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FORUM REVIEW ARTICLE

Distinct Folding Pathways of Two Homologous Disulfide Proteins: Bovine Pancreatic Trypsin Inhibitor and Tick Anticoagulant Peptide

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Abstract

The folding pathways of disulfide proteins vary substantially (Arolas *et al.*, *Trends Biochem Sci* 31: 292–301, 2006). The diversity is mainly manifested by (a) the extent of heterogeneity of folding intermediates, (b) the extent of presence of native-like intermediates, and (c) the variation of folding kinetics. Even among structurally similar proteins, the difference can be enormous. This is demonstrated in this concise review with two structurally homologous kunitz-type protease inhibitors, bovine pancreatic trypsin inhibitor and tick anticoagulant peptide, as well as a group of cystine knot proteins. The diversity of their folding mechanisms is illustrated with two different folding techniques: (a) the conventional method of disulfide oxidation (oxidative folding), and (b) the novel method of disulfide scrambling (Chang, *J Biol Chem* 277: 120–126, 2002). This review also highlights the convergence of folding models concluded form the conventional conformational folding and those obtained by oxidative folding. *Antioxid. Redox Signal.* 14, 127–135.

Introduction

The pathway and mechanism of protein folding are inherently complex. This has been known from results of both conventional conformational folding (1,6,7,32,37,54) as well as oxidative folding of disulfide proteins (3,45). These two approaches differ by their methods of monitoring the folding. The mechanism of conformational folding is typically tracked by spectra of fluorescence, circular dichroism, infrared, and nuclear magnetic resonance (NMR) (34,35,40,55). The pathway of oxidative folding is followed and defined by disulfide bonding (26). Despite the distinct physicochemical signals, mechanism of both conformational and oxidative folding exhibit a high degree of diversity.

Diversity of protein conformational folding

There are two favored models of conformational folding. The framework model (40,41,48) proposes that secondary structures (α-helix, β-strand, *etc.*) form first during the early stage of folding. This is followed by docking and packing of preformed secondary structural units to form the native tertiary structure. The hydrophobic collapse model (6,31,39,57) 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.

The framework model and the hydrophobic collapse model apparently correspond to two extreme folding models. Results obtained from conformational folding of many proteins have shown that extreme mechanisms fitting either model are rare. 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. Uversky and Fink (58) have analyzed data on the conformational properties of 41 native and partially folded states and concluded a valid correlation between the increase of secondary structure and the decrease of hydrodynamic volume during folding. Essentially, they found little evidence among analyzed proteins for either

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compact intermediates lacking secondary structure or unfolded intermediates comprising highly ordered secondary structure. These studies have led to the proposal of a third folding model, the nucleation–condensation model (29,36), 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.

Diversity of protein oxidative folding

The pathway and mechanism of protein oxidative folding exhibit comparable extent of diversity as those observed in the conformational folding. This has been reviewed recently (3,45). There are two well-characterized extreme models of disulfide folding pathways, represented by two 3-disulfide proteins, bovine pancreatic trypsin inhibitor (BPTI) and hirudin, respectively. The folding pathway of the BPTI model (27,28,38,60) is characterized by a limited number of folding intermediates adopting exclusively native-like structures; a folding mechanism that resembles that of framework model. In contrast, the folding pathway of the hirudin model (11,15,23,24) is illustrated by a rapid accumulation of fully oxidized, compact isomers as intermediates; a folding mechanism that bears a resemblance to that of hydrophobic collapse model. In addition, there are protein models that exhibit folding properties of both BPTI and hirudin. The folding mechanisms of these proteins, including epidermal growth factor (22,62) and RNase A (46,49,53,61), are consistent with the nucleation–condensation model (29,36). Thus, the compatibility between the models of conformational folding and oxidative folding supports a unified, although diverse mechanism of protein folding, independent of the distinct physicochemical signals utilized to monitor the folding.

Scope of this review

In this concise review, we intent to further highlight the diversity of protein folding mechanism by presenting the divergent folding pathways of structurally similar proteins. It will narrow the subjects to small homologous disulfide proteins elucidated by the technique of disulfide oxidation (26) and disulfide scrambling (18,14).

Methods for Analysis of Folding Pathway of Disulfide Proteins

The folding pathway of disulfide proteins can be elucidated by three distinct methods.

Conformational folding

This is the most commonly applied folding technique (7,32,37), for both disulfide and nondisulfide containing proteins. In this approach, native disulfide bonds are kept intact throughout the folding experiments. The step of unfolding is achieved by using denaturant, extreme pH, or temperature. The folding is then initiated by removal of denaturant (*e.g.*, by gel filtration, dilution, or dialysis), pH adjustment, or temperature jump. Most unfolded proteins usually refold spontaneously to form the native structure. The pathway of protein refolding is then observed and characterized by the mechanism of restoration of selective physicochemical signals that differentiate between the native and unfolded states. These signals include spectra of fluorescence, circular dichroism, infrared, and NMR (34,35,40,55).

