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Diversity of Folding Pathways and Folding Models of Disulfide Proteins

JUI–YOA CHANG

ABSTRACT

Comprehensive understanding of the mechanism of protein folding requires the elucidation of both a folding pathway and a folding model. This entails characterization of the properties and structures of folding intermediates populated along the folding pathway, as well as the formation and interplay of secondary structures and tertiary structures along the course of folding. Using the conventional unfolding–refolding technique, there are limitations of acquiring these data in detail because of the inherent difficulty of trapping and analysis of folding intermediates. The technique of oxidative folding, in contrast, permits trapping, isolation, and further structural characterization of folding intermediates at any stage of the folding process. In this brief review, we present the potential of the technique of oxidative folding for concurrent analysis of both folding pathways and folding models. *Antioxid. Redox Signal.* 10, 171–177.

INTRODUCTION

PROTEINS FOLD via **diverse pathways** (2, 32) which are characterized mainly by the heterogeneity and structures of folding intermediates that populated along the pathway of folding. The two extreme ends of this diversity are represented by proteins that fold via selected intermediates adopting exclusively native-like structures, and proteins that fold via highly heterogeneous intermediates comprising mostly non-native structures (2). Proteins also fold by **distinct models** (17) which are distinguished by the interplay between the secondary and tertiary structures (compactness) along the course of folding. For some proteins, 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**) (4, 27, 28, 34). For other proteins, 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**) (18, 26, 38). Yet, for the majority of proteins, compaction of protein and formation of secondary structure occur almost in parallel during the course of folding (**nucleation–condensation model**) (20, 39). The scope of diversity of protein folding pathways and protein folding models have long been debated subjects (17).

For a comprehensive understanding of the mechanism of protein folding, it is imperative to elucidate both the folding pathway and folding model of each investigated protein. It is important to experimentally demonstrate the connection between the structure/property of folding intermediates and the interplay of secondary and tertiary structures that guide the formation of these intermediates. For instance, do proteins that fold by a framework model also display limited intermediates adopting mainly native-like structures because of the hierarchical formation of preformed structural units? Or do proteins folding by a hydrophobic collapse model also fold via heterogeneous intermediates adopting mainly non-native like structures because of the nonspecific packing of at the early stage of folding? These are issues at the center of understanding the mechanism of protein folding.

Currently, the detailed pathways of protein folding have been mainly derived from the studies of oxidative folding of disulfide proteins (2, 15, 22, 32, 40). On the other hand, most in-depth models of protein folding are concluded from the analy-

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

sis of nondisulfide proteins or disulfide proteins in the presence of intact native disulfide bonds (17, 20). Cases of protein folding with both a folding pathway and a folding model elucidated in detail are rare. In this article, we will (a) review the diversity of disulfide folding pathways and protein folding models; (b) describe the approach for concurrent analysis of folding pathways and folding models; and (c) present cases of oxidative folding that are consistent with the framework model and hydrophobic collapse model.

DISULFIDE PROTEINS FOLD VIA DIVERSE PATHWAYS

The mechanism of protein folding is characterized by a folding pathway. **Oxidative folding** is one of the well-established techniques for studying the pathway of protein folding (2, 15, 16, 22, 40). It is applicable to proteins that contain two disulfide bonds or more. Using this method, a disulfide protein is first reduced and denatured in the presence of reducing agent (*e.g.*, 20–50 m*M* dithiothreitol) and denaturant (*e.g.*, 6 *M* GdmCl or 8 *M* urea). After exclusion of the reductant and denaturant by gel filtration, dilution, or dialysis, the reduced and denatured protein is then allowed to refold in alkaline buffer (pH 7.3–8.8) in the presence of redox agent that promotes disulfide bonds formation and shuffling (16). Folding intermediates of oxidative folding are subsequently trapped in a time-course fashion noncovalently by sample acidification (*e.g.*, 2% aqueous trifluoroacetic acid (TFA) of formic acid) or covalently by chemical modification (e.g., carboxymethylation). Trapped folding intermediates are typically separated by reversed phase HPLC or ion-exchange chromatography and analyzed for their structures by chemical methods, circular dichroism (CD), or nuclear magnetic resonance (NMR). The pathway of refolding is subsequently tracked and analyzed by the mechanism of formation of folding intermediates (*disulfide isomers*) containing either native or non-native disulfide bonds. For instance, a protein that contains three disulfide bonds can potentially adopt 75 different disulfide isomers (15 one-disulfide, 45 two-disulfide, and 15 three-disulfide). Among them are seven isomers that contain exclusively native disulfide bonds (3 one-disulfide, 3 two-disulfide and 1 three-disulfide). All these 75 isomers could potentially be present during the oxidative folding. The disulfide folding pathway is characterized and defined by the heterogeneity and structures of these isomers that accumulate along the process of oxidative folding that leads to the formation of the single native isomer containing the three native disulfide bonds.

