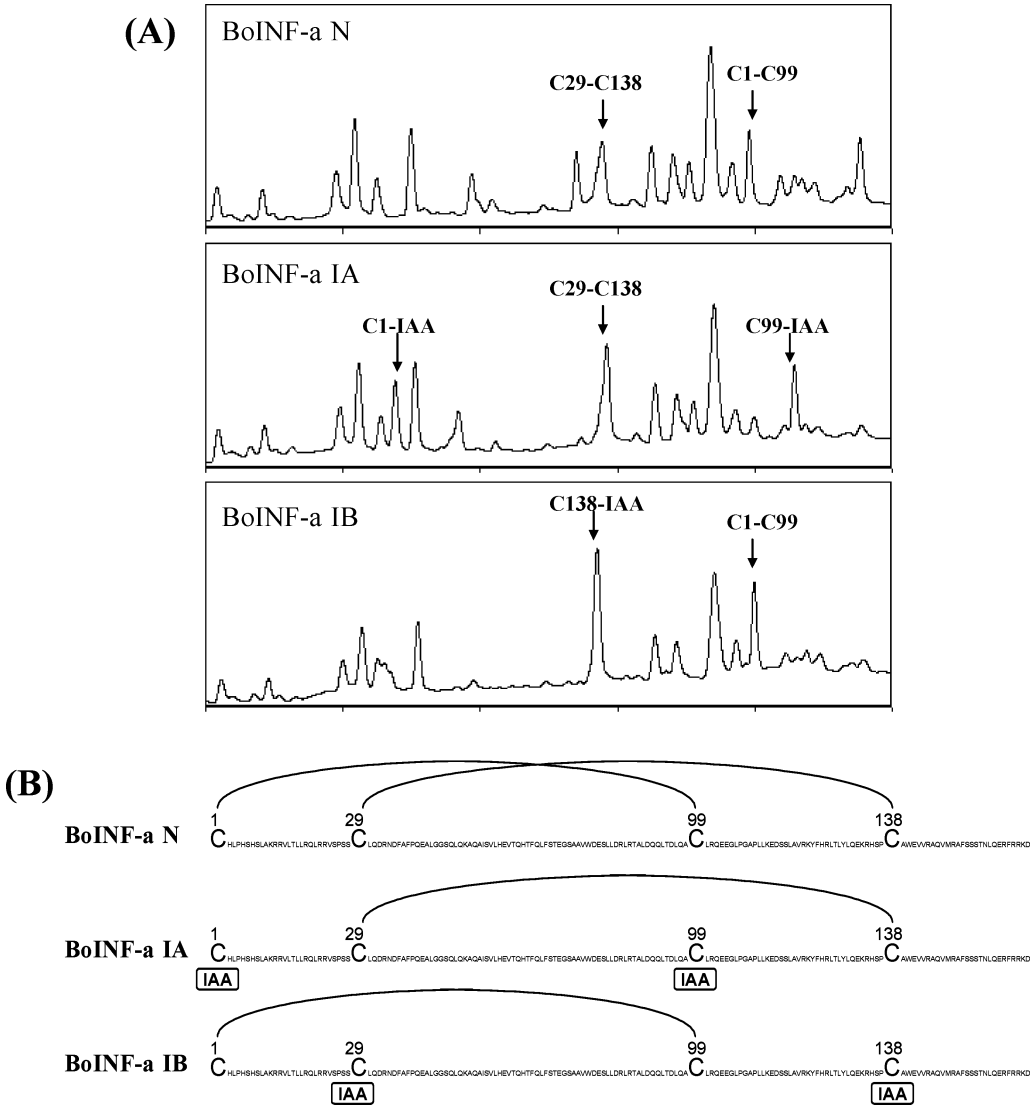

FIGURE 3: Elucidation of the disulfide structures of major folding intermediates of BoINF-α. (A) IA and IB were purified from HPLC, modified with iodoacetamide, freeze-dried, and digested with trypsin. The native BoINF-α was also digested with trypsin in parallel as a control. Tryptic peptides were separated by HPLC and analyzed by Edman sequencing as well as MALDI mass spectrometry. (B) The data obtained from the analysis of tryptic peptides lead to the conclusion that there are two native disulfide bonds present in BoINF-α-IA ($Cys^{29}-Cys^{138}$) and BoINF-α-IB ($Cys^{1}-Cys^{99}$).

suggesting a greater constraint of forming the $Cys^{29}-Cys^{138}$ disulfide bond than the formation of the $Cys^{1}-Cys^{99}$ disulfide bond, which is consistent with the fact that IB is ~4-fold more populated than IA as folding intermediates (Figure 2A). (c) Unlike IB, IA is unable to convert to the native BoINF-α quantitatively. This is also consistent with the results of oxidative folding starting with the fully reduced BoINF-α (Figure 2A). The fact that IA folds ~4-fold faster than IB yet there is a residual IA that is unable to attain the native structure also suggests the likelihood of the presence of non-native one-disulfide isomers that are coeluted with IA.