Oxidative folding

Application of this method is limited to disulfide containing proteins. In this approach, disulfide bonds of native proteins (N-protiens) are first reduced and denatured by a reducing agent (*e.g.*, dithiothreitol [DTT]) and a denaturant (*e.g.*, guanidine hydrochloride [GdmCl]). After removal of the reductant and denaturant (*e.g.*, by gel filtration or sample dilution), the reduced and denatured protein is allowed to refold in the presence of redox buffer *via* disulfide formation (oxidation) that leads to the formation of the species containing the intact native disulfide bonds. The disulfide folding pathway is then characterized by heterogeneity, structures, and kinetic properties of disulfide isomers accumulated along the folding pathway, which are fractionated, quantified, and analyzed by high-performance liquid chromatography (HPLC). This technique, pioneered by Creighton (26–28), has been applied to the extensive elucidation of folding pathways of numerous disulfide containing proteins (3,45), including among many others, BPTI (27,28,38,60), ribonuclease A (46,49), and hirudin (11,24).

Scrambling folding

Application of this method is also limited only to the disulfide-containing proteins. It can be considered as a hybrid of conformational folding and oxidative folding. In this approach, a native protein is unfolded in the presence of denaturant and thiol catalyst (*e.g.*, 0.1 m*M* β-mercaptoethanol). Under these conditions, the native protein unfolds by shuffling its native disulfide bonds and converts to a mixture of fully oxidized scrambled isomers that are amenable to fractionation, isolation, and further structural analysis (20,12). These unfolded isomers are collectively termed as X-isomers. The heterogeneity of X-isomers is determined by the number of disulfide bonds. For proteins that contain 3- and 4-disulfide bonds, there are 14 and 104 possible unfolded states of X-isomers, respectively. The folding experiment is initiated by incubation of isolated X-isomers in the alkaline buffer containing a thiol catalyst. Under these conditions, all X-isomers refold (also *via* disulfide shuffling) to form the native structure. The folding pathway is then characterized by the heterogeneity, structures, and kinetic properties of X-isomers accumulated along the folding pathway (18,14).

The technique of scrambling folding differs from that of oxidative folding in one major aspect—the nature of the starting material of the folding experiment. The starting material of oxidative folding is fully reduced protein (R-isomer or R-protein), which can be generated by using reducing agent alone, in the absence of denaturant or heat (reductive unfolding). In contrast, preparation of the starting material of scrambling folding (X-isomers or X-protein) requires strong denaturing conditions (*e.g.*, 6 *M* GdmCl), similar to that used in preparation of the starting material of conformational folding. In addition, GdmCl-unfolded proteins often still comprise heterogeneous conformational isomers possessing diverse states of free energy (33,56). This has been verified in the production of X-isomers using the method of disulfide

scrambling (20,12). These diverse X-isomers can be fractionated and isolated. Thus, unlike the method of oxidative folding, scrambling folding can be initiated with a structurally defined X-isomer that possesses the highest free energy among all denatured isomers (16,18,14).

BPTI and Tick Anticoagulant Peptide Are Two Structurally Homologous Disulfide Proteins

BPTI and tick anticoagulant peptide (TAP) both belong to the family of kunitz-type protease inhibitors (42). TAP is a factor-Xa-specific inhibitor isolated from the blood-sucking tick *Ornithodoros moubata* (59). The two proteins are nearly identical in size (58 a.a. for BPTI *vs.* 60 a.a. for TAP). They also share modest sequence homology (44), the same disulfide pattern (52), similar content of secondary structures (2), and almost superimposable three-dimensional structures (Fig. 1) (44). Despite the overall similarity of their structures, BPTI and TAP fold *via* very different pathways. This will be illustrated here using the two folding techniques specific for disulfide proteins, folding *via* disulfide oxidation and disulfide scrambling.

Diversity of Folding Pathways of BPTI and TAP Elucidated by the Method of Disulfide Oxidation (Oxidative Folding)

BPTI is the first and most extensively investigated model for elucidation of disulfide folding pathway. The details of BPTI folding, pioneered by Creighton, have been documented and reviewed in a number of articles (27,28,38,60). In short, oxidative folding of BPTI proceeds *via* a limited number of intermediates comprising mainly native disulfide bonds. Out of the 74 possible disulfide isomers (15-1SS, 45-2SS, and 14-3SS) that may serve as folding intermediates, only five (two 1SS and three 2SS) were shown to accumulate along the folding pathway of BPTI and all adopt native disulfide bonds. Folding of BPTI proceeds *via* two predominant native 1SS intermediates (Cys^{30}-Cys^{51} and Cys^{5}-Cys^{55}), followed by formation of two 2SS kinetic traps (Cys^{30}-Cys^{51}, Cys^{14}-Cys^{38} and Cys^{5}-Cys^{55}, Cys^{14}-Cys^{38}) and one 2SS intermediate (Cys^{5}-Cys^{55}, Cys^{30}-Cys^{51}), which serves as a direct precursor of the native BPTI (Cys^{5}-Cys^{55}, Cys^{30}-Cys^{51}, Cys^{14}-Cys^{38}). The flow chart of folding pathway of BPTI and the original HPLC data of intermediates analysis are shown in Figure 2A and 2B, respectively. The unique folding pathway of BPTI clearly indicates that stable subdomain structures dictate the formation of native-like intermediates and limit the heterogeneity of folding intermediates.