This technique of oxidative folding, first applied to the study of bovine pancreatic trypsin inhibitor (BPTI) (15, 22, 40) and ribonuclease A (35, 37, 41), was subsequently employed by many different laboratories to characterize the folding mechanism of numerous disulfide proteins (1, 3, 5–8, 10–12, 14, 19, 24, 25, 33, 36, 43). The results obtained so far have revealed a broad diversity and versatility of the mechanism of oxidative folding, which is displayed by both folding pathways and folding kinetics. Even among small proteins with comparable size and the same number of disulfide bonds, folding pathways may vary significantly. These differences are mainly manifested by: (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 (15, 22, 40) and hirudin (7, 14) (both 3-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 1- and 2-disulfide intermediates that adopt native disulfide bonds and native-like structures. Out of 74 possible disulfide isomers, only five to six 1- and 2-disulfide intermediates were shown to populate along the folding pathway of BPTI and all of them were found to adopt native disulfide bonds (40). By contrast, the folding intermediates of hirudin consist of a highly heterogeneous population of 1- and 2-disulfide species. Well-populated folding intermediates adopting native disulfides are absent in the case of hirudin. Scrambled 3-disulfide isomers, not found in BPTI, were shown to serve as folding intermediates of hirudin (14). Then there are protein models that feature folding pathways exhibited by both BPTI and hirudin. Epidermal growth factor (EGF) (12), tick anticoagulant peptide (8), and leech carboxypeptidase inhibitor (LCI) (36) are three examples. For example, the folding pathway of TAP (3 disulfides) comprises heterogeneous populations of 1-disulfide, 2-disulfide, and 3-diuslfide (scrambled) intermediates, as well as two predominant 1- and 2-disulfide intermediates containing exclusively native disulfide bonds.

PROTEINS ALSO FOLD VIA DISTINCT MODELS

The mechanism of protein folding is also defined by distinct models (17). 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 for establishing a general model of protein folding. Two major models have been put forward to account for the mechanism of protein folding. The **framework model** (4, 27, 28, 34) proposes that secondary structures (α-helix and β-strand, etc.) form first during the early stage of folding, which is followed by docking and packing of preformed secondary structural units to form the native tertiary structure. The **model of hydrophobic collapse** (18, 26, 38) 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 organization of tertiary structure is initiated.

Results of studies accumulated so far, nonetheless, have shown 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 another word, folding of most proteins follows the middle-of-the-road between the **framework**