Content of the α-Helical Structure of the Folding Intermediates of BoINF-α. The disulfide isomers of BoINF-α, including the native (N), IA, IB, fully reduced (R), and reduced carboxymethylated (R-CM) intermediates, were all purified from HPLC, freeze-dried, and reconstituted in aqueous solution containing 0.5% (by volume) trifluoroacetic acid, and their far-UV CD spectra were recorded (Figure 5A). Samples were analyzed in the acidic solution to prevent any disulfide formation of IA, IB, and fully reduced isomers. The native BoINF-α comprises five segments of α-helical structure and exhibits double minima at 208 and 222 nm, characteristics for α-helical-rich proteins. The unexpected finding is that all four other non-native BoINF-α forms also display a similar α-helical structure content. The states of disulfide structures of IA, IB, and BoINF-α-R were verified by HPLC analysis of the same samples immediately following CD measurement (Figure 5A, inset).

The α-helical structure of BoINF-α-R appears to fold instantly after removal of the denaturant and before formation of any detectable disulfide bond. This is further demonstrated by the dilution experiment shown in Figure 5B. In the presence of GdnCl (6 M) and DTT (3 mM), the structure of BoINF-α-R is unordered. Upon 10-fold dilution of the GdnCl to 0.6 M and within approximately 1 min, BoINF-α-R was shown to exhibit α-helical structure content almost similar to that of native BoINF-α.

Differentiation of the Stability of α-Helical Structure of the Native and Reduced Carboxymethylated BoINF-α. BoINF-α-N and BoINF-α-RCM exhibit a comparable content of α-helical structure (Figure 5), but their stability differs.

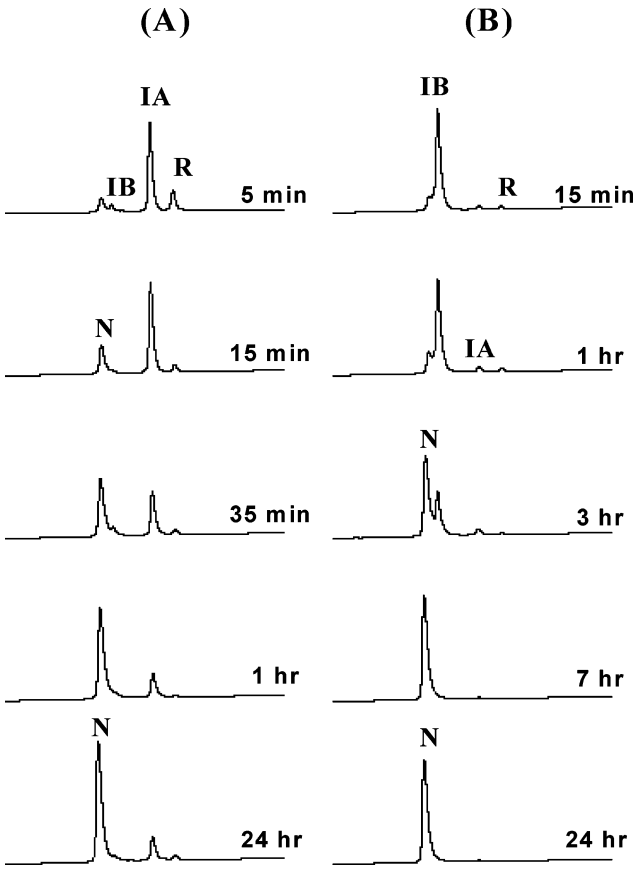

FIGURE 4: Stop−go folding of the two major folding intermediates of BoINF-α. IA and IB were isolated from HPLC, freeze-dried, and reconstituted in Tris-HCl buffer (0.1 M, pH 8.4) to resume the folding. The final protein concentration was 0.5 mg/mL. The process of folding was quenched by acidification and analyzed by HPLC as described in the legend of Figure 2.

To assess their relative stability, these two proteins were incubated in the presence of increasing concentrations of GdnCl overnight, and their CD spectra were recorded (Figure 6). The results show that the α-helical structure of BoINF-α-RCM is significantly less stable that that of BoINF-α-N. The midpoint unfolding of the α-helical structure of BoINF-α-N occurs at ∼3.2 M GdnCl. In contrast, the midpoint disruption of the α-helical structure of BoINF-α-RCM takes place at a GdnCl concentration of <1 M.