Thus, the folding mechanism of BPTI is congruent with the framework model (29), in which secondary structures (α-helix, β-strand, *etc.*) form early during the folding, which is followed by docking and packing of preformed secondary structural units to form the native tertiary structure.

The folding pathway of TAP differs significantly from that of BPTI. The folding intermediates and folding pathway of TAP are far more complex than that of BPTI (Fig. 2A, C). The folding intermediates consist of a highly heterogeneous population of 1SS and 2SS isomers (16,13). Although one 1SS (Cys^{33}-Cys^{55}) and one 2SS (Cys^{33}-Cys^{55}, Cys^{15}-Cys^{39}) intermediate containing exclusively native disulfide bonds are also predominant along the pathway (16), they are among at least 25–30 species of well populated intermediates identified. Most importantly, scrambled 3SS X-TAP isomers, not observed with BPTI folding, were shown to serve as essential folding intermediates of TAP (Fig. 2C) (13). Among the 14 possible X-TAP isomers, four have been found to predominate at the late stage of oxidative folding (see fractions g, f, k, and d in Fig. 2C). Their disulfide structures were determined and shown in Figure 4. Indeed, the folding pathway of TAP resembles that of hirudin (11,24). The mechanism consists of an initial stage of nonspecific packing that leads to the formation of scrambled 3SS isomers as folding intermediates. This is followed by consolidation of scrambled 3SS isomers to reach the native structure *via* disulfide shuffling.

Thus, the folding mechanism of TAP is more compatible with the hydrophobic collapse model (29,31), in which a rapid hydrophobic collapse 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.

Diversity of Folding Pathways of BPTI and TAP Elucidated by the Method of Disulfide Scrambling

The technique of disulfide scrambling permits folding experiments to be initiated with an isolated X-isomer that represents the most extensively unfolded state and possesses the highest free energy among all unfolded X-isomers (18,14). Such X-isomers can be identified through systematic unfolding experiments with increasing strength of denaturant using

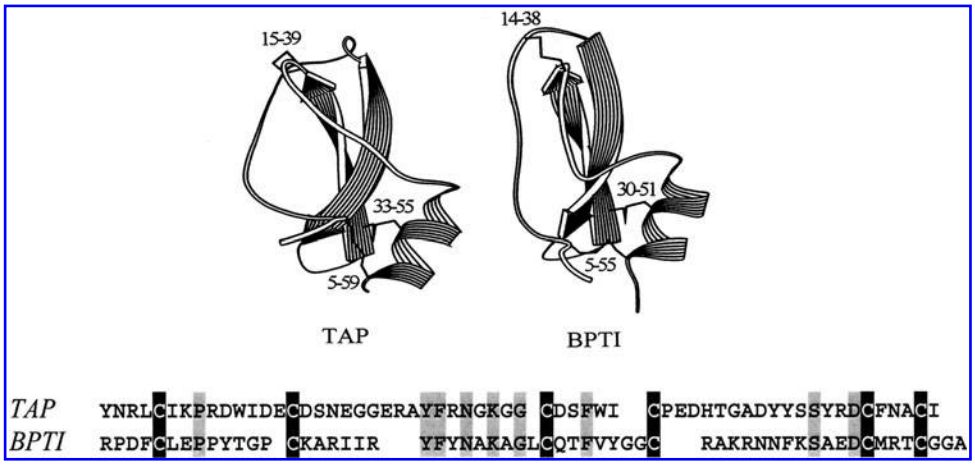

FIG. 1. Structures of BPTI and TAP. (Top) Schematic presentation of three-dimensional structures of native TAP and BPTI. Both proteins have a similar content of secondary structures (α-helix and β-sheet) and adopt comparable three-dimensional conformation (44). The three corresponding native disulfides are numbered. (Bottom) Amino acid sequence homology of TAP and BPTI. BPTI, bovine pancreatic trypsin inhibitor; TAP, tick anticoagulant peptide.