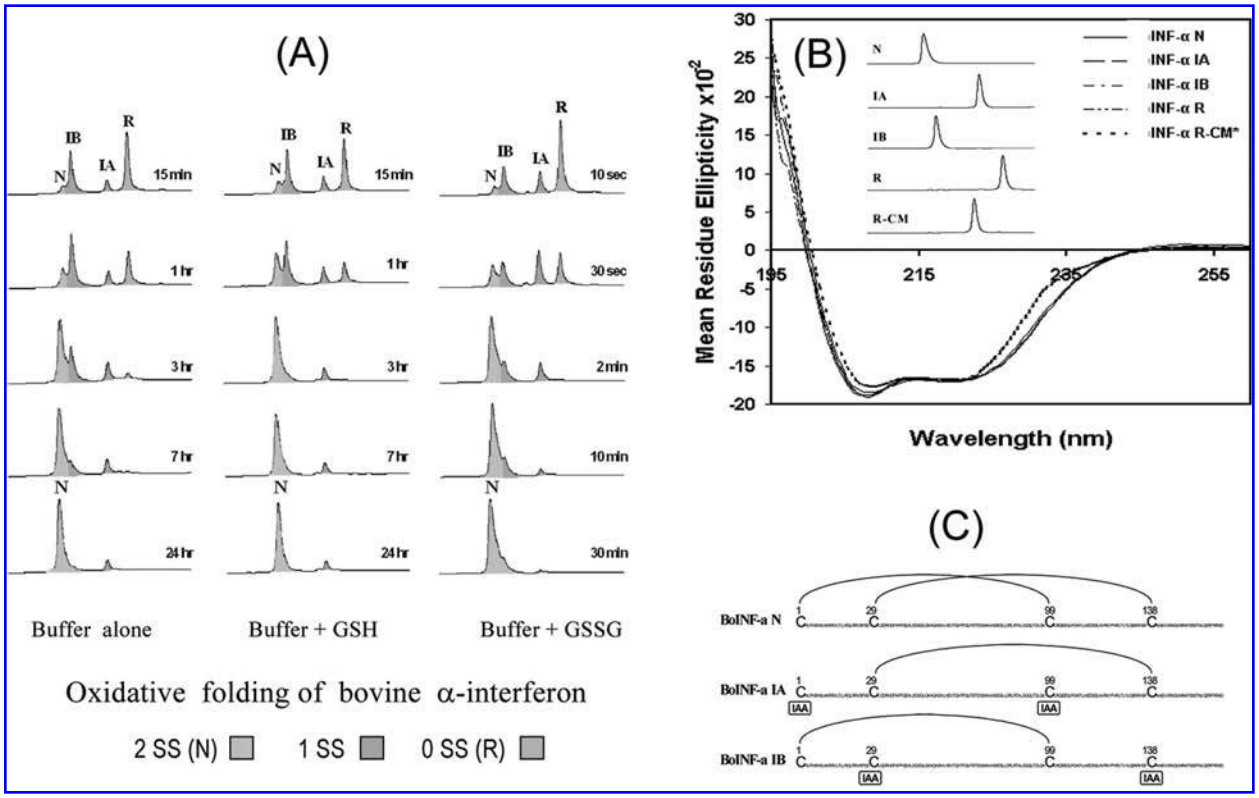

FIG. 1. Oxidative folding of INF-α. (**A**) HPLC profiles of the intermediates of oxidative folding of reduced denatured INF-α. Folding was performed at 22°C in the Tris-HCl buffer (0.1 *M*, pH 8.4) alone; in the same Tris-HCl buffer containing GSH (1 m*M*); and in the same Tris-HCl buffer containing GSSG (0.5 m*M*). Intermediates of folding were withdraw at different time points, quenched with an equal volume of 4% aqueous TFA and analyzed by reverse-phase HPLC using the following conditions. Solvent A was water containing 0.1% trifluoroacetic acid. Solvent B was acetonitrile/water (9:1 vol/vol) containing 0.086% trifluoroacetic acid. The gradient was 40–60% B linear in 60 min. The flow rate was 0.5 ml/min. Column was Vydac C18 for peptides and proteins, 4.6 mm $\times$ 5 μm. Column temperature was 23°C. "R" and "N" indicate the elution positions of the fully reduced and the native INF-α, respectively. "IA" and "IB" denote two major 1-disulfide folding intermediates. (**B**) Far UV CD spectra of five disulfide isomers of INF-α. They were purified by HPLC, reconstituted in 0.5% aqueous TFA with a final protein concentration of 0.5 mg/ml, and analyzed for their CD spectra using a JASCO J-715 Spectropolarimeter. Samples were immediately analyzed by HPLC following CD recording (*inset*). "N" is native INF-α. "IA" and "IB" are two predominant 1-disulfide folding intermediates. "R" is fully reduced INF-α with four free cysteines. "RCM" is reduced-carboxymethylated INF-α. (**C**) The disulfide connectivity of major folding intermediates of INF-α. IA and IB were purified from HPLC, modified with iodoacetamide, freeze dried, and digested with trypsin. The native INF-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. The data obtained from the analysis of tryptic peptides lead to the conclusion of two native disulfide bonds present in INF-α-IA (Cys^{29}–Cys^{138}) and INF-α-IB (Cys^{1}–Cys^{99}) (30).