BoINF-α-RCM Exhibits a Hydrodynamic Volume Larger than That of BoINF-α-N. The structural properties of BoINF-α-N and BoINF-α-RCM were further analyzed by nondenaturing gel electrophoresis, which is useful for the evaluation of the size and shape of proteins. Using this technique, the mobility of a protein moving in an electric field through the gel depends on its net charge, size, and shape. In general, the mobility is directly correlated to its net charge and inversely related to its size and shape. For a protein, unfolding and expansion of the conformation tend to reduce the mobility.

The relative mobility of BoINF-α-N and BoINF-α-RCM in the 15% nondenaturing gel is shown in Figure 7. BoINF-α-RCM exhibits a mobility slower than that of BoINF-α-N (Figure 7). The difference in mobility could have been more significant if one takes into consideration the six additional charges (carboxymethylated Cys) of BoINF-α-RCM, which may have considerably accelerated the mobility of BoINF-α-RCM through the gel. The data thus confirm that BoINF-α-RCM has a shape more extended than that of BoINF-α-N.

DISCUSSION

Protein Folding Pathway and Protein Folding Model. Disulfide proteins fold via diverse pathways (*21*, *22*) which are characterized mainly by the heterogeneity of folding intermediates and the absence or presence of nativelike folding intermediates. At the two extreme ends of this diversity are proteins that fold via selected intermediates which adopt exclusively nativelike structures (*23*−*25*) and proteins that fold via highly heterogeneous intermediates which comprise mostly non-native structures (*12*, *14*, *26*−*28*). Proteins also fold by distinct models (*1*, *7*) which are distinguished by the interplay between the secondary and tertiary structures (compactness) along the course of folding. For some proteins (*29*), secondary structures fold early during the folding; this is followed by docking and packing of preformed secondary structural units to form the native tertiary structure (framework model). For other proteins (*8*, *12*, *14*, *26*), a rapid hydrophobic collapse (interaction) accounts for the major driving force of folding; this is followed by searching and fine-tuning of conformation in a confined volume to reach the native structure (hydrophobic collapse model). Yet, for the majority of proteins, compaction of the protein and formation of secondary structure occur almost in parallel during the course of folding (*9*) (nucleation−condensation model). The scope of the diversity of the protein folding pathway and protein folding model has long been a debated subject (*1*, *21*, *22*). More importantly, there is a pressing need to experimentally demonstrate the connection between the folding pathway and folding model. Theoretically, a protein that folds via a framework model is likely to become limited folding intermediates, adopting mostly nativelike structures, due to the hierarchical packing of preformed structural units. In contrast, a protein that folds via a hydrophobic collapse model is more likely to become heterogeneous folding intermediates comprising mainly non-native structures, because of the nonspecific packing of the polypeptide chain during the early stage of folding. The demonstration of the link between the folding pathway and folding model is essential to a more thorough understanding of the mechanism of protein folding. However, experimental data supporting such connections are scarce.

The key to the discrimination between these three different folding models (*1*) depends heavily on our ability to measure the state of secondary structure and tertiary structure (or compactness) at different stages of folding. Conclusions presented by Uversky and Fink (*9*) were derived from proteins studied mainly by the conventional unfolding−refolding technique. Using this conventional method, trapping and isolation of folding intermediates are unfeasible. Therefore, only a few intermediates present at the unfolding−refolding equilibrium were assessed with respect to their secondary structure and compactness. A more comprehensive correlation of these two structural properties, and thus a better characterization of folding model, can be achieved if more folding intermediates at different stages of folding can be trapped, isolated, and analyzed. In this respect, the technique of oxidative folding fulfills the need (*20*). As illustrated in