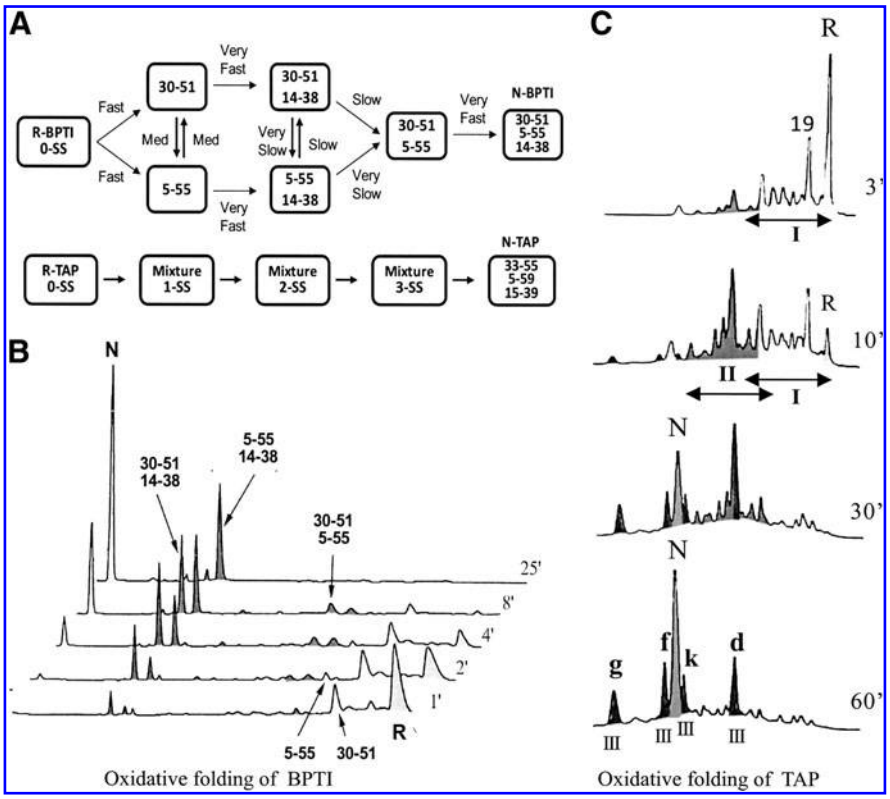

FIG. 2. Pathway of oxidative folding of BPTI and TAP. (A) The disulfide folding pathways of BPTI (taken from Ref. 60) and TAP. All five predominant folding intermediates of BPTI, including two 1SS and three 2SS isomers, comprise native disulfide bonds. About 25 fractions of heterogeneous 1SS, 2SS, and 3SS intermediates were identified in the pathway of TAP folding. **(B)** HPLC analysis of acid-trapped intermediates of BPTI oxidative folding (taken from Ref. 60). Folding experiment was carried out at 25°C in the Tris-HCl buffer (pH 8.7) containing oxidized glutathione (0.15 m*M*). The disulfide bonds are presented by sequence positions of cysteines. **(C)** HPLC analysis of acid-trapped intermediates of TAP oxidative folding (16,13). Folding experiment was carried out at 22°C in the Tris-HCl buffer (pH 8.4) containing oxidized glutathione/reduced glutathione (0.5 m*M*/1.0 m*M*). The distribution of 1SS(I), 2SS(II), and 3SS(III) intermediates are indicated with double-headed arrows. The disulfide structures of four major 3SS(III) intermediates, X-TAP-d, X-TAP-k, X-TAP-f, and X-TAP-g, are presented in Figure 4. N, native protein; 1SS, 1-disulfide isomer; 2SS, 2-disulfide isomer; 3SS, 3-disulfide isomer; HPLC, high-performance liquid chromatography; R, fully reduced protein.