and **hydrophobic collapse** models. Uversky and Fink analyzed data on the conformational properties of 41 native and partially folded states and concluded there is a valid correlation between the increase of secondary structure and the decrease of hydrodynamic volume during folding (39). Essentially, they found no evidence among analyzed proteins for 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 of increasing length, the secondary structure were observed only after residues that make long range interaction appear (21). These studies has led to the proposal of a new unifying mechanism of folding, the **nucleation–condensation model** (17, 20), which invokes the importance of interplay and 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, most studies aimed at elucidating folding model are not accompanied by thorough analysis of folding pathway.

So far, our knowledge relating to the folding model has been largely acquired by the ***conventional methods*** of unfolding–refolding. Using this conventional approach, the protein first unfolds in the presence of strong denaturant (*e.g.*, 8 *M* urea or 6 *M* GdmCl) or by extreme pH and temperature. Following the removal of denaturant (*e.g.*, by gel filtration, dilution, or dialysis), pH jump, or temperature jump, denatured proteins usu-

ally refold spontaneously to form the native structure. The pathway of protein refolding is then monitored by the mechanism of restoration of some physicochemical signals that distinguishes the native and unfolded states. The most commonly used signals are spectra of fluorescence, circular dichroism, infrared, ultraviolet, and NMR coupled with amide proton exchange. This method is most versatile and can be practically applied to study folding behaviors of any protein. Unlike the technique of oxidative folding, it does not permit, in most cases, isolation of folding intermediates.

CONCURRENT ANALYSIS OF FOLDING PATHWAYS AND FOLDING MODELS USING THE TECHNIQUE OF OXIDATIVE FOLDING

The interaction and formation of secondary and tertiary structures during the folding has bearings on the pathway of protein folding. Theoretically, a protein that folds by a *framework model* is likely to undergo limited folding intermediates adopting mostly native-like structures, due to the hierarchical packing of preformed structural units. In contrast, a protein folding by the *hydrophobic collapse model* is more likely to exhibit heterogeneous folding intermediates comprising mainly non-native structures, because of the nonspecific packing of polypeptide chain during the early stage of folding. Demonstration of the link between the folding pathway and folding model is essential to a thorough understanding of the mechanism of protein folding. However, experimental data supporting such connections are largely absent.

The key to the discrimination between these three different folding models (*framework model*, *hydrophobic collapse model,* and *nucleation–condensation model*) (17, 20, 39) is our technical capability to measure the state of secondary structure and tertiary structure (or compactness) of a protein at different stages of folding. Conclusions and reviews presented by Uversky and Fink (39) 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 few intermediates present at the equilibrium unfolding/refolding were measured for their secondary structure and compactness. A more comprehensive correlation of these two structural properties, and thus a better characterization of the 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 requirement. The method of oxidative folding provides advantages of analyzing the folding intermediates not equally shared by the conventional unfolding–refolding technique. (a) The chemical process of oxidative folding can be quenched at any time point by sample acidification or chemical modification. (b) Both acid and chemically trapped folding intermediates (disulfide isomers) can be isolated by liquid chromatography for further structural characterization. (c) Acid-trapped intermediates are amenable to stop/go folding experiments. They can be isolated and reconstituted in the folding buffer to permit continuation of folding. This allows identification of their kinetic role in the pathway of folding. For each protein, folding intermediates can be trapped at different stages of folding as desired. These intermediates can be analyzed for their heterogeneity, structure, and kinetic property to establish the folding pathway. The same set of intermediates can be further analyzed for their amount of secondary structure, degree of compactness, and content of tertiary structure in order to classify the folding model.

