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Diverse Pathways of Oxidative Folding of Disulfide Proteins: Underlying Causes and Folding Models

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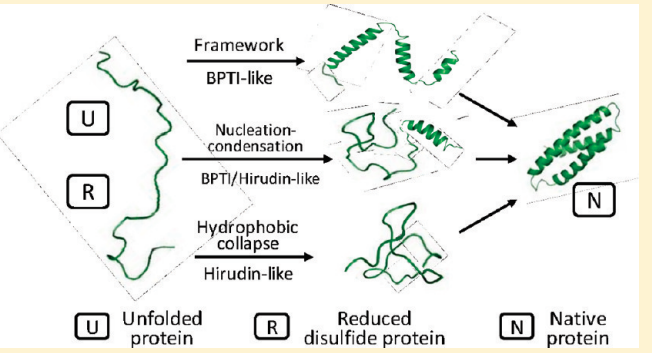
ABSTRACT: The pathway of oxidative folding of disulfide proteins exhibits a high degree of diversity, which is manifested mainly by distinct structural heterogeneity and diverse rearrangement pathways of folding intermediates. During the past two decades, the scope of this diversity has widened through studies of more than 30 disulfide-rich proteins by various laboratories. A more comprehensive landscape of the mechanism of protein oxidative folding has emerged. This review will cover three themes. (1) Elaboration of the scope of diversity of disulfide folding pathways, including the two opposite extreme models, represented by bovine pancreatic trypsin inhibitor (BPTI) and hirudin. (2) Demonstration of experimental evidence accounting for the underlying mechanism of the folding diversity. (3) Discussion of the convergence between the extreme models of oxidative folding and models of conventional conformational folding (framework model, hydrophobic collapse model).



Most experiments of protein folding have been conducted by the approach of conformational folding.[1−3] This is achieved by unfolding proteins with strong denaturant, extreme pH, or elevated temperature. Following the removal of denaturant, a pH jump, or temperature adjustment, unfolded proteins usually refold spontaneously to the native structure. The mechanism of protein refolding is then elucidated by the restoration of selective physicochemical signals that distinguishes the native and unfolded states along the process of folding. The most commonly used signals are spectra of fluorescence, circular dichroism, infrared, and NMR.[4−6] This approach is applicable to study folding of proteins with or without disulfide bonds. Most knowledge of the mechanism of protein folding available to date has been generated using the conformational folding approach.[2,3,7−14] However, this approach has its limitations. (a) Conformational folding does not allow trapping and isolation of folding intermediates. The transient intermediates of conformational folding are usually heterogeneous, short-lived, and highly dynamic. They are very difficult to trap and isolate. Therefore, their structural and kinetic properties have been typically analyzed collectively. As a result, minor yet kinetically relevant intermediates may be overlooked. (b) The structural heterogeneity of the starting material of folding is largely undefined. The folding experiments are usually initiated with denaturant unfolded proteins. However, even under strong denaturing conditions (e.g., 6 M GdmCl), many proteins may still retain residual structures.[15−21] This is crucial because the extent of conformational heterogeneity of the starting material of folding experiment has significant implications[22] for the folding landscape[23−25] as well as the interpretation of observed early stage folding mechanism.[7,26,27]

Some of the limitations of conformational folding may be overcome by using the method of oxidative folding, which is applied to study only disulfide containing proteins. In this approach, a protein is first unfolded in the presence of a reducing agent (e.g., dithiothreitol) and a denaturant (e.g., 6 M GdmCl). After removal of reductant and denaturant, the fully reduced protein [R] is allowed to refold in buffer solution containing redox agents to form the native protein [N] (Figure 1). The folding intermediates are quenched by sample acidification or chemical reaction and subsequently analyzed by HPLC for the characterization of disulfide isomers presented along the process of folding. The method of oxidative folding differs from conformational folding and offers more. (a) The folding experiment is initiated with a structurally (disulfide structure) defined protein isomer. (b) Folding intermediates can be trapped, isolated, and structurally characterized. (c) Acid-trapped folding intermediates can be subjected to stop/go folding to evaluate their kinetic roles in the folding pathway. (d) The folding kinetics can be controlled via adjusting the speed of disulfide formation and disulfide shuffling by redox agents.

The technique of oxidative folding, pioneered by Creighton[28−30] to study bovine pancreatic trypsin inhibitor (BPTI), has been subsequently applied to the elucidation of folding pathways of

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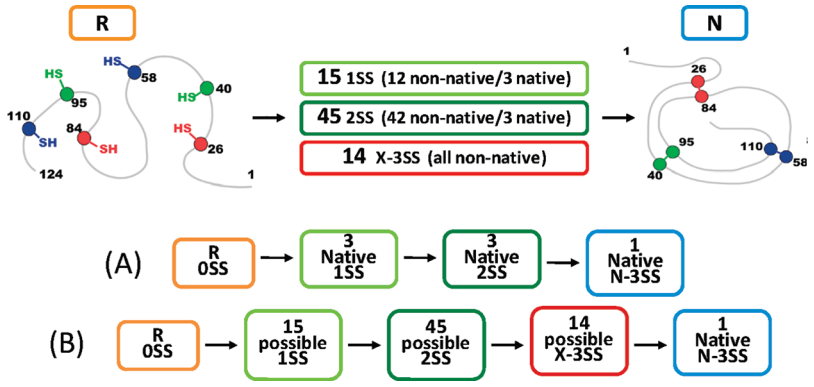

Figure 1. Hypothetical pathways of oxidative folding of a 3-disulfides protein. Along the folding pathway from the fully reduced isomer [R] to the native structure [N], there are 74 possible disulfide isomers that may serve as intermediates. They include 15 1SS isomers (three are native), 45 2SS isomers (three are native), and 14 3SS isomers (all non-native, also known as X-3SS). Two extreme models of disulfide folding pathway are depicted here. (A) One extreme model is defined by the native intermediates, in which folding intermediates comprise only 1SS and 2SS isomers adopting exclusively native disulfide bonds. The folding pathway of BPTI (see Figure 2) resembles this model. (B) An opposite extreme model is defined by the high heterogeneity of folding intermediates, which include most of 74 possible 1SS, 2SS, and X-3SS isomers. The folding pathway of hirudin (Figure 3) is close to this model.

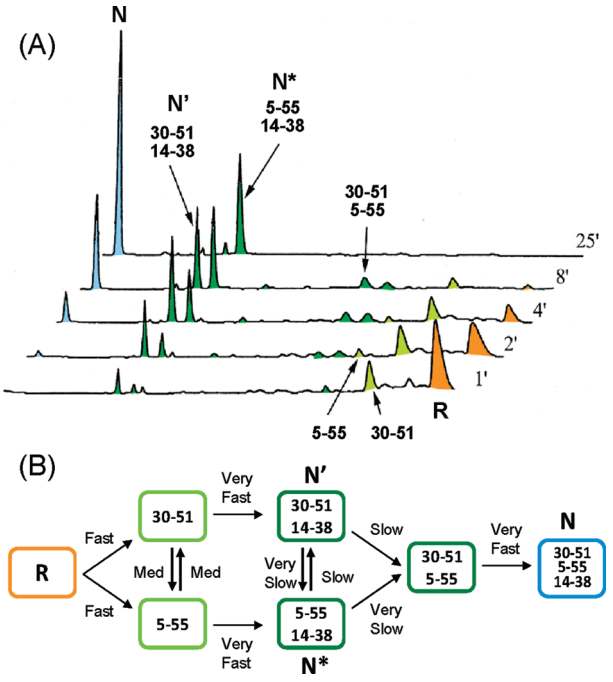

Figure 2. (A) HPLC profile of major BPTI folding intermediates. The folding experiment was carried out at 25 °C, in the Tris-HCl buffer (0.1M, pH 8.7) containing GSSG (0.15 mM).[34] Folding intermediates were trapped by sample acidification and analyzed by RP-HPLC. (B) The major pathway of oxidative folding of BPTI. Five major intermediates containing exclusively native disulfide bonds were identified along the folding pathway. Adapted from ref 34.

numerous disulfide containing proteins. The list of these proteins is given in Table 1 and will be the subjects of this review.

■ SCOPE OF DIVERSITY OF DISULFIDE FOLDING PATHWAY; THE EXAMPLE OF A 3-DISULFIDES PROTEIN

Oxidative folding of a 3-disulfides (3SS) protein is illustrated in Figure 1. The folding is initiated with the fully reduced protein [R] (6 Cys) and ended with the structure containing three native disulfide bonds [N]. Along the pathway, there are 74 disulfide isomers which may serve as the folding intermediates. They consist of 15 1SS isomers (three are native), 45 2SS isomers (three are native) and 14 3SS isomers (X-3SS, all contain at least two non-native disulfide bonds). The disulfide folding pathway is basically defined by the heterogeneity and the structural and kinetic property of these isomers accumulated along the course of oxidative folding. According to this scheme, there are two extreme scenarios of disulfide folding pathway. (a) One extreme model is represented by limited number of folding intermediates consisting of exclusively native disulfide bonds (e.g., three native 1SS isomers and three native 2SS isomers) (Figure 1A). (b) Another extreme model is represented by highly heterogeneous intermediates including the vast majority of 74 possible disulfide isomers (Figure 1B). As will be reviewed in this article, the diversity is enormous. Disulfide folding pathways resembling both extreme models as well as behaving in-between have been well characterized. Among them, BPTI and hirudin are best characterized disulfide proteins representing the (a) and (b) extreme models, respectively.

Review articles dealing with the diversity of disulfide folding pathway have been published previously.[31,32] This review focuses on in vitro folding and differs from the published articles in three aspects. (a) It will elaborate the unique folding property of various models, case by case, including specific kinetic properties of their folding intermediates. (b) It will present experimental data supporting the hypothesis for the underlying cause of the diversity of disulfide folding pathway. (c) It will discuss the convergence of folding models derived from oxidative folding and conformational folding.