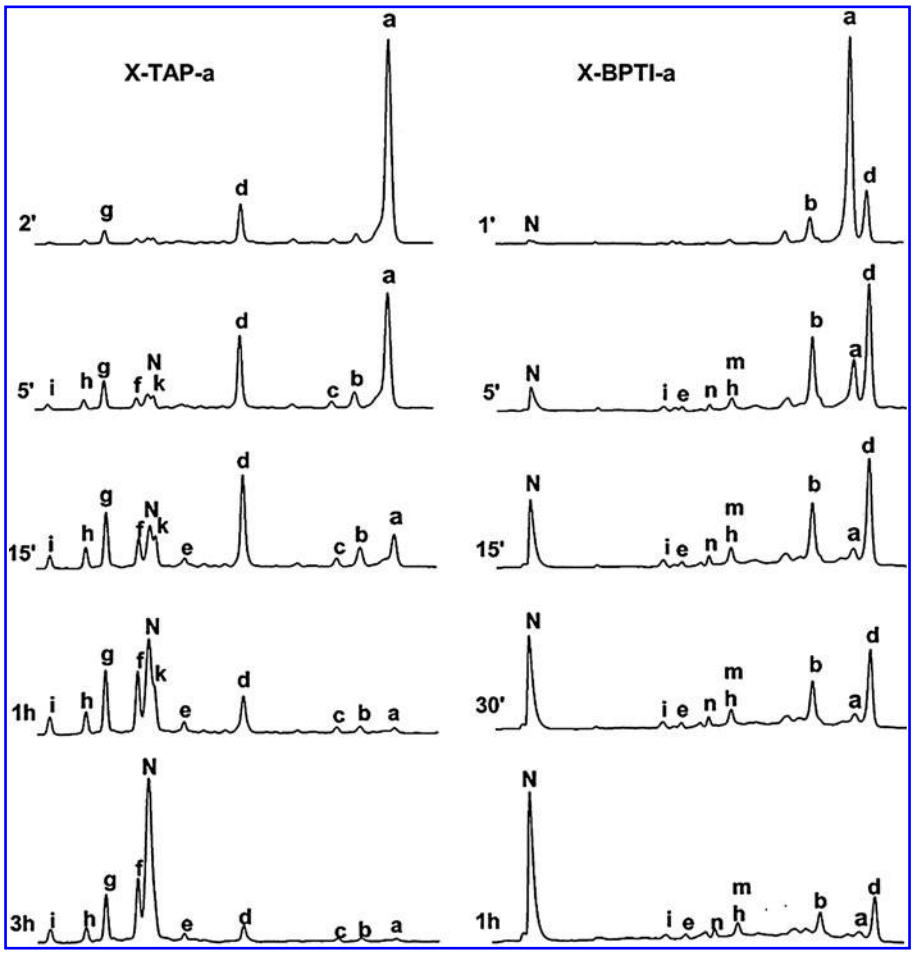

FIG. 3. Pathways of folding of X-TAP-a and X-BPTI-a through disulfide scrambling. Folding experiments were carried out at 22°C in Tris-HCl buffer (pH 8.4) containing cysteine, 0.5 m*M* for X-TAP-a, and 0.1 m*M* for X-BPTI-a. The protein concentration was 0.5 mg/ml. Intermediates of folding were trapped by sample acidification (4% trifluoroacetic acid) and analyzed by HPLC (18). "N" indicates the elution position of the native protein. All folding intermediates are marked with lower case.

the technique of disulfide scrambling. In the cases of BPTI and TAP, these two specific X-isomers were identified as X-BPTI-a (17) and X-TAP-a (12), which share the same beads-form disulfide pattern (Fig. 4). X-BPTI-a and X-TAP-a were allowed to refold in parallel under identical conditions *via* disulfide shuffling to reach the native BPTI and TAP. Folding intermediates were trapped by acid quenching and analyzed by HPLC. The results are illustrated in Figure 3. Of the 13 possible X-isomers that may serve as folding intermediates, 7 were found in the folding pathway of X-BPTI-a → N-BPTI and 9 were found in the pathway of X-TAP-a → N-TAP. All folding intermediates have been isolated and characterized (18). Their disulfide structures are also shown in Figure 4.

Folding activity of both proteins appears to initiate at the C-terminal domain, resulting in rapid formation of "X-TAP-d" and "X-BPTI-d" during the early stage of folding (Fig. 3). However, this is essentially the only observed similarity. There are several striking dissimilarities that distinguish the folding pathway of X-BPTI-a from that of X-TAP-a. (a) One of the predominant BPTI intermediates X-BPTI-b (Cys^5-Cys^{14}, Cys^{30}-Cys^{51}, Cys^{38}-Cys^{55}) contains a native disulfide bond Cys^{30}-Cys^{51}, and constitutes about 34% of the total BPTI folding intermediates. The counterpart, X-TAP-b (Cys^5-Cys^{15},Cys^{33}-Cys^{55},Cys^{39}-Cys^{59}), is barely detectable in TAP folding. This difference may be due to the unique BPTI subdomain structure that stabilizes Cys^{30}-Cys^{51}, a similar mechanism that favors the formation of predominant 1SS intermediate (Cys^{30}-Cys^{51}) during the oxidative folding of BPTI (Fig. 2B). The same factor apparently does not exist in TAP structure. (b) Three X-TAP isomers (X-TAP-f, X-TAP-g, and X-TAP-k) sharing a stable non-native disulfide bond Cys^{15}-Cys^{33} are shown to act as kinetic traps of TAP folding. Their counterparts are completely absent in the BPTI folding pathway. (c) Folding intermediates of BPTI are energetically compartmentalized (Fig. 5), whereas most folding intermediates of TAP are interconvertible and exist in equilibrium (Fig. 6). This is demonstrated by the energy booth accommodating isomers X-BPTI-e, n, and m (18). Stop/go folding experiments show that X-BPTI-e, X-BPTI-n, and X-BPTI-m equilibrate rapidly among each other (Fig. 5), but these three isomers are unable to cross energy barriers and convert to X-BPTI-b, X-BPTI-d, or X-BPTI-i. Structurally, X-BPTI-e, n, and m share Cys^5-Cys^{30}, implying that stability of this non-native disulfide bond accounts for the energy compartment of these three isomers.

The remarkable diversity of folding mechanism between X-BPTI-a and X-TAP-a may provide an invaluable model in further understanding why structurally homologous proteins fold differently. Specifically, detailed NMR analysis can be