OXIDATIVE FOLDING OF BOVINE α-INTERFERON, A CASE OF FRAMEWORK MODEL

Intermediates of oxidative folding of INF-α comprise exclusively native disulfide bonds

α-Interferon (NF-α) is a single polypeptide protein containing 166 amino acids, two native disulfide bonds (Cys^{1}–Cys^{99}; Cys^{29}–Cys^{138}) and five stretches of α-helical structure (29, 31, 42). The pathway of oxidative folding of INF-α has been investigated (30). Reduced-denatured INF-α was allowed to refold in the Tri-HCl buffer (pH 8.4) in the absence and presence of redox agents (GSH, GSSH). Folding intermediates were quenched at different time points by sample acidification and analyzed directly by HPLC (Fig. 1A). Of the six 1-disulfide isomers and two 2-disulfide isomers that may serve as folding intermediates of INF-α, only two fractions of intermediates were observed. They are designated as IA and IB. The pattern of folding intermediates is not affected by the presence of redox agent. IA and IB were isolated, modified with iodoacetamide, and analyzed by MALDI mass spectrometry. The results reveal that both IA and IB are 1-disulfide isomers of INF-α.

Carboxymethylated IA and IB were further treated with trypsin. Tryptic peptides were isolated by HPLC and analyzed by Edman amino acid sequencing and mass spectrometry in order to identify peptides that contain either disulfide bonds or carboxymethylated cysteine. The results show that IA comprises Cys^{29}–Cys^{138} and IB contains Cys^{1}–Cys^{99}, both are native disulfide bonds of INF-α (Fig. 1C). Thus, of the eight possible 1- and 2-disulfide isomers, only two native-like 1-disulfide isomers INF-α (Cys^{1}–Cys^{99}) and INF-α (Cys^{29}–Cys^{138}) predominate as intermediates along the folding pathway (30).

Secondary structures of Bo-INF-α fold rapidly before formation of disulfide bond

Aside from predominance of native-like folding intermediates, the α-helical structure of INF-α was shown to fold almost quantitatively before any detectable disulfide bond is formed (30). This is demonstrated by the observation that fully reduced INF-α (starting material of oxidative folding) and reduced carboxymethylated INF-α both exhibit content of α-helical structure indistinguishable form that of native INF-α (Fig. 1B). The two major folding intermediates, IA and IB, also display similar content of α-helical structures (Fig. 1B). In a separate experiment, it was shown that α-helical structures of fully reduced INF-α fold completely within 1 min as soon as the denaturant (GdmCl) is diluted from 6 to 1 *M*.

The folding mechanism of INF-α is thus consistent with the *framework model*, in which secondary structures fold first, followed by docking (compaction) of pre-formed secondary structural elements. This accounts for the predominance of native-like intermediates populated along the folding pathway of INF-α.

OXIDATIVE FOLDING OF HIRUDIN, A CASE OF HYDROPHOBIC COLLAPSE MODEL

Intermediates of oxidative folding of hirudin are highly heterogeneous

Hirudin is a leech-derived thrombin-specific inhibitor and a small β-sheet protein (23). It consists of a structurally defined N-terminal core domain (49 amino acids) stabilized by the three native disulfide bonds and a disordered C-terminal tail (16 amino acids). The pathway of oxidative folding of the hirudin core domain has been investigated in detail in our laboratories (7, 14). Reduced denatured hirudin was allowed to refold in the Tri-HCl buffer (pH 8.4) in the absence and presence of GSH or GSSG. The process of folding was quenched at different time points by sample acidification and analyzed by HPLC (Fig. 2A). The data reveal an exceedingly high heterogeneity of folding intermediates of hirudin. Of the 60 possible 1- and 2-disulfide isomers that may serve as folding intermediates, at least 30 fractions have been identified. Among the 14 possible 3-disulffide scrambled isomers (designated as X-hirudins), 11 are present as folding intermediates and structurally characterized (Fig. 2A and C).

These results thus indicate that folding of hirudin undergoes an initial stage of nonspecific packing which leads to the for-