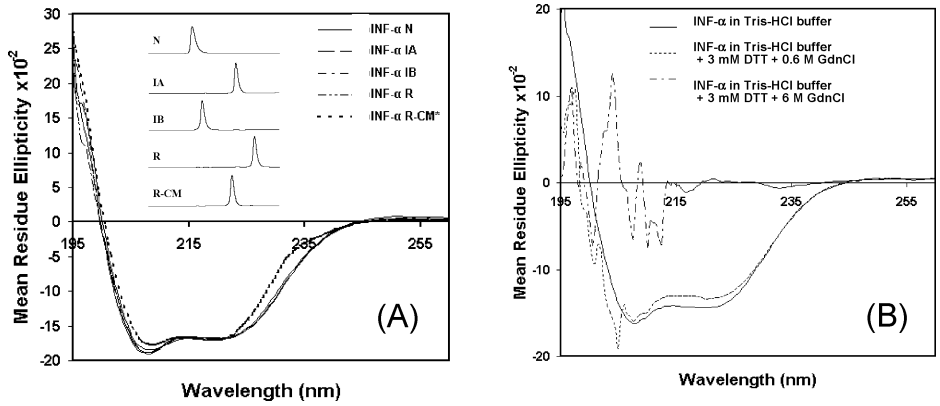

FIGURE 5: (A) Far-UV CD spectra of five disulfide isomers of BoINF-α. They were purified by HPLC and reconstituted in 0.5% aqueous TFA with a final protein concentration of 0.5 mg/mL, and their CD spectra were analyzed using a JASCO J-715 spectropolarimeter. Samples were immediately analyzed by HPLC following CD recording (inset). N represents native BoINF-α. IA and IB are two predominant one-disulfide folding intermediates (see Figure 3). R represents fully reduced BoINF-α with four free cysteines. R-CM is reduced carboxymethylated BoINF-α. (B) Rapid formation of the α-helical structure of reduced denatured BoINF-α. The native BoINF-α (1 mg/mL) was incubated in Tris-HCl buffer (10 mM, pH 7.3) containing 30 mM DTT and 6 M GdnCl for 30 min at 22 °C. The sample was then divided into two parts. One part was diluted with 9 volumes of the same Tris-HCl buffer (10 mM, pH 7.3) containing 6 M GdnCl, and its CD spectrum was recorded. The final concentrations of DTT and GdnCl were 3 mM and 6 M, respectively. The second part was diluted with 9 volumes of the same Tris-HCl buffer (10 mM, pH 7.3), and its CD spectrum was immediately recorded (the process took less than 1 min). The final concentrations of DTT and GdnCl were 3 mM and 0.6 M, respectively. The spectra of native BoINF-α dissolved in the same Tris-HCl buffer with the same final protein concentration (0.1 mg/mL) are also presented.

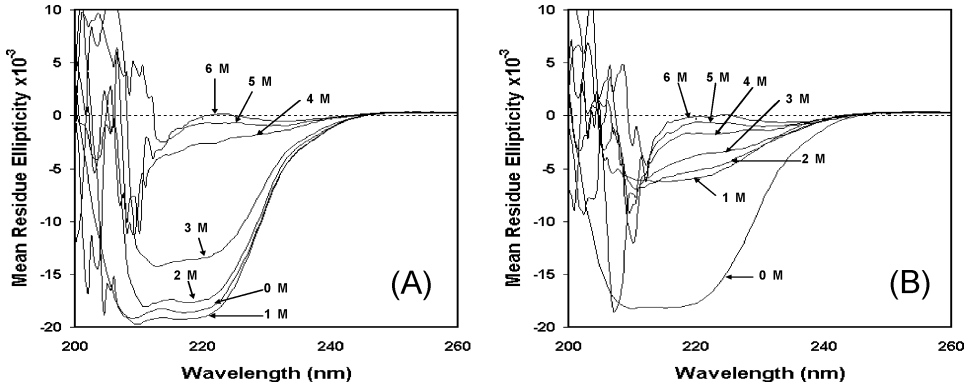

FIGURE 6: Distinction of the stability of α-helical structure between the native and reduced carboxymethylated BoINF-α. BoINF-α-N (A) and reduced carboxymethylated BoINF-α-RCM (B) were dissolved in Tris-HCl buffer (10 mM, pH 7.3) containing different concentrations of GdnCl (1−8 M). The samples were incubated for 16 h at 23 °C before their CD spectra were recorded. The parameters of the CD spectrophotometer are described in the text.

the analysis of the disulfide folding pathway of BoINF-α described above (Figure 2), folding intermediates of disulfide proteins can be trapped and analyzed at any time point during the course of oxidative folding, thus providing ample folding intermediates for characterization of their structural properties.