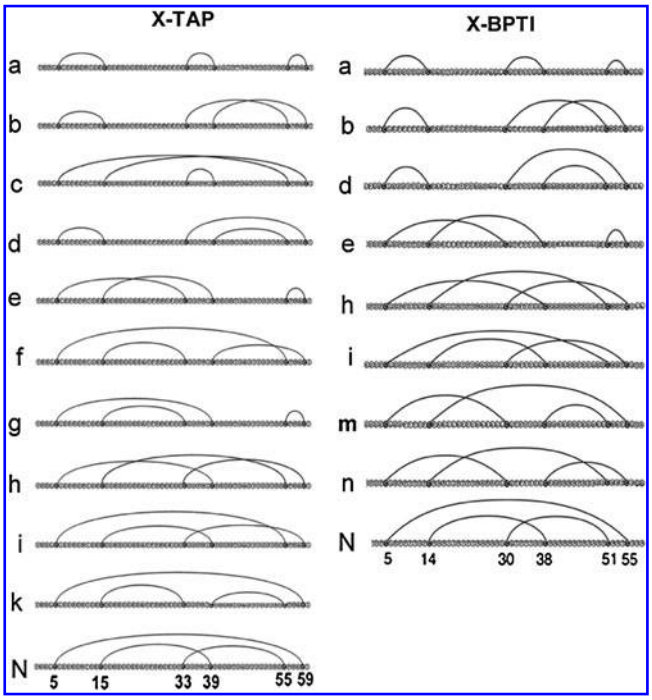

FIG. 4. Disulfide structures of isomers of X-TAP and X-BPTI. These isomers have been identified along the folding pathways of X-TAP-a and X-BPTI-a.

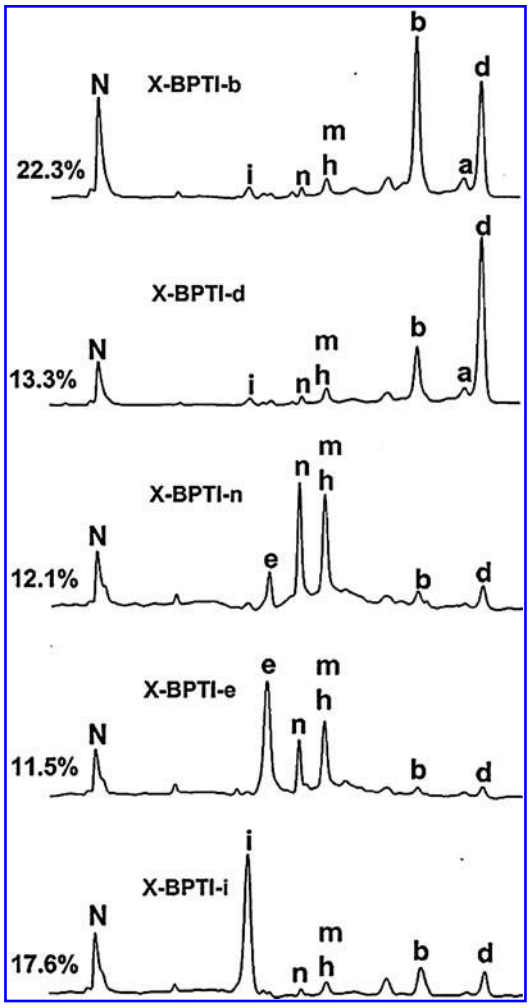

FIG. 5. Stop/Go folding experiments of isolated X-BPTI isomers. Five 3SS folding intermediates of X-BPTI-a (isomers b, d, n, e, and i) were purified by HPLC, freeze-dried, and allowed to resume the folding in Tris-HCl buffer (pH 8.4) containing cysteine (0.1 m*M*). Folding was performed at 22°C for 15 min, trapped by sample acidification (4% trifluoroacetic acid), and analyzed by HPLC (18). "N" indicates the elution position of the native BPTI. Numbers given at the left-hand side indicate the recovery of native BPTI.

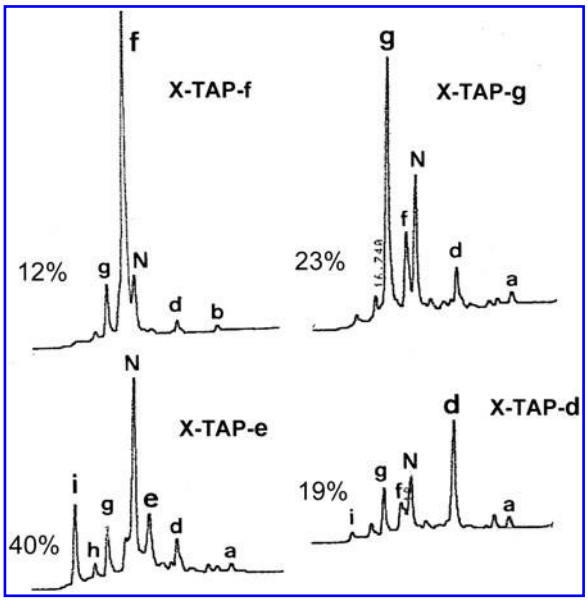

FIG. 6. Stop/Go folding experiments of isolated X-TAP isomers. Four 3SS folding intermediates of X-TAP-a (isomers d, e, f, and g) were purified by HPLC, freeze-dried, and allowed to continue the folding in Tris-HCl buffer (pH 8.4) containing cysteine (1 m*M*). Folding was performed at 22°C for 10 min, trapped by sample acidification, and analyzed by HPLC (18). "N" indicates the elution position of the native TAP. Numbers given at the left-hand side indicate the recovery of native TAP.

carried out with X-isomers that constitute the predominant intermediate (X-BPTI-b), the kinetic traps (X-TAP-f, X-TAP-g, and X-TAP-k), and the energy compartments (X-BPTI-e, X-BPTI-n, and X-BPTI-m). The results should provide structural basis as to how these intermediates are stabilized.

A Group of Homologous Cystine Knot Protease Inhibitors also Exhibit Diversity of Disulfide Folding Pathways

It is apparent that BPTI and TAP will not be the only examples. MCoTI-II, EETI-II, kalata B1, and AAI all belong to a group of homologous cystine knot protease inhibitors, comprising roughly 30 amino acids and 3 disulfide bonds. They share some sequence homology and similar disulfide pattern [1–4,2–5,3–6] (Fig. 7). The disulfide folding pathways of these four small proteins have been investigated in detail in the laboratory of Craik and Cemazar (8,10,25,30,43). MCoTI-II, EETI-II, and kalata B1-fold *via* pathways resembling that of BPTI, with an instant accumulation of single predominant 2SS intermediate comprising two native disulfide bonds. However, their folding kinetics are different. For MCoTI-II and EETI-II, both native 2SS intermediates fold to form corresponding native proteins directly (8), similar to the role of BPTI[Cys^5-Cys^{55}, Cys^{30}-Cys^{51}] in BPTI folding (Fig. 2). In the case of kalata B1, the native 2SS intermediate needs to unfold and revert back to the state of 1SS isomer in order to reach native kalata B1 (8). In a sharp contrast, AAI folds *via* the pathway similar to that of TAP. Oxidative folding of AAI is characterized by a high heterogeneity of 1SS, 2SS, and 3SS intermediates. Among the 14 possible 3SS X-isomers of AAI, seven have been isolated and structurally

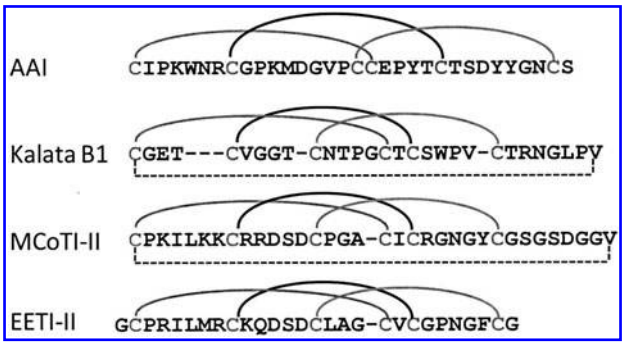

FIG. 7. Amino acid sequence and disulfide pattern of four cystine-knot protease inhibitors. Note that MCoTI-II and kalata B1are cyclic peptides (8).

characterized by Cemazar and colleagues (10). These results demonstrate that the diversity of disulfide folding pathways of structurally homologous proteins may be a common phenomenon.

The Underlying Cause for the Diversity of Disulfide Folding Pathway: A Protein May Fold *via* BPTI-Like Pathway or TAP-Like Pathway Depending on the Stability of Its Subdomain

The predominance of 1SS intermediate (Cys^{30}-Cys^{51}) (Fig. 2) and X-BPTI-b (Cys^5-Cys^{14}, Cys^{30}-Cys^{51}, Cys^{38}-Cys^{55}) (Fig. 3) along the folding pathways of BPTI suggest that structural elements stabilizing the subdomain comprising Cys^{30}-Cys^{51} bond play a critical role in dictating the folding pathway of BPTI. Similar structural features are apparently much less evident in the folding of TAP. Indeed, the stable subdomain of BPTI may also account for the distinct pathways of reductive unfolding of BPTI and TAP. The experiment of reductive unfolding is designed to evaluate the relative stability and interdependency of disulfide bonds in the native protein. The three native disulfide bonds of TAP were reduced by DTT in an all-or-none manner without accumulation of partially reduced intermediates, whereas those of BPTI were reduced in a sequential and stepwise manner, with preferential cleavage of Cys^{14}-Cys^{38} at a very low concentration of DTT (21,63). We have previously shown that there exists a striking correlation between the mechanism(s) of reductive unfolding and that of oxidative folding (21). Those with their native disulfide bonds reduced in a collective all-or-none manner tend to exhibit both a high degree of heterogeneity of folding intermediates and the accumulation of fully oxidized scrambled isomers along the folding pathway, such as TAP and hirudin. A sequential reduction of the native disulfide bonds is associated with the presence of predominant intermediates with native-like structures, such as BPTI.

To corroborate the hypothesis that the extent of stability of subdomain structure accounts for the divergent folding pathways of BPTI and TAP, we have analyzed oxidative folding of a 3-disulfide variant of α-lactalbumin (αLA-IIIA) (Fig. 8) (19,51). αLA-IIIA lacks one native disulfide bond Cys^6-Cys^{120} of αLA, which is reduced and carboxymethylated (see Fig. 8C). The remaining three native disulfide bonds of αLA-IIIA (Cys^{28}-Cys^{111}, Cys^{61}-Cys^{77}, Cys^{73}-Cys^{91}) adopt the disulfide pattern [1–6,2–4,3–5] similar to that of BPTI and TAP. Most importantly, αLA-IIIA also retains a *β*-sheet domain

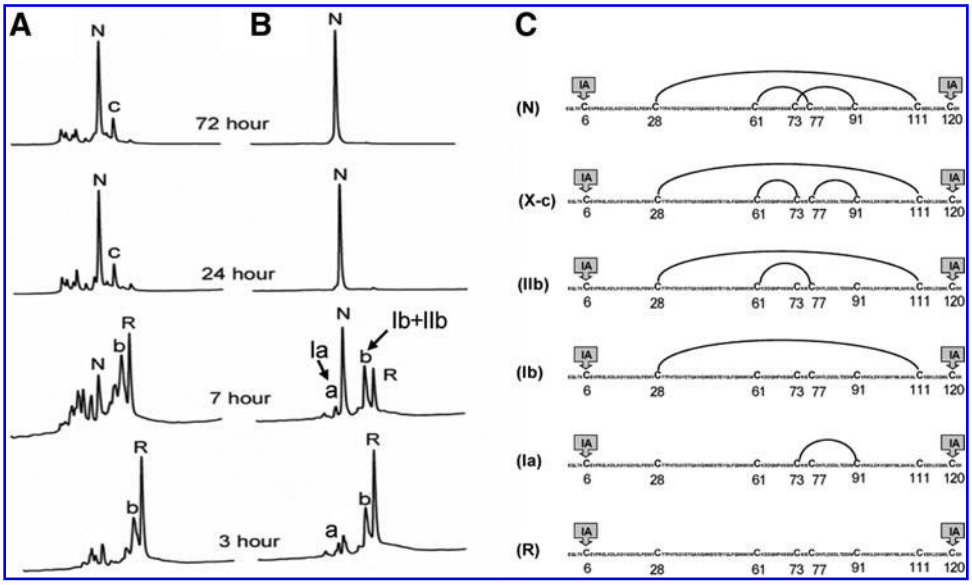

FIG. 8. Oxidative folding of a 3-disulfide variant of αLA, designated as αLA-IIIA (51). **(A)** Folding of fully reduced αLA-IIIA in Tris-HCl buffer (pH 8.4). **(B)** Folding of fully reduced αLA-IIIA in Tris-HCl buffer (pH 8.4) containing $CaCl_2$ (5 m*M*). Folding intermediates were trapped by sample acidification and analyzed by HPLC. Fraction "a" consists of single 1SS (Ia) intermediate. Fraction "b" consists of one 1SS (Ib) intermediate and one 2SS (IIb) intermediate. Fraction "c" contains single 3SS X-αLA-IIIA isomer (34). **(C)** Disulfide structures of isomers of αLA-IIIA. Cys^6 and Cys^{120} were coupled to IA. αLA, α-lactalbumin; IA, iodoacetate.

surrounding Cys^{61}-Cys^{77}, Cys^{73}-Cys^{91} that can be stabilized upon calcium binding. We demonstrated that αLA-IIIA can fold *via* either BPTI-like pathway or TAP-like pathway, depending on whether its subdomain is stabilized (19,50,51).

Fully reduced αLA-IIIA (R) was allowed to refold with and without stabilization of its *β*-sheet domain by calcium binding. The results showed that in the absence of $CaCl_2$ (Fig. 8A), the folding pathway of αLA-IIIA resembles that of TAP (16,13) and hirudin (11,24), which is characterized by a high heterogeneity of folding intermediates and the accumulation of scrambled 3SS isomers along the pathway. The fractions appeared in the 24 and 72 h samples are all 3SS isomers of αLA-IIIA. Among them, the disulfide structure of X-αLA-IIIA-c is known (Fig. 8C) (50). In the presence of $CaCl_2$ (Fig. 8B), αLA-IIIA folded *via* BPTI-like pathway (27,38,60), with limited intermediates comprising exclusively native disulfide bonds. Two 1SS and one 2SS intermediates connected by native disulfide bonds were identified (Fig. 8C).

Concluding Remarks

This diversity of disulfide folding pathway has been broadened during the past decade through analysis of oxidative folding of numerous small disulfide-rich proteins (3,45). It has been further highlighted by the significant differences of folding mechanisms observed among structurally similar proteins demonstrated in this review. It has been proposed (21) and experimentally demonstrated (19,51) that one of the underlying causes of the diversity is the relative stability of protein subdomains. This information, together with elucidation of new NMR structural data of several native-like folding intermediates (2a,4,5,9,47), has provided a more detailed picture of folding mechanism of disulfide proteins. Finally, it is important to stress that the diversity of protein oxidative folding is fundamentally consistent with that of conformational folding. The folding models of BPTI and TAP are compatible with the framework model and hydrophobic collapse model (29), respectively.

Acknowledgments

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Abbreviations Used

1SS = 1-disulfide isomer
2SS = 2-disulfide isomer
3SS = 3-disulfide isomer
αLA = α-lactalbumin
BPTI = bovine pancreatic trypsin inhibitor
DTT = dithiothreitol
GdmCl = guanidine hydrochloride
HPLC = high-performance liquid chromatography
IA = iodoacetate
N = native protein
NMR = nuclear magnetic resonance
R = fully reduced protein
TAP = tick anticoagulant peptide

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