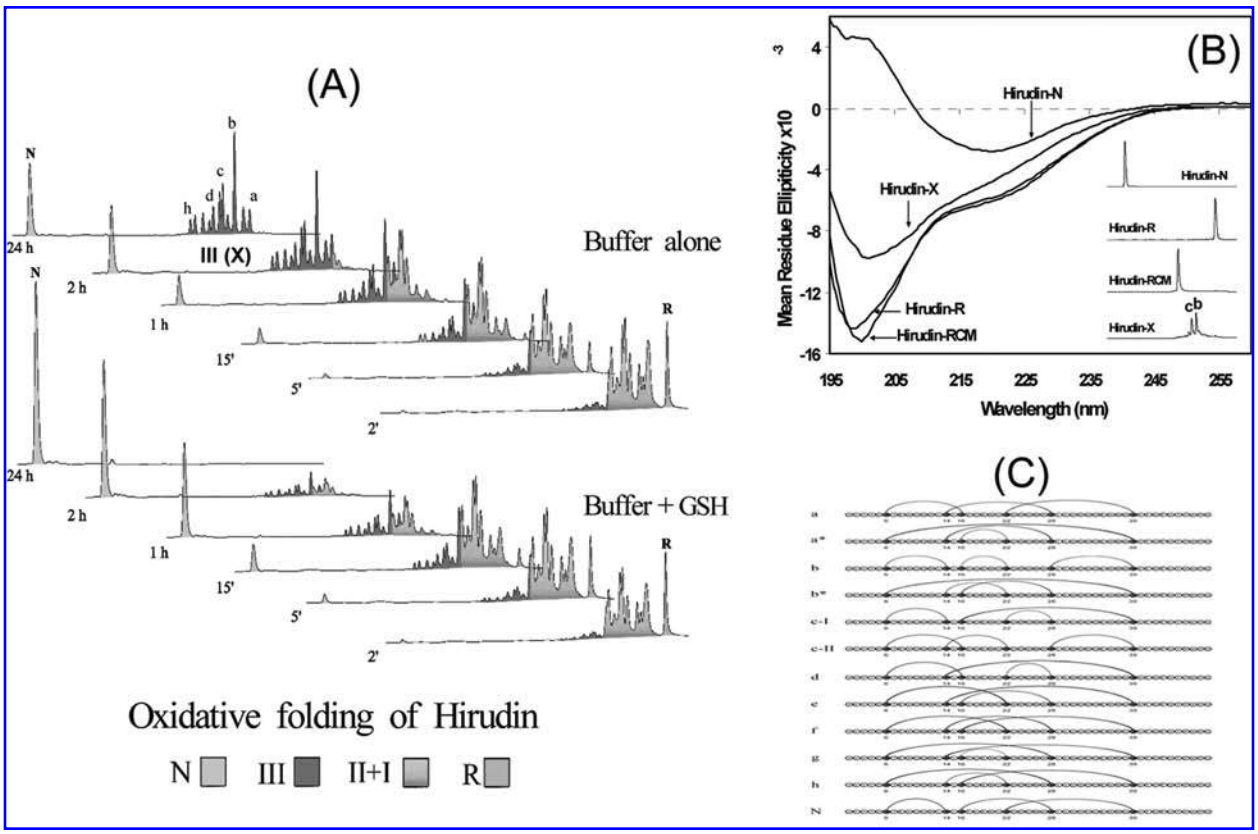

FIG. 2. Oxidative folding of hirudin core domain. (**A**) HPLC profiles of the intermediates of oxidative folding of hirudin. Folding was performed at 22°C in the Tris-HCl buffer (0.1 *M*, pH 8.4) alone; and in the same Tris-HCl buffer containing GSH (1 m*M*) Intermediates of folding were withdraw at different time points, quenched with an equal volume of 4% aqueous TFA, and analyzed by reverse-phase HPLC using the following conditions. Solvent A was water containing 0.1% trifluoroacetic acid. Solvent B was acetonitrile/water (9:1 vol/vol) containing 0.086% trifluoroacetic acid. The gradient was 14–36% B linear in 60 min. The flow rate was 0.5 ml/min. Column was Vydac C18 for peptides and proteins, 4.6 mm $\times$ 5 μm. Column temperature was 22°C. "R" and "N" indicate the elution positions of the fully reduced and the native hirudin, respectively. I + II indicate collectively 1- and 2-disulfide intermediates. Scrambled 3-disulfide intermediates (III) are also designated as X-hirudins and denoted from *a* to *h*. (**B**) Far UV CD spectra of five disulfide isomers of hirudin. They were purified by HPLC, reconstituted in 0.5% aqueous TFA with a final protein concentration of 0.5 mg/ml, and analyzed for their CD spectra using a JASCO J-715 Spectropolarimeter. Samples were immediately analyzed by HPLC following CD recording (*inset*). "N" is native hirudin. "R" is fully reduced hirudin with six free cysteines. "RCM" is reduced-carboxymethylated hirudin. "X" is mixture of X-hirudins. (**C**) The disulfide connectivity of 11 isomers of X-hirudins. Determination of disulfide connectivity of these X-hirudins has been documented (9, 13).

mation of heterogeneous X-hirudins as essential folding intermediates. This is followed by disulfide shuffling and reorganization of X-hirudins to attain the native hirudin (7). The final stage of X-hirudins ➪ N-hirudin conversion requires the presence of free thiols as catalyst. When folding of hirudin was performed in the buffer alone, air oxygen catalyzes disulfide formation in a slow motion and free cysteines of 1- and 2-disulfide intermediates act as thiol catalyst to promote X-hirudins ➪ N-hirudin conversion during the early stage of folding. As the folding progresses, more disulfide bonds are formed and free cysteines are depleted, therefore X-hirudins become entrapped, unable to convert to the N-hirudin due to the absence of thiol catalyst (Fig. 2A, top panel). The inclusion of GSH (1 m*M*) in the folding buffer facilitates disulfide shuffling of X-hirudins and ensures the completion of hirudin folding (Fig. 2A, bottom panel). When folding was carried out in the same buffer containing GSSG (2 m*M*), a rapid formation of X-hirudins and a slow X-hirudins ➪ N-hirudin conversion was observed. Numerous disulfide proteins display properties of oxidative folding similar to that of hirudin (6, 8, 11).

The formation of secondary structure and tertiary structure of hirudin occurs at the final stage of oxidative folding

Disulfide isomers of hirudin, including the fully reduced hirudin (R-hirudin, the starting material of oxidative folding), the reduced-carboxymethylated hirudin (RCM-hirudin), a mixture of X-hirudins and the N-hirudin were analyzed for their secondary structure by far UV CD scanning (Fig. 2B). The CD spectrum of N-hirudin displays a single minimum at ~218 nm, a unique CD signal for proteins rich in β-sheet structure. In contrast, both R- and RCM-hirudins exhibit a strong dichroic band at 198–200 nm, consistent with the random-coil structure. X-Hirudins, the essential folding intermediates, also exhibit a strong minimum near 200 nm. These data thus indicate that formation of the secondary structure (β-sheet) occurs mainly at the final stage of hirudin folding, concurrent with the restructuring of X-hirudins and the appearance of native tertiary structure of hirudin.

The folding mechanism of hirudin thus represents an example that is compatible with or close to the *hydrophobic collapse model*, in which hydrophobic interaction accounts for the major force driving the rapid and nonspecific packing of the polypeptide during the early stage of folding, followed by fine tuning and restructuring of the tertiary and secondary structures within the confined volume to reach the native structure.

CONCLUDING REMARKS

We have briefly reviewed the current status of diversity of disulfide folding pathways and protein folding models, and pointed out that a comprehensive understanding of protein folding will require thorough characterization of both folding pathway and folding model. We have also suggested that the method of oxidative folding represents a practical tool to attain both set of structural information because of the feasibility of trapping a large numbers of folding intermediates for further characterization. Using this approach, our laboratory has shown the connections between folding pathway and folding model of two distinct proteins, hirudin and bovine INF-α. It is also understandable that a more meticulous characterization of folding pathway and folding model will demand application of an array of contemporary technologies, including stop-flow CD and NMR.

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ABBREVIATIONS

BPTI, bovine pancreatic trypsin inhibitor; CD, circular dichroism; CI2, chymotrypsin inhibitor 2; EGF, epidermal growth factor; GdmCl, guanidinium hydrochloride; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high performance liquid chromatography; Hir, hirudin; INF-α, α-interferon; LCI, leech carboxypeptidase inhibitor; MALDI, matrix-assisted laser desorption ionization; RCM, reduced and carboxymethylated; TAP, tick anticoagulant peptide.

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Address reprint requests to:
Jui–Yoa Chang
Institute of Molecular Medicine
1825 Pressler Street
Houston, TX 77030

E-mail: Rowen.Chang@uth.tmc.edu

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