For a more valid comparison of disulfide folding pathways, it is important that they were concluded from studies using comparable techniques. It is therefore relevant to mention that most protein models cited and discussed in this review were derived from studies utilizing: (a) reduced/oxidized glutathione (GSH/GSSG) as the main redox agents in promoting the folding; (b) acid quenching for trapping of folding intermediates; and (c) reversed phase HPLC for analysis of trapped folding intermediates. Exceptions will be pointed out explicitly. The method of quenching is most crucial because distribution of rapidly interconverting disulfide isomers may shift depending on the method of quenching free Cys. For instance, chemical quenching (alkylation) may yield more intermediates than

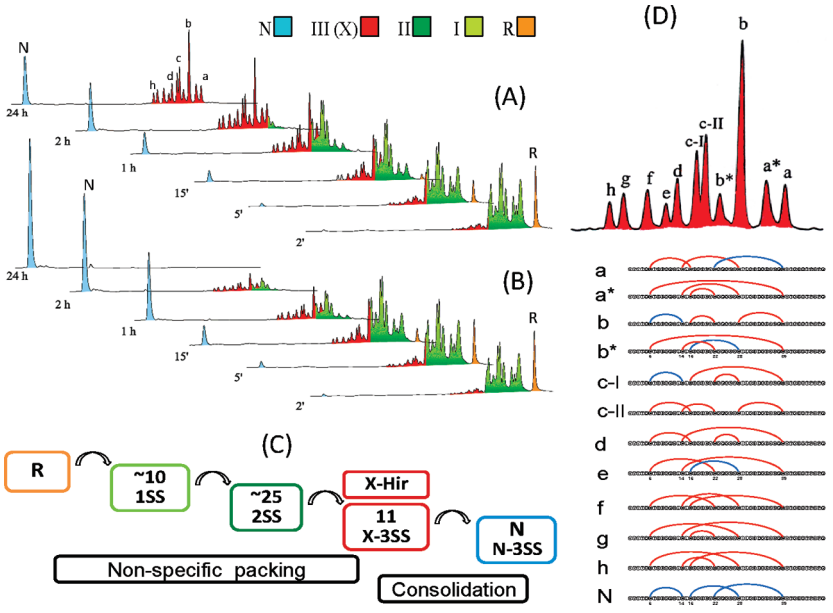

Figure 3. Oxidative folding of hirudin core domain elucidated by HPLC analysis of acid trapped folding intermediates. (A) Folding performed in Tris-HCl buffer (0.1 M, pH 8.4) without redox agent. Formation of disulfide bond is catalyzed by air oxidation. (B) Folding performed in Tris-HCl buffer (0.1 M, pH 8.5) containing GSH (1 mM). Folding intermediates were quenched with an equal volume of 4% aqueous trifluoroacetic acid and analyzed by reverse-phase HPLC.[58] The pathway of folding is characterized by a sequential flow of R-hirudin via diverse 1SS(I), 2SS(II), and X-3SS(III) isomers to from N-hirudin. Each group of intermediate further includes highly heterogeneous isomers. Eleven of the 14 possible X-3SS isomers were identified.[60] GSH serves to catalyze conversion of X-3SS intermediates to form N-hirudin. In the absence of GSH, about 75% of hirudin was trapped as X-3SS hirudin, unable to convert to N-hirudin. (C) Pathway of oxidative folding of hirudin. An initial stage of nonspecific packing (disulfide oxidation) via heterogeneous 1SS and 2SS isomers leads to the formation of eleven scrambled X-3SS isomers as intermediates. A final stage of consolidation (disulfide shuffling) converts X-3SS isomers to the native hirudin. (D) The composition and disulfide connectivity of 11 X-3SS isomers.[59]

acid quenching. This has been shown in the studies of BPTI[33−35] and epidermal growth factor (EGF).[36−38]

THE PATHWAY OF OXIDATIVE FOLDING OF BPTI: ONE EXTREME MODEL

The folding pathway of BPTI represents an extreme model defined by the predominance of native intermediates. The structure of BPTI comprises 58 amino acids and three native disulfide bonds [Cys^{30}-Cys^{51},Cys^{5}-Cys^{55},Cys^{14}-Cys^{38}]. It is the first and most extensively investigated protein for the elucidation of disulfide folding pathway.[33−35,39−48] The pathway of BPTI folding was shown to proceed via selected 1SS and 2SS intermediates to form the native structure. Specifically, scrambled X-3SS isomer has not been observed in BPTI folding (Figure 2). The original studies, using the method of chemical trapping (alkylation) at pH 8.4,[28,33,35] identified two major 1SS intermediates (one is native) and five major 2SS intermediates (three are native). A subsequent study using the technique of acid trapping at pH 7.3[34] confirmed the majority of native intermediates found in the original finding. However, intermediates with non-native disulfide bonds were detected at much lower concentration than previously reported. The discrepancy is most likely due to the difference of trapping techniques. It is likely that chemical alkylation may shift the distribution of rapidly interconverting intermediates and generate additional disulfide isomers.[35] We have similarly observed this difference in the case of EGF folding,[36,37] in which iodoacetate trapped intermediates are more heterogeneous than those trapped by acidification. The disulfide folding pathway of BPTI based upon results obtained from analysis of acid trapped intermediates is outlined in Figure 2.[34] The intermediates include five of the six native 1SS and 2SS isomers of BPTI. The initial prevalence of two native 1SS intermediates [Cys^{30}-Cys^{51}] and [Cys^{5}-Cys^{55}] was followed by a rapid formation of native [Cys^{14}-Cys^{38}] and accumulation of two native 2SS intermediates, [Cys^{30}-Cys^{51},Cys^{14}-Cys^{38}][N′] and [Cys^{5}-Cys^{55},Cys^{14}-Cys^{38}][N*] as kinetic traps. Slow rearrangement of these two native-like 2SS isomers leads to the third native 2SS intermediate [Cys^{30}-Cys^{51}, Cys^{5}-Cys^{55}] which then rapidly form N-BPTI via oxidation of the last native disulfide bond [Cys^{14}-Cys^{38}].

The predominance of native intermediates (Figure 2) should in no way diminish the importance of two non-native intermediates identified in the Creighton's BPTI model, in which [Cys^{30}-Cys^{51},Cys^{5}-Cys^{38}] and [Cys^{30}-Cys^{51},Cys^{5}-Cys^{14}] were shown to be critical in the rearrangement pathway of BPTI folding.[33,35,39] A similar role of these two minor non-native intermediates was subsequently demonstrated by Weissman and Kim using the methods of acid quenching and stop/go folding.[46]

The predominance of intermediates taking on exclusively native disulfide bonds also indicates that the mechanism of BPTI folding is dictated by a sequential formation of stable, native-like subdomains along the pathway.[43] This has been substantiated by NMR analysis of peptide and mutated protein models of BPTI containing only [Cys^{30}-Cys^{51}],[42,45] [Cys^{5}-Cys^{55}],[44,49] or [Cys^{30}-Cys^{51}, Cys^{14}-Cys^{38}],[50] all revealing the existence of native-like domain structures. Indeed, the unique stability of BPTI subdomains may contribute to the extraordinary conformational stability of native BPTI. The protein remains essentially intact under 6 M GdmCl, as measured by the method of disulfide scrambling,[51] a novel technique for analyzing protein unfolding and refolding based on the reversible conversion between native (N) and fully oxidized scrambled (X) protein isomers.[22]

Table 1. Mode of Reductive Unfolding and Oxidative Folding of Small Disulfide-Rich Proteins[a]

proteins[b]	size (a.a.)	(no. of SS)	reductive unfolding	ref	oxidative folding	ref
BPTI	**58**	**(3SS)**	**sequential**	124, 141	**BPTI**	34
LDTI	46	(3SS)	sequential	73	BPTI-like	73
IGF-I	70	(3SS)	sequential	78	BPTI-like	79
IFN-α	166	(2SS)	ND[c]		BPTI-like	81
MCoTI-II	34	(3SS)	sequential	85	BPTI-like	83
EETI-II	32	(3SS)	ND		BPTI-like	83
NTX-α62	62	(4SS)	ND		BPTI-like	142
hirudin	**65**	(3SS)	**all-or-none**	122	**hirudin**	58
PCI	39	(3SS)	all-or-none	124	hirudin-like	87
LCI	66	(4SS)	all-or-none	97	hirudin-like	96
ACI	67	(5SS)	all-or-none	90	hirudin-like	90
TCI	75	(6SS)	all-or-none	99[d]	hirudin-like	99
proinsulin	86	(3SS)	all-or-none	93	hirudin-like	93
CTX-III	60	(4SS)	all-or-none	102	hirudin-like	101
LA5	40	(3SS)	all-or-none	92	hirudin-like	92
conotoxins	~24	(3SS)	ND		hirudin-like	105
AAI	32	(3SS)	all-or-none	95	hirudin-like	95
ProBPTI	71	(3SS)	ND		BPTI-hirudin-like	63
TAP	60	(3SS)	all-or-none	124	BPTI-hirudin-like	68
EGF	53	(3SS)	sequential	124	BPTI-hirudin-like	37
SLPI	107	(8SS)	sequential	143	BPTI-hirudin-like	110
αLA (+$CaCl_2$)	122	(4SS)	sequential	130	BPTI-like	131
αLA (− $CaCl_2$)	122	(4SS)	all-or-none	130[e]	hirudin-like	131

[a] All proteins were analyzed using comparable techniques. For reductive unfolding, proteins were reduced by DTT in the absence of denaturant. For oxidative folding, intermediates were trapped by sample acidification and analyzed by reversed phase HPLC. [b] BPTI is bovine pancreatic trypsin inhibitor. LDTI is leech derived tryptase inhibitor. IGF-I is insulin-like growth factor-I. IFN-α is biovine α-interferon. MCoTI-II is *Momordica cochinchinensis* trypsin inhibitor II. EETI-II is *Ecballium elaterium* trypsin inhibitor II. NTX-α62 is neurotoxin-α62. Hirudin is leech derived thrombin inhibitor. PCI is potato carboxypeptidase inhibitor. LCI is leech carboxypeptidase inhibitor. ACI is ascaris carboxypeptidase inhibitor. TCI is tick carboxypeptidase inhibitor. CTX-III is cardiotoxin-III. LA5 is ligand binding module 5 of low density lipoprotein receptor. AAI is *Amaranthus*α-amylase inhibitor. TAP is tick anticoagulant peptide. EGF is human epidermal growth factor. SLPI is secretory leucocyte protease inhibitor. αLA is bovine α-lactalbumin. [c] Not determined. [d] Reductive unfolding is initiated with separate N- and C-terminal domains. [e] Reductive unfolding is initiated with αLA-IIIA.

THE PATHWAY OF OXIDATIVE FOLDING OF HIRUDIN: AN OPPOSITE EXTREME MODEL

The folding pathway of hirudin represents another extreme model defined by highly heterogeneous non-native intermediates. Hirudin is a thrombin specific inhibitor.[52] The structure of native hirudin comprises a structured N-terminal core domain (residues 1−49) and a disordered C-terminal tail (residues 50−65),[53] which bind selectively to the catalytic site and fibrinogen recognition site of α-thrombin, respectively.[53,54] The core domain of hirudin is stabilized by 24 identified hydrogen bonds, two stretches of β-sheet, and three native disulfide bonds.[55] Without disruption of the native disulfide bonds, hirudin was shown to be an extremely stable molecule.[56]

The folding pathway of hirudin[57,58] was shown to undergo a sequential flow of 1SS, 2SS, and X-3SS intermediates to form the native structure (Figure 3A,B). Each group of intermediates further includes highly heterogeneous isomers. To our estimation,[58] at least 50−60% of the 74 possible isomers are present along the pathway of hirudin folding. Among them, 11 of 14 possible X-3SS isomers have been isolated and characterized (Figure 3D).[59,60] The mechanism of hirudin folding consists of two stages (Figure 3C). An initial stage of nonspecific packing (via disulfide oxidation) leads to the formation of diverse X-3SS isomers. A second stage of consolidation (via disulfide shuffling) converts X-3SS isomers to the N-hirudin. The stage of consolidation requires free thiol as catalyst. When folding was performed in the buffer alone (Figure 3A), conversion of X-3SS isomers to the N-hirudin was initially catalyzed by free Cys of 1SS and 2SS intermediates. As folding progresses, free Cys continues to diminish; therefore, X-3SS intermediates accumulate and become stuck, unable to undergo disulfide shuffling to reach the native structure. In the presence of supplementing Cys, GSH, or β-mercaptoethanol, quantitative recovery of N-hirudin can be achieved (Figure 3B).

One remarkable feature of the hirudin model is that kinetics of the two-stage mechanism can be adjusted separately.[61] This is illustrated in Figure 4. (a) The phase of disulfide formation (nonspecific packing) is accelerated by GSSG or Cys-Cys (cystine). For instance, when folding of hirudin was carried out in the buffer containing 2 mM of Cys-Cys, disulfides form rapidly, and the only detectable intermediates after 2 min of folding are X-3SS isomers (Figure 4A). (b) The phase of disulfide shuffling (consolidation) is promoted by GSH, Cys, protein disulfide isomerase (PDI), or β-mercaptoethanol. Among them, PDI is the most effective agent. PDI (EC 5.3.4.1) is an enzyme in the endoplasmic reticulum in eukaryotes that catalyzes the formation and breakage of disulfide bonds. For example, when folding was carried out in the buffer containing 10 μM of PDI, the only detectable intermediates throughout the entire folding process are 1SS and 2SS isomers (Figure 4B). (c) The time scale

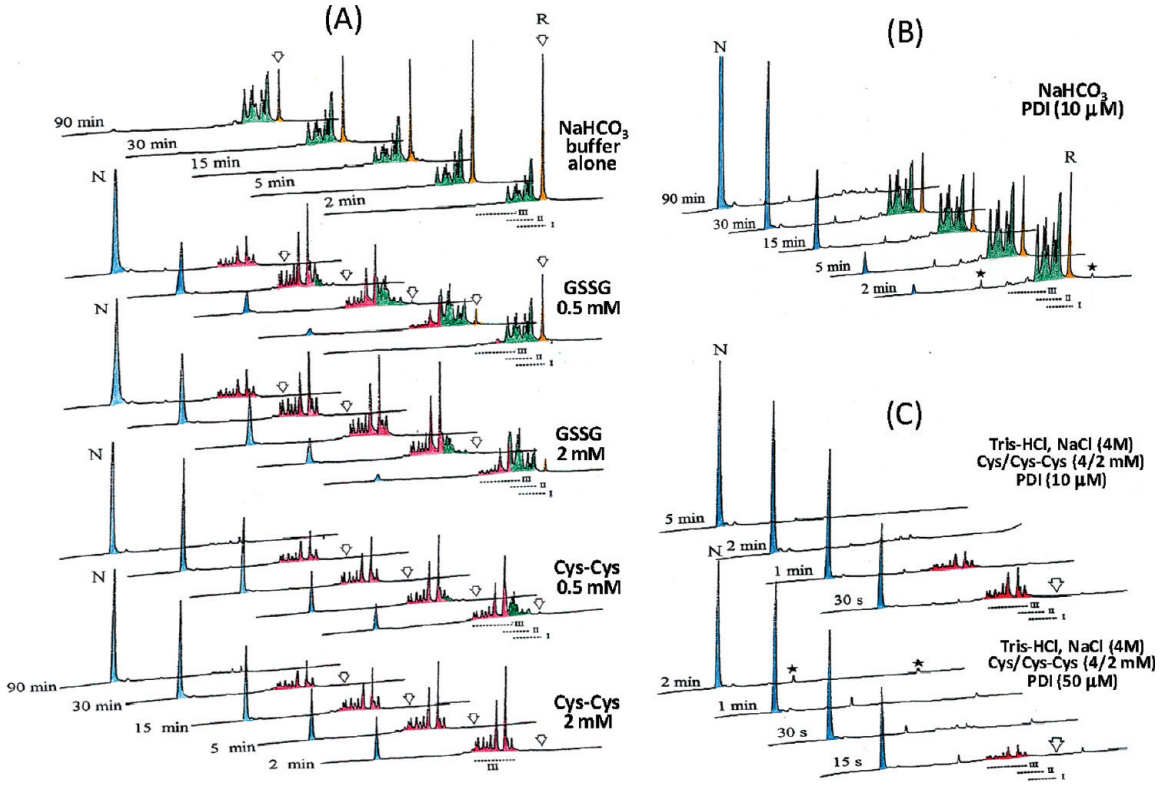

Figure 4. Controlling the kinetics of hirudin folding.[61] (A) Acceleration of disulfide formation. The kinetics of disulfide formation can be accelerated by GSSG or Cys-Cys (cystine). In the $NaHCO_3$ buffer (0.1 M, pH 8.3) alone (top panel), the folding is slow. After 90 min, the folding intermediates still comprise only 1SS and 2SS species. When folding was performed in the same buffer containing Cys-Cys (2 mM) (bottom panel), the only detectable intermediates after 2 min folding are X-3SS isomers. (B) Acceleration of disulfide shuffling. The kinetics of disulfide shuffling is best catalyzed by PDI. When R-hirudin folds in the buffer ($NaHCO_3$) containing PDI (10 μM), predominant intermediates throughout the entire folding process are heterogeneous 1SS and 2SS isomers. X-3SS isomers are barely detectable due to their rapid conversion to N-hirudin. Two minor peaks marked with stars are derived from PDI sample. (C) Acceleration of both disulfide formation and disulfide shuffling. In the Tris-HCl buffer containing NaCl (4 M), Cys/Cys-Cys (4 mM/2 mM) and PDI (50 μM), quantitative oxidative folding of hirudin can be achieved within 30 s.

of completing the folding can be reduced from 24 h to less than 30 s, if an optimized concoction of redox buffer is employed. For instance, when folding was carried out in the sodium bicarbonate buffer containing only β-mercaptoethanol (0.25 mM), the folding is slow and quantitative recovery of native hirudin was achieved within approximately 24 h. In contrast, when folding was performed in Tris-buffer containing NaCl (4 M), Cys/Cys-Cys (4 mM/2 mM), and PDI (50 μM), complete folding was accomplished within 30 s (Figure 4C), a 2880-fold increase of folding kinetic.[61]

In summary, the folding pathway of hirudin differs from that of BPTI by three major characteristics: (a) the presence of high heterogeneity of folding intermediates; (b) the absence of predominant intermediates containing native disulfide bonds; and (c) the presence of fully oxidized X-3SS isomers as compact folding intermediates. An alternative pathway of hirudin folding[62] suggests that yet to be identified native 2SS isomers serve as a direct precursor of N-hirudin.

THE PATHWAY OF OXIDATIVE FOLDING OF PRO-BPTI: A BPTI-HIRUDIN HYBRID MODEL

In vivo, BPTI is synthesized as a precursor protein (proBPTI) that contains additional 13 amino acid residues (TPGCDTSN QAKAQ) attached to the N-terminus of matured BPTI. Correctly folded proBPTI has been purified from bovine platelets.[63] The folding pathway of fully reduced proBPTI was investigated,[63] and the results were compared to that of matured BPTI shown in Figure 2. There are two significant differences: (a) The efficiency of proBPTI folding is greater than that of BPTI. This increased folding efficiency is partly due to the presence of free Cys within the pro-sequence which serves to catalyze the shuffling of disulfide bonds; (b) BPTI and proBPTI fold by a pathway similar to the state of 2SS intermediates, yet via distinct pathways from 2SS intermediates to the native structure (Figure 5). In the BPTI folding, the rate-limiting steps involve very slow conversion of [N′] and [N*] to [Cys^{30}-Cys^{51},Cys^{5}-Cys^{55}], which then form [N] rapidly (Figure 2). In the proBPTI folding, the counterpart [proN′] folds to form [proN] more efficiently. It achieves this by converting to a mixture of non-native X-3SS isomers (abbreviated by III or X), which then rearrange intramolecularly via disulfide shuffling to reach the final native state, [proN] (Figure 5). In contrast, [proN*] hardly oxidize or rearrange under the experimental time scale.[63] Thus, the mechanism of the final stage of proBPTI folding resembles that of hirudin. It is concluded that proBPTI folding is predominantly BPTI-like, with the final stage of hirudin-like folding. The fact that [proN′] folds more efficiently than [N′] underscores the significance of compact X-isomers as intermediates in the pathway of protein oxidative folding.

These two remarkable features of proBPTI oxidative folding need to be further elaborated. (a) The presence of an extra internal Cys, similar to that existing in the proBPTI sequence, serves as a

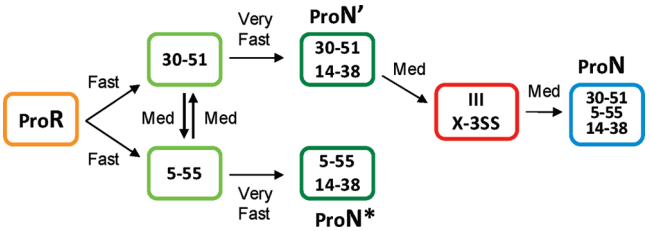

Figure 5. The disulfide folding pathway of proBPTI. The folding pathway of proBPTI differs from that of BPTI at the final stage of folding. In the BPTI folding (Figure 2), [N′] rearranges and converts to [30−51;5−55] which then rapidly forms [N] via oxidation of the third disulfide bond. In the proBPTI folding, [proN′] undergoes a group of [X-3SS] which then forms [proN] via disulfide shuffling. Adapted from ref 63.

thiol catalyst to promote disulfide shuffling and folding efficiency. This was also demonstrated in the folding of a hirudin variant with $Asp^{33} \rightarrow Cys^{33}$ mutation (Cys^{33}-hirudin).[64] Because of the presence of an extra Cys, reduced Cys^{33}-hirudin is capable of folding via X-3SS intermediates to form the native structure in the buffer without thiol catalyst. (b) The kinetic property of [proN′] is not alone. A major 2SS kinetic trap of EGF folding, EGF-II (see below), also exhibits the same property.[37]

THE PATHWAY OF OXIDATIVE FOLDING OF TICK ANTI-COAGULANT PEPTIDE (TAP): ANOTHER HIRUDIN-BPTI HYBRID MODEL

Tick anticoagulant peptide (TAP) is a potent and specific inhibitor of factor Xa.[65] TAP and BPTI share roughly identical size (60 a.a. vs 58 a.a.), the same disulfide pattern,[66] similar content of secondary structures, and nearly superimposable 3D conformations[67] (Figure 6A). Despite the overall similarity of their structures, their folding pathways display remarkable differences[68] (Figure 6B).

The folding pathway of TAP exhibits characteristics resembling both BPTI and hirudin models. (a) Similarity to BPTI model: Two major native 1SS and 2SS intermediates, TAP-[Cys^{33}-Cys^{55}] and TAP-[Cys^{33}-Cys^{55},Cys^{15}-Cys^{39}], structural counterparts of BPTI-[Cys^{30}-Cys^{51}] and BPTI-[Cys^{30}-Cys^{51},Cys^{14}-Cys^{38}][N′] (Figure 2), were also found in TAP folding (Figure 6B,C).[69] (a) Dissimilarity to BPTI model: However, these two native intermediates of TAP do not proceed to form N-TAP the way BPTI folding does. As folding progresses, the majority of these two native intermediates unfolds and rearranges via X-3SS isomers as a major route to reach N-TAP. (c) Similarity to hirudin model: Folding intermediates of TAP consist of heterogeneous 1SS and 2SS isomers (Figure 6C).[69] At least 15−18 well populated intermediates were identified. Specifically, X-3SS isomers, a hallmark of hirudin model, were shown to serve as major folding intermediates of TAP. Among the 14 possible X-3SS isomers, seven have been characterized,[70] and four were shown to populate along the pathway of TAP.[68] Most importantly, folding of TAP also undergoes a two-stage mechanism. When folding was performed in the presence of Cys-Cys or GSSG, which promote disulfide formation, X-3SS isomers accumulate rapidly and become the major kinetic trap of TAP folding.[68]

Thus, despite the prevalence of two native 1SS and 2SS isomers as transient intermediates, TAP folding exhibits almost all characteristics displayed by hirudin folding (Figures 3 and 4).

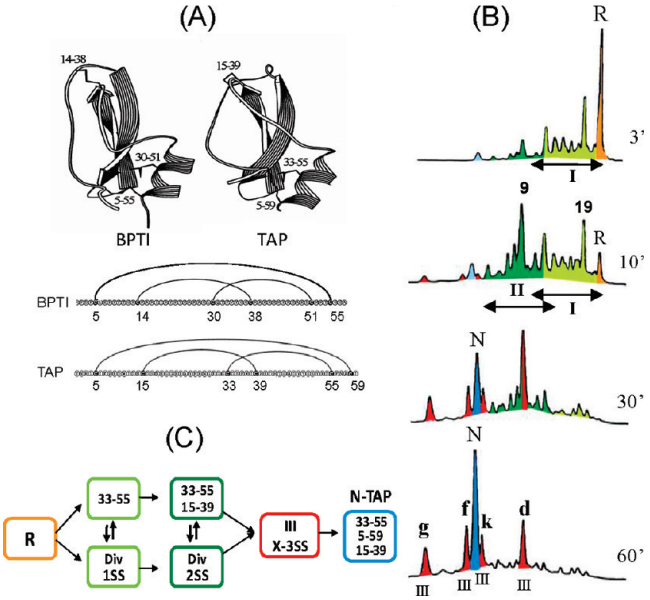

Figure 6. Oxidative folding of TAP, a Kunitz-type inhibitor structurally homologous to BPTI. (A) TAP and BPTI share similar 3D conformation and disulfide pattern. (B) HPLC profile of TAP folding intermediates. The folding experiment was carried out at 23 °C, in the Tris-HCl buffer (0.1M, pH 8.4) containing GSSG/GSH (0.5 mM/1.0 mM). Folding intermediates were trapped by sample acidification and analyzed by RP-HPLC.[68] I, II and III indicate 1SS, 2SS and X-3SS isomers. Fractions 19 and 9 contain two native 1SS and 2SS isomers, TAP-[Cys^{33}-Cys^{55}] and TAP-[Cys^{33}-Cys^{55},Cys^{15}-Cys^{39}], respectively. (C) The major disulfide folding pathway of TAP undergoes X-3SS isomers and resembles mostly hirudin model.

In summary, it is concluded that the folding pathway of TAP is predominantly hirudin-like.

DISULFIDE PROTEINS THAT FOLD VIA BPTI-LIKE PATHWAY

A number of documented disulfide folding pathways are consistent with the BPTI model (Table 1). Their folding pathways share two major characteristics: (a) the predominance of limited numbers of folding intermediates containing native disulfide bonds and adopting native-like structures; and (b) the relative absence of fully oxidized X-isomers as folding intermediates. However, they may differ by distinct kinetic properties of their native intermediates exhibited along the folding pathway. This will be summarized latter in this article.

The most evident case is leech derived tryptase inhibitor (LDTI, 46 a.a., 3SS).[71,72] It folds via mainly native 1SS and 2SS intermediates. Three species of 2SS isomers (IIa, IIb, and IIc) (Figure 7A), each containing two native disulfide bonds, were shown to predominate during the LDTI folding. Stop/Go folding experiments demonstrated that only IIa is an immediate precursor of native structure and oxidizes directly to N-LDTI. NMR structures of acid-trapped and isolated IIa, IIb, and IIc reveal global folds similar to that of the native protein, including a native-like canonical inhibitory loop.[73] The disulfide folding pathway of LDTI illustrated in Figure 7A is strikingly similar to that of BPTI, with one crucial difference. The direct precursor of N-BPTI, BPTI-[Cys^{30}-Cys^{51},Cys^{5}-Cys^{55}], does not accumulate during the folding (Figure 2), whereas the direct precursor of native LDTI (IIa) acts as a kinetic trap, presumably due to the

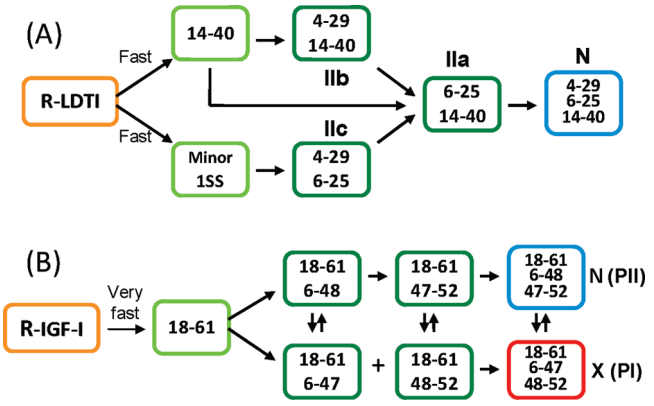

Figure 7. (A) The disulfide folding pathway of leech derived tryptase inhibitor (LDTI).[72,73] Intermediates identified along the folding pathway of LDTI contain only native disulfide bonds. (B) The disulfide folding pathway of IGF-I.[78,79] Note that under physiological conditions, IGF-I folds into two 3SS isomers, PI and PII, with comparable thermodynamic stability.[74,75] PII is the biologically active form (N-IGF-I) and PI is a scrambled X-isomer. Both LDTI and IGF-I fold by the BPTI model.

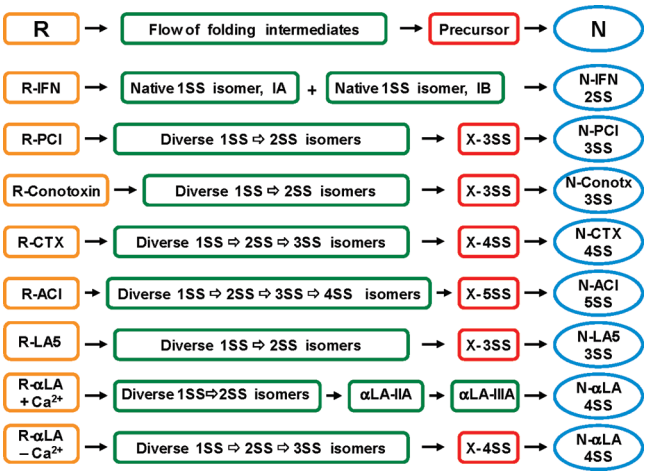

Figure 8. Folding pathway of various disulfide proteins.

significant energy barrier it needs to overcome in order to reach the native state.

Another example is insulin-like growth factor-I (IGF-I, 70 a.a., 3SS). IGF-I is unique in which oxidative folding of reduced IGF-I yields two 3SS products with similar thermodynamic stability[74−77] (Figure 7B). These two folded 3SS isomers are designated as PI and PII,[76] wherein PII is the biologically active form [N-IGF-I] [Cys^{18}-Cys^{61},Cys^{6}-Cys^{48},Cys^{47}-Cys^{52}] and PI is a scrambled X-isomer [Cys^{18}-Cys^{61},Cys^{6}-Cys^{47},Cys^{48}-Cys^{52}]. Oxidative folding of IGF-I has been investigated by various laboratories.[76,78,79] A key step in the IGF-I folding pathway is the rapid formation of a stable native 1SS intermediate [Cys^{18}-Cys^{61}] as a kernel of folding. The major pathway leading to the formation of N-IGF-1 includes two native 2SS intermediates, [Cys^{18}-Cys^{61},Cys^{6}-Cys^{48}] and [Cys^{18}-Cys^{61},Cys^{47}-Cys^{52}]. Furthermore, kinetic analysis revealed that rearrangement of [Cys^{18}-Cys^{61},Cys^{6}-Cys^{48}] to form [Cys^{18}-Cys^{61},Cys^{47}-Cys^{52}] followed by the last step oxidation of Cys^{6} and Cys^{48} represents the productive route to generate N-IGF-I (PII).

Oxidative folding of bovine α-interferon (IFN-α, 166 a.a., 2SS) is another clear case of BPTI model. Native IFN-α comprises five stretches of α-helical structure and two disulfide bonds [Cys^{1}-Cys^{99}; Cys^{29}-Cys^{138}].[80] There are six possible 1SS isomers (two are native) and two X-2SS isomers that may serve as folding intermediates. However, of the eight possible isomers, only two native 1SS isomers, IFN-α-[Cys^{1}-Cys^{99}][IB] and IFN-α-[Cys^{29}-Cys^{138}][IA], were shown to populate along the folding pathway. [IA] and [IB] do not exist in equilibrium and convert to the N-IFN-α independently (Figure 8).[81] More importantly, α-helical structures formed almost quantitatively before formation of disulfide bond and compaction of the protein ever occurred. This was demonstrated by the observation that fully reduced R-IFN-α (starting material of oxidative folding) and reduced-carboxymethylated RCM-IFN-α both exhibit content of α-helical structure indistinguishable from that of N-IFN-α.[81] Furthermore, the stability of α-helical structure of RCM-IFN-α is significantly lower than that of N-IFN-α, indicating that secondary structures developed during the early stage of folding can be further stabilized by formation of the native disulfide bonds and the compact tertiary structure.[81] Thus, the folding mechanism of IFN-α is also consistent with an extreme example of *framework model*, in which secondary structures fold first, followed by docking (compaction) of preformed secondary structural elements to attain the native structure.[14]

In addition, three homologous cystine knot protease inhibitors (~30 a.a., 3SS), namely, *Momordica cochinchinensis* trypsin inhibitor II (MCoTI-II), *Ecballium elaterium* trypsin inhibitor II (EETI-II), and kalata B1,[82−86] were shown to fold via a BPTI-like pathway with a rapid accumulation of single predominant 2SS intermediate containing two native disulfide bonds. There is, however, a difference between them. For MCoTI-II and EETI-II, both native 2SS intermediates convert to their corresponding native proteins directly,[85] similar to the role of BPTI-[Cys^{5}-Cys^{55},Cys^{30}-Cys^{51}] in BPTI folding (Figure 2). In the case of kalata B1, the native 2SS intermediate needs to unfold and revert to the state of 1SS isomer in order to reach native kalata B1.[83,85] NMR structural analysis have shown that stable 2SS intermediates of MCoTI-II,[86] EETI-II,[82] and kalata B1[84] all adopt native-like fold.

■ DISULFIDE PROTEINS THAT FOLD VIA HIRUDIN-LIKE PATHWAY

These proteins are listed in Table 1 and their folding pathways are depicted in Figures 8 and 10. They include potato carboxypeptidase inhibitor (PCI, 3SS),[87−89] ascaris carboxypeptidase inhibitor (ACI, 5SS),[90,91] ligand binding module five of LDL receptor (LA5, 3SS),[92] human proinsulin (HPI, 3SS),[93] *Amaranthus* α-amylase inhibitor (AAI, 3SS),[94,95] leech derived carboxypeptidase inhibitor (LCI, 4SS),[96,97] tick carboxypeptidase inhibitor (TCI, 6SS),[98−100] cardiotoxin (CTX-III, 4SS),[101,102] and a group of conotoxins (3SS).[103−107] Their folding pathways exhibit three common features: (a) the presence of heterogeneous non-native intermediates, including fully oxidized X-isomers; (b) the absence of predominant intermediates containing native disulfide bonds; and (c) the rapid accumulation of X-isomers in the presence of GSSG or Cys-Cys.

An outstanding case is PCI (39 a.a., 3SS) oxidative folding[87] illustrated in Figure 9. Folding intermediates of PCI consist of highly heterogeneous 1SS, 2SS, and X-3SS isomers. When folding of PCI was conducted in the buffer without redox agent, about 65% of the protein was stuck as X-3SS isomers, unable to convert to N-PCI (Figure 9A). This can be surmounted if thiol

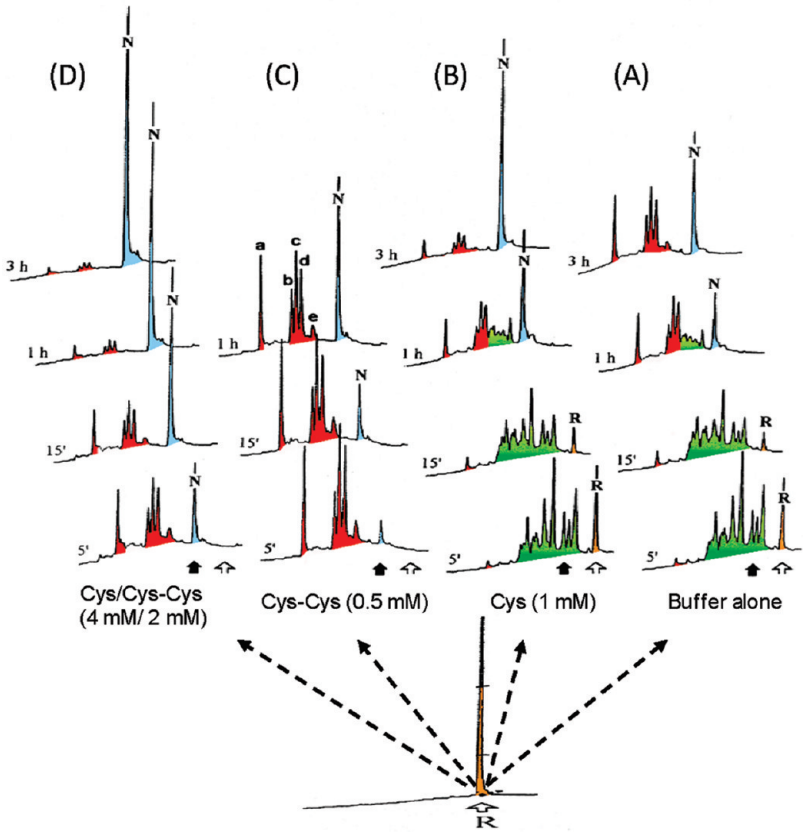

Figure 9. Oxidative folding of PCI (3SS) in the presence of selected redox agents.[87] The Cys/Cys-Cys system is used here as a demonstration. The GSH/GSSG system has a similar effect. (A) In the Tris-HCl buffer (0.1 M, pH 8.5) without redox agent. (B) In the Tris-HCl buffer containing Cys (1 mM). (C) In the Tris-HCl buffer containing Cys-Cys (0.5 mM). (D) In the Tris-HCl buffer containing Cys/Cys-Cys (4 mM/2 mM). Open and solid arrows indicate the elution positions of R-PCI and N-PCI, respectively. The disulfide species are colored in a similar fashion as those shown in Figure 3.

catalyst (e.g., 1 mM Cys) is included in the folding (Figure 9B). In the presence of Cys-Cys (0.5 mM) which accelerates disulfide formation, folding intermediates rapidly accumulated as X-3SS isomers (Figure 9C). In essence, the mechanism of PCI folding shown in Figure 9A,B is indistinguishable from that of hirudin folding revealed in Figure 3A,B. Furthermore, point mutations at the C-terminal tail or deletion of up to three C-terminal residues of PCI resulted in a lower efficiency of the final stage of reshuffling process.[88] There is another feature of PCI folding, in which PCI appears to be thermodynamically unable to fold quantitatively. Even under optimized folding conditions, about 3−5% of PCI was still recovered as X-3SS isomers (Figure 9D). This conclusion has also been corroborated by unfolding experiments of native PCI under physiological conditions.[89]

In the case of LCI (66 a.a., 4SS), oxidative folding proceeds via diverse 1SS, 2SS, 3SS isomers and reaches a rate limiting step in which a mixture of a heterogeneous population of X-4SS isomers and two native-like 3SS isomers (designated as LCI-IIIA and LCI-IIIB) accumulate as kinetic traps (Figure 10A).[96] LCI-IIIA and LCI-IIIB exist in equilibrium and each contains three native disulfide bonds. LCI-IIIA is shown to structurally and functionally resemble N-LCI, whereas LCI-IIIB bears resemblance to scrambled isomers.[97] Interestingly, LCI-IIIA and LCI-IIIB do not form the fourth corresponding native disulfide bonds directly to attain N-LCI. Stop/Go kinetic analysis reveals that for LCI-IIIA and LCI-IIIB to reach N-LCI, major structural rearrangements via heterogeneous X-4SS isomers are required (Figure 11).[97] Thus, X-4SS isomers are direct precursors of N-LCI, similar to the hirudin folding. The kinetic property of LCI-IIIA and LCI-IIIB is markedly similar to that of EGF-II in EGF folding (Figure 12) and [proN′] in proBPTI folding (Figure 5), which are also required to unfold and undergo X-isomers in order to reach native structures.

TCI (75 a.a., 6SS) consists of structurally similar N-terminal (Nt) and C-terminal (Ct) domains, each containing three disulfide bonds arranged in an identical pattern.[98] Recombinant TCI, TCI-Nt (residue 1−36), and TCI-Ct (residues 37−75) were expressed and their folding pathways investigated separately. Oxidative folding of TCI-Nt and TCI-Ct abides by the hirudin model.[99] They both undergo heterogeneous 1SS, 2SS, and X-3SS isomers to reach their respective native 3SS structures. In the folding of intact TCI, both Nt and Ct domains appear to refold independently, which leads to the formation of two native 3SS intermediates, IIIa and IIIb, among minor 3SS isomers (Figure 10B).[99] TCI-IIIa and TCI-IIIb adopt natively folded Nt and Ct domains, respectively.[100] For TCI-IIIa and TCI-IIIb to continue the folding, they all have to undergo heterogeneous 4SS, 5SS intermediates to arrive at X-6SS isomers as main precursors of N-TCI (Figure 10B).[99] Similar to hirudin folding, conversion of X-6SS to form N-TCI requires the presence of thiol catalyst. When folding of fully reduced TCI was conducted in the buffer alone, more than 80% of the protein was stuck as X-6SS isomers.

It is relevant to mention that the kinetic properties of TCI-IIIa and TCI-IIIb most likely differ from that of LCI-IIIA, LCI-IIIB,[97]

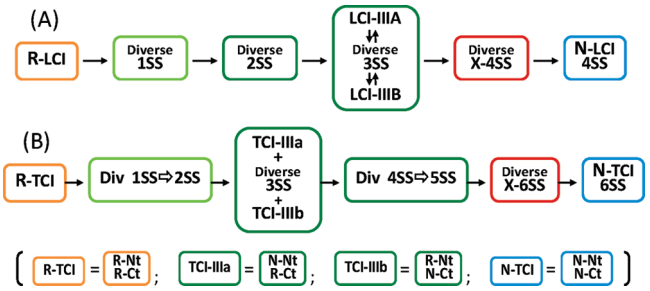

Figure 10. (A) The disulfide folding pathway of leech carboxypeptidase inhibitor (LCI).[96,97] The two native 3SS intermediates, LCI-IIIA and LCI-IIIB, need to undergo X-4SS isomers in order to form N-LCI, a rearrangement pathway similar to that of EGF-II shown in Figure 10. (B) The disulfide folding pathway of tick carboxypeptidase inhibitor (TCI).[98,99] The two 3SS intermediates, TCI-IIIa and TCI-IIIb, each comprises three native disulfide bonds at the N-terminal domain (Nt) and C-terminal domain (Ct), respectively. They both undergo diverse 4SS and 5SS intermediates to reach X-6SS isomers as direct precursors of N-TCI, presumably without unraveling of the already formed native disulfide bonds.

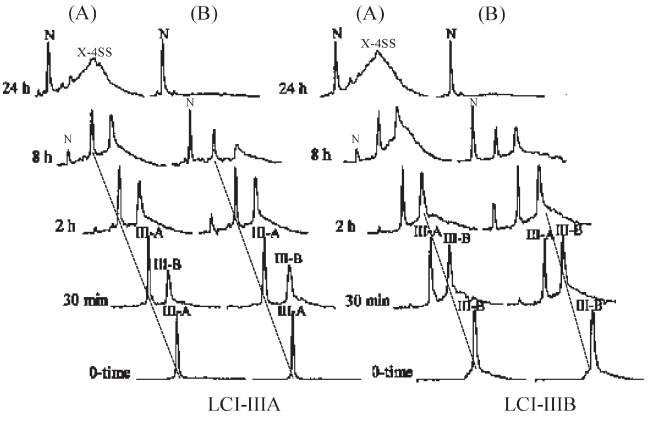

Figure 11. Stop/Go folding experiments of LCI-IIIA and LCI-IIIB, two native 3SS intermediates of LCI oxidative folding.[97] HPLC isolated LCI-IIIA and LCI-IIIB were freeze-dried and reconstituted in the Tris-HCl buffer (pH 8.4) to allow continuation of the folding in the absence (A) and presence (B) of 0.25 mM β-mercaptoethanol. Folding intermediates were then trapped by sample acidification and analyzed by HPLC. Note that in the absence of β-mercaptoethanol, the majority of LCI was stuck as X-4SS isomers, unable to form N-LCI, a phenomenon similar to the folding of hirudin and PCI shown in Figures 3A and 9A. Adapted from ref 97.

EGF-II,[37] or proBPTI-[proN′],[63] which all need to undergo unraveling of existing native-like structures in order to continue the folding. Although both TCI-IIIa and TCI-IIIb end up as heterogeneous X-6SS isomers before reaching N-TCI, such X-6SS isomers could comprise either native Nt and scrambled Ct, or native Ct and scrambled Nt. In another words, TCI-IIIa and TCI-IIIb most likely preserve their already formed native domain structures en route to N-TCI.

■ DISULFIDE PROTEINS FOLD VIA BPTI/HIRUDIN-LIKE PATHWAY

These proteins fold via pathways that exhibit characteristics of both BPTI and hirudin models. Aside from Pro-BPTI[63] and TAP,[68,69] human epidermal growth factor (EGF, 53 a.a., 3SS),[36−38]

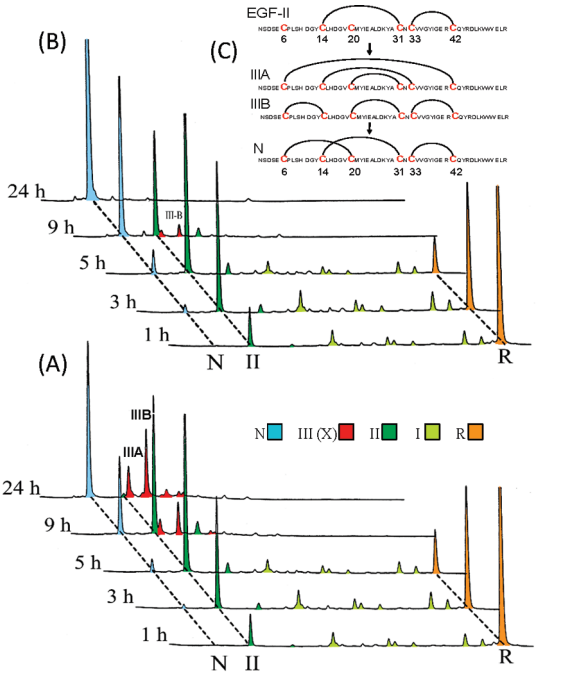

Figure 12. Oxidative folding of human EGF.[37] (A) Folding carried out in the Tris-HCl buffer (0.1 M, pH 8.4) without thiol compound. (B) Folding carried out in the same Tris-HCl buffer containing β-mercaptoethanol (0.25 mM). Folding intermediates were quenched by sample acidification and analyzed by RP-HPLC. Five minor 1SS intermediates are colored in light green. II indicates the major 2SS kinetic trap, EGF-II. IIIA and IIIB are two major species of X-3SS isomers. (C) The disulfide structures of EGF-II, IIIA, IIIB, and the rearrangement pathway for EGF-II to undergo IIIA and IIIB to reach N-EGF.

secretory leucocyte protease inhibitor (SLPI, 107 a.a., 8SS),[108−110] and ribonuclease A (RNaseA, 124 a.a., 4SS) [111−115] are three well documented examples.

EGF (53 a.a., 3SS) is a β-sheet protein comprising three distinct loops (A, B, and C) connected correspondingly by three native disulfide bonds, [Cys^6-Cys^{20}, Cys^{14}-Cys^{31}, Cys^{33}-Cys^{42}]. The connection of Cys^6 and Cys^{20} forming the N-terminal A-loop is essential for the biological activity of EGF.[116] Oxidative folding of EGF was shown to undergo six minor 1SS intermediates and rapidly accumulates a single native 2SS intermediate (designated as EGF-II) as a major kinetic trap (Figure 12).[36−38] EGF-II contains two of the three native disulfide bonds of EGF, [Cys^{14}-Cys^{31} and Cys^{33}-Cys^{42}]. However, EGF-II does not proceed to form N-EGF by direct oxidation of the third native disulfide [Cys^6-Cys^{20}]. Stop/Go folding experiments showed that a major route for EGF-II to reach the native structure is via a rearrangement pathway through X-3SS isomers (EGF-IIIA and EGF-IIIB), which then form N-EGF by disulfide shuffling (Figure 12C).[37] Thus, complete folding of EGF requires thiol catalyst. When folding of EGF was conducted in the buffer alone (Figure 12A), about 45% of the protein was trapped as X-3SS isomers, unable to convert to N-EGF, similar to the mechanism of hirudin folding (Figure 3A) and PCI folding (Figure 9A). The kinetic property of EGF-II bears a striking resemblance to that of proBPTI-[proN′], which also undergoes a rearrangement pathway via X-3SS isomers in order to attain the native structure (Figure 5).[63] The property of EGF-II also resembles that of LCI-IIIA and LCI-IIIB (Figure 10A).[97]

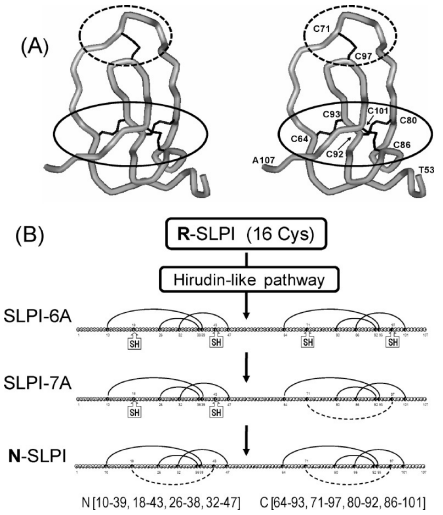

Figure 13. Oxidative folding of secretory leucocyte protease inhibitor (SLPI).[110] (A) 3D structure of the C-terminal domain of SLPI residues 53−107.[109] The three native disulfide bonds (Cys^{64}-Cys^{93}, Cys^{80}-Cys^{92} and Cys^{86}-Cys^{101}) at the center of the domain are enclosed by a solid line. The disulfide bond (Cys^{71}-Cys^{97}) at the enzyme binding loop is circled by a dashed line. (B) The SLPI folding consists of two stages. The first stage of [R-SLPI]⟹[SLPI-6A] folding resembles the hirudin model and the second stage of [SLPI-6A]⟹[SLPI-7A]⟹[N-SLPI] bears a resemblance to the BPTI model. N-SLPI comprises two structurally homologous domains (N- and C-terminus). Each domain is connected by four disulfide bonds. The solid lines connect six disulfide bonds located at the center of the two domains. The dashed lines link two disulfides situated at the enzyme binding loops. SLPI-7A lacks one disulfide bond (Cys^{18}-Cys^{43}) at the N-terminal domain. SLPI-6A lacks both disulfides at the enzyme binding loops (Cys^{18}-Cys^{43} and Cys^{71}-Cys^{97}). The connectivity of four native disulfide bonds at both N- and C-terminal domains are indicated.

The folding pathway of SLPI (107 a.a., 8SS) displays a unique revelation of its own. SLPI also consists of two structurally homologous domains (N- and C-terminal) with approximately equal size and identical disulfide bridging pattern.[108,109] Each domain comprises three disulfide bonds at its domain center (Figure 13A, cycled by solid line) and a fourth disulfide bond at the ligand-binding loop (Figure 13A, cycled by dashed line). Despite an exceedingly large number of possible folding intermediates (∼46 million SS isomers) that may be generated by 16 Cys, oxidative folding of SLPI turns out to be surprisingly efficient. *Complete folding of SLPI can be achieved in a buffer solution using air oxidation alone, a phenomenon facilitated by the unique folding mechanism of SLPI.*[110] Essentially, the folding pathway of SLPI can be dissected into two distinct phases as illustrated in Figure 13B. (a) Folding from [R-SLPI] to [SLPI-6A] obeys the hirudin model. It undergoes heterogeneous isomers to arrive at diverse 6SS intermediates, which then rearrange via disulfide shuffling to form the native 6SS intermediate [SLPI-6A], which lacks the two native disulfide bonds at both ligand binding loops of N- and C-terminal domains . Because 6SS isomers still have four free Cys to serve as thiol catalyst, this stage of reaction can be achieved in the buffer alone. (b) Folding from [SLPI-6A]⟹[SLPI-7A]⟹[N-SLPI] involves spontaneous oxidation of the last two disulfide bonds at the ligand-binding sites. This stage of reaction is akin to the last step of BPTI folding,

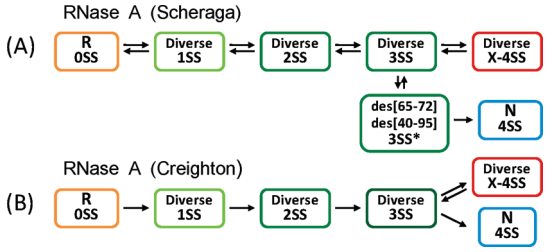

Figure 14. Two disulfide folding pathway of RNase A proposed by Scheraga (A)[111] and Creighton (B).[29] Both models depict a sequential flow of heterogeneous 1SS, 2SS, 3SS to X-4SS isomers. The difference is the scheme of how N-4SS formed. The Creighton model suggests a general route of [3SS]⟹[N-4SS], whereas the Scheraga model asserts the [3SS]]⟹[3SS*]]⟹[N-4SS] pathway, in which [3SS*] includes two native 3SS isomers, des-[40−95] and des-[65−72]. Note that [3SS*] was identified by a rather unique method. Unlike most crucial folding intermediates which were isolated from acid-quenched intermediates ensemble and identified by stop/go folding, [3SS*] was detected via steady-state distribution of intermediates ensembles mediated by 200 mM of oxidized DTT.[117]

which also involves the oxidation of the last native disulfide bond [Cys^{14}-Cys^{38}] near the ligand-binding site.

Oxidative folding of RNase A (124 a.a., 4SS) has been thoroughly investigated by the laboratory of Scheraga.[111−115] However, analytical methods utilized by Scheraga to study RNase A differ from those applied to other protein models described in this review. (a) Unlike most protein models in which folding was promoted by the GSH/GSSG redox system, folding of RNase A was assisted and mediated by oxidized and reduced DTT. (b) Unlike most protein models in which folding intermediates were quenched by sample acidification and analyzed by reversed phase HPLC, folding intermediates of RNase A were quenched by chemical modification and analyzed by ion-exchange HPLC. (c) Unlike most protein models in which kinetic traps were identified via Stop/Go folding experiments of isolated intermediates, those of RNase A were identified via steady-state distribution of diverse intermediates mediated by oxidized DTT.[117,118]

A kinetic model of RNase A folding pathway proposed by Scheraga and colleagues is shown in Figure 14A.[111] A pre-equilibrium (quasi-steady-state) is first established among the reduced protein [R] and unstructured 1SS-4SS ensembles in the presence of 200 mM oxidized DTT. Following removal of oxidized DTT, 3SS ensembles rearrange to form two structured 3SS* species, which comprise des-[40−95] and des-[65−72].[117] These two structured intermediates contain three native disulfide bonds but lack the Cys^{40}-Cys^{95} and Cys^{65}-Cys^{72} disulfide bonds, respectively. Formation of des-[40−95] and des-[65−72] (conversion of 3SS to 3SS*) represents the rate-limiting step of RNase A folding. The native protein is regenerated primarily through two pathways, corresponding to the reshuffling of unstructured 3SS species to form the two structured 3SS* species followed by the formation of the native protein. The des-[40−95] pathway is the major pathway for regenerating the native protein under normal conditions, accounting for about 80% of the recovery of native protein. An earlier proposed folding pathway of RNase A reported by Creighton[29,119] is also shown in Figure 14B. The pathway was derived from analysis of time-course; iodoacetate trapped folding intermediates conducted in the presence of GSSG. In addition, the folding pathway of onconase (ONC, 104 a.a., 4-disulfides), a RNase A homologue, was also analyzed by similar techniques.[120,121]

THE UNDERLYING MECHANISM FOR THE DIVERSITY OF DISULFIDE FOLDING PATHWAY

The scope of the diversity of disulfide folding pathways has been demonstrated with various documented protein models. Among them, divergent folding pathways were also observed between structurally homologous proteins. This provides perhaps the most useful information to reveal the underlying cause of the folding diversity. An enlightening case is the difference of folding between BPTI [33−35] and TAP.[68,69] Despite their substantial structural similarity (Figure 6), TAP folds mostly via a hirudin-like pathway. This discrepancy can be elucidated by the nature of their folding intermediates. Folding of BPTI is dominated by two native 1SS and 2SS intermediates, BPTI-[Cys^{30}-Cys^{51}] and BPTI-[Cys^{30}-Cys^{51},Cys^{14}-Cys^{38}]. Their counterparts also exist in TAP folding. However, TAP-[Cys^{33}-Cys^{55}] and TAP-[Cys^{33}-Cys^{55},Cys^{15}-Cys^{39}] appear to be *less stable* than BPTI-[Cys^{30}-Cys^{51}] and BPTI-[Cys^{30}-Cys^{51},Cys^{14}-Cys^{38}]. Unlike the two native 1SS and 2SS intermediates of BPTI which persist along the folding pathway (Figure 2), the two native intermediates of TAP unravel, rearrange, and convert to X-3SS isomers as a major route to reach native TAP (Figure 6), a pathway resembling the hirudin model. Thus, the extent of stability of protein subdomains encompassing native intermediates likely represents an important origin of folding diversity. Indeed, BPTI folding is a classical example in which native subdomain structures dictate the folding pathway.[43] NMR structural analysis has shown that most native intermediates are stabilized by local native-fold. They include intermediates isolated from the folding of BPTI,[42,44,45,49,50] LDTI,[73] LCI,[97] EETI-II,[82] MCoTI-II,[86] and TCI.[100]

The suggestion that stability of subdomain structure dictates the folding diversity is also substantiated by the correlated mechanism of reductive unfolding and oxidative folding (Table 1). The technique of reductive unfolding[40,122,123] is used to measure the stability of native disulfide bonds against reduction by a reducing agent such as DTT. Because stability of a disulfide bond is generally connected to its adjacent structures, its ability to resist the reduction therefore should reflect the stability of a subdomain surrounding this disulfide bond. In this approach, reduction of disulfide bonds of a native protein may occur either in a *sequential* or *all-or-none* manner.[122,124] A sequential reduction is indicative of differential stability of protein subdomains. For instance, BPTI was reduced in a sequential manner, with selective reduction of Cys^{14}-Cys^{38} first, followed by reduction of the remaining two disulfide bonds.[40,124] In contrast, all three native disulfide bonds of hirudin and TAP were reduced in an all-or-none fashion.[122]

We have previously hypothesized[124] that proteins with their disulfide bonds reduced in a "sequential" manner tend to fold via a BPTI-like pathway, whereas those with their disulfide bonds reduced in an "all-or-none" manner are inclined to fold by a hirudin-like pathway. This hypothesis has been further validated with data published in the past decade. There is a striking correlation between the mode of reductive unfolding and oxidative folding for various proteins (Table 1). Almost without exception, the BPTI-like pathway is associated with "sequential" reduction and the hirudin-like pathway is connected to "all-or-none" reduction. Those which fold in-between BPTI and hirudin pathways are associated with either "sequential" or "all-or-none" reductive unfolding.

These results taken together suggest that one of the major determinants for the folding diversity is the relative structural stability of protein subdomains. The results also imply that for a given disulfide protein, the pathway of oxidative folding can be somehow predicted from the mechanism of reductive unfolding.

A PROTEIN MAY FOLD VIA EITHER THE BPTI-MODEL OR HIRUDIN-MODEL, DEPENDING ON WHETHER ITS SUBDOMAIN STRUCTURE IS BEING STABILIZED

The notion that diversity of disulfide folding pathway is associated with the structural stability of protein subdomains needs to be verified experimentally. For this purpose, α-lactalbumin (αLA) provides an ideal protein model.. The structure of bovine αLA comprises an α-helical domain and a β-sheet calcium binding domain, each connected by two native disulfide bonds, [Cys^{6}-Cys^{120},Cys^{28}-Cys^{111}] and [Cys^{61}-Cys^{77},Cys^{73}-Cys^{91}], respectively. The structure of the β-sheet domain is known to be stabilized upon binding to calcium.[125−127] Oxidative folding of reduced αLA was shown to be highly calcium-dependent.[125,128,129] Our laboratory has also conducted a systematic investigation of oxidative folding of αLA.[130] The results confirm that folding pathway of αLA is dictated by calcium.[130,131] (a) In the presence of calcium, reduced αLA folds via BPTI-like pathway. The folding is characterized by an initial formation of diverse 1SS and 2SS isomers, which rearrange quickly to form a predominant 2SS intermediate, designated as αLA-IIA. This is followed by conversion of αLA-IIA(2SS)⇒αLA-IIIA(3SS)⇒N-αLA(4SS) (Figure 8). Both αLA-IIA and αLA-IIIA are native intermediates. αLA-IIA has two native disulfide bonds at the β-sheet domain (Cys^{61}-Cys^{77},Cys^{73}-Cys^{91}). αLA-IIIA contains three of the four native disulfides (Cys^{28}-Cys^{111},Cys^{61}-Cys^{77},Cys^{73}-Cys^{91}). The kinetics of this folding pathway is dependent on the redox agents. Without GSSG, the final step of folding, αLA-IIIA(3SS)⇒N-αLA(4SS), represents the major rate-limiting step.[130] (b) In the absence of calcium, reduced αLA folds via a hirudin-like pathway. The folding pathway is characterized by a sequential flow from R-αLA through heterogeneous 1SS, 2SS, 3SS intermediates to arrive at X-4SS isomers, followed by conversion of X-4SS isomers to reach N-αLA via disulfide shuffling (Figure 8). In cases in which folding was carried out in the absence of thiol catalyst (which promotes disulfide shuffling) or in the presence of excessive GSSG (which promotes rapid disulfide formation), X-4SS isomers will accumulate and their conversion to N-αLA will become the major rate-limiting step of αLA folding.[130,132]

A more vivid demonstration is provided by the folding experiments initiated with αLA-IIA (Figure 15).[131] αLA-IIA is a predominant intermediate when folding of reduced αLA was carried out in the presence of calcium. It includes a structured β-sheet (calcium binding) domain linked by two native disulfide bonds [Cys^{61}-Cys^{77}, Cys^{73}-Cys^{91}] and four free Cys (Cys^{6}, Cys^{28}, Cys^{111}, and Cys^{120}). How αLA-IIA carries on the folding will depend on whether its structured β-sheet domain is being stabilized by calcium. In the absence of calcium (Figure 15A), αLA-IIA unfolds a significant portion (∼85%) of its existing native-like β-domain and folds via heterogeneous intermediates, including X-4SS isomers, to reach N-αLA, a pathway that resembles the hirudin model. In the presence of calcium (Figure 15B), αLA-IIA folds efficiently via two native 3SS intermediates (αLA-IIIB and αLA-IIIA) to attain N-αLA, a pathway conspicuously similar to the BPTI model. Stop/Go folding experiments have shown that αLA-IIIB and αLA-IIIA do not exist in equilibrium. The conversion of [αLA-IIA]⇒[αLA-IIIB]⇒[αLA-IIIA]⇒[N-αLA] is essentially a spontaneous, one-way process.

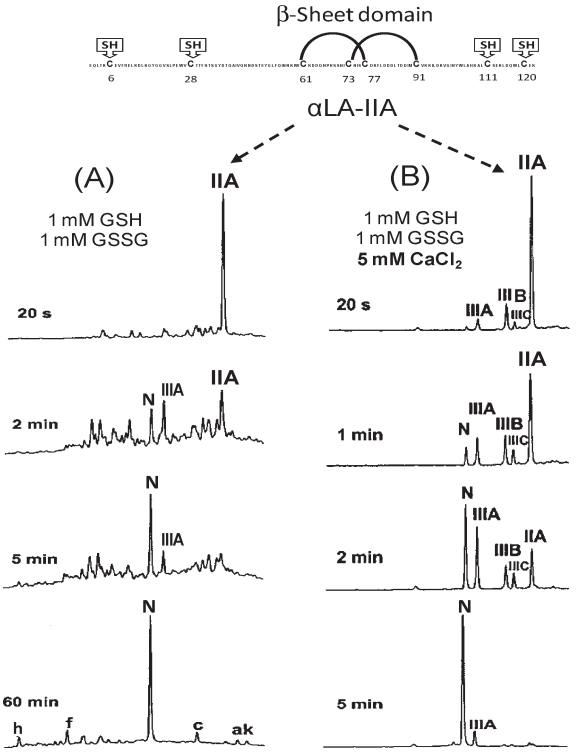

Figure 15. Oxidative folding initiated with αLA-IIA.[131] (A) Folding performed in Tris-HCl buffer (pH 8.4) containing GSSG/GSH (1 mM/1 mM). Without $CaCl_2$, αLA-IIA folds via a hirudin-like pathway, which requires unraveling of a significant portion of the two existing native disulfide bonds in the β-sheet domain. The presence of fully oxidized X-4SS isomers as folding intermediates is indicated in the 60 min sample by a, k, c, f, h (see ref 132 for their disulfide structures). (B) Folding performed in Tris-HCl buffer (pH 8.4) containing GSSG/GSH (1 mM/1 mM) and $CaCl_2$ (5 mM). By stabilization of the β-sheet domain with $CaCl_2$, αLA-IIA folds via a BPTI-like pathway, with selected intermediates containing mainly native disulfide bonds. The disulfide structures of αLA-IIIA and αLA-IIIB are given in Figure 17. The minor intermediate, αLA-IIIC, takes on two native disulfide bonds [Cys^{61}-Cys^{77}, Cys^{73}-Cys^{91}] and one non-native disulfide bond [Cys^{6}-Cys^{28}].[131]

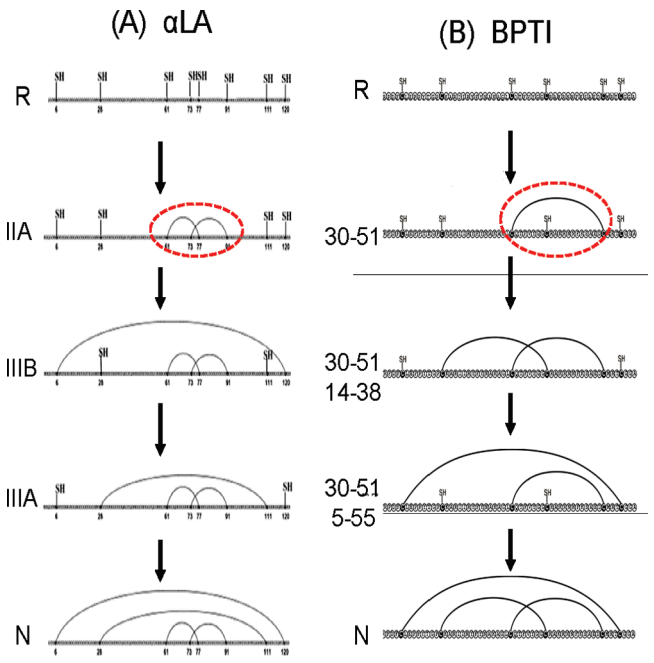

Figure 16. The similarity of the folding pathway between calcium bound αLA (A) and BPTI (B). Folding of αLA was performed in Tris-HCl buffer (pH 8.4) containing GSSG/GSH (1 mM/1 mM) and $CaCl_2$ (5 mM).[131] Folding of BPTI was carried out in Tris-HCl buffer (pH 8.7) containing GSSG (0.15 mM).[34] The stable structural kernels of both αLA and BPTI folding are circled with a red line.

The similarity between the folding of BPTI and calcium bound αLA is illustrated in Figure 16. Both BPTI-[Cys^{30}-Cys^{51}] and [αLA-IIA] formed early in the folding and play a parallel role in their respective folding pathways. Each contains native disulfide bond(s) stabilized subdomain and acts as a kernel in the folding pathway. The rearrangement pathway of [αLA-IIIB]⟹[αLA-IIIA]⟹[N-αLA] is also strikingly similar to that of BPTI-[Cys^{30}-Cys^{51},Cys^{14}-Cys^{38}][N′]⟹BPTI-[Cys^{30}-Cys^{51},Cys^{5}-Cys^{55}]⟹[N-BPTI].

SUMMARY OF THE DIVERSITY OF DISULFIDE FOLDING PATHWAY

The folding pathways of 28 disulfide proteins have been reviewed and elaborated in this article. Among them, about 35% fold by the BPTI model, 45% by the hirudin model and 20% exhibit folding properties of the BPTI−hirudin hybrid model (Table 1). Aside from structural variety of their folding intermediates, a fascinating diversity is displayed by the diverse rearrangement pathways of native intermediates accumulated along the folding pathway. These native intermediates, observed in all three different models, are typically one native disulfide bond short of forming [N]. However, they may fold via four different routes to complete the folding. *Path (a) Converting to [N] directly by oxidation of the last native disulfide bonds.* These intermediates include BPTI-[Cys^{30}-Cys^{51},Cys^{5}-Cys^{55}],[34] LDTI-IIa,[73] IGF-I-[Cys^{18}-Cys^{61},Cys^