The Folding Mechanism of BoINF-α Is Compatible with the Framework Model. In this study, we have investigated the folding mechanism of BoINF-α using the technique of oxidative folding. The results show that the pathway of BoINF-α folding is predominated by the intermediates containing exclusively native disulfide bonds. Among the eight disulfide isomers that may serve as intermediates, two one-disulfide isomers containing native disulfide bonds (Cys^{29}−Cys^{138} and Cys^{1}−Cys^{99}) were found to constitute more than 95% of the total folding intermediates. The mechanism of the initial stage of BoINF-α folding is also dictated by the rapid formation of nativelike secondary structures, before any packing and disulfide formation of BoINF-α occur. As expected, α-helical structures disconnected from tertiary structure are significantly less stable than those embedded in the native protein. The folding mechanism of BoINF-α is thus compatible or close to the case of the framework model, in which nativelike secondary structures form first during the early phase of folding, followed by compaction of preformed structural units to reach the native structure. This explains why folding intermediates of BoINF-α adopt solely native disulfide bonds.

The folding mechanism of BoINF-α contrasts sharply with that of hirudin (a β-sheet protein stabilized by three disulfide bonds) and other disulfide proteins that has also been characterized by the method of oxidative folding and was shown to include a folding mechanism close to the hydrophobic collapse model (*12*, *14*, *15*). Unlike BoINF-α, folding intermediates of hirudin are comprised of a highly heterogeneous isomer; most of them contain non-native disulfide bonds. The starting material and the compacted intermediates of hirudin folding are also devoid of β-sheet structure (R. J.-Y. Chang, unpublished data), which indicate that formation of secondary and tertiary structure occurs concurrently during the final stage of hirudin folding within a confined volume.

The diversity of folding mechanisms between BoINF-α and hirudin may in part be due to the difference in their

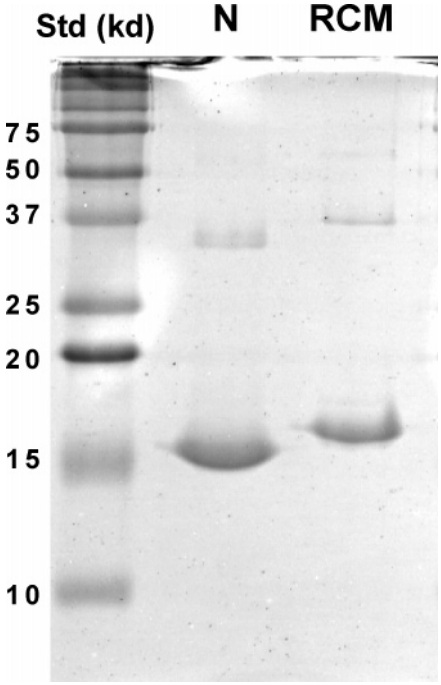

FIGURE 7: Comparison of the hydrodynamic volumes of BoINF-α-N and BoINF-α-RCM using nondenaturing gel electrophoresis. Native BoINF-α-N and reduced carboxymethylated BoINF-α-RCM (5 μg per lane) were dissolved in the sample buffer without β-mercaptoethanol and heat treatment. The samples were analyzed by 15% polyacrylamide gel electrophoresis.

secondary structures. α-Helical structures are formed out of intrastrand contacts. Their formation therefore is more likely to be independent of the compaction of protein and oxidation of disulfide bonds. If α-helical structures take shape early in the folding, docking, and packing of these preformed structural units, they are prone to be hierarchical and folding intermediates are prone to adopt nativelike structures. This is what has been observed in the case of BoINF-α folding. In contrast, formation of β-sheet structures is invariably due to interstrand contacts and consequently is more likely to be dependent on the compactness of the protein and the formation of disulfide bonds. In the absence of preformed structural units, nonspecific packing via hydrophobic interaction is likely to be the dominant force of folding and folding intermediates are likely to be heterogeneous. Indeed, proteins that fold via the framework model investigated so far are entirely helical proteins (*1*), similar to BoINF-α. On the other hand, cardiotoxin-III, an all-β-sheet protein, also folds via a mechanism indistinguishable from that of hirudin (*15*).

However, the scope of the diversity of the protein folding pathway and folding model is extensive (*1*, *7*, *21*, *22*). Many more proteins with a diverse composition of secondary structures need to be investigated to determine whether folding properties observed with BoINF-α and hirudin are generally applicable. At the core of important questions that remain to be answered are the following. (a) To what extent do α-, β-, and α/β-structures dictate the folding model and folding pathway of proteins? (b) To what extent do folding models associate with the folding pathways?

Oxidative folding has proven to be a very useful method for simultaneous analysis of the folding pathway and folding model of a disulfide protein. The major advantage is that folding intermediates can be trapped, isolated, and structurally characterized at different and numerous stages of folding (*20*). For each protein, the same set of trapped folding intermediates can be utilized for characterization of the folding pathway and folding model. This includes concurrent analysis of their conformational heterogeneity, disulfide structure, kinetic property, secondary structure, compactness, and tertiary structure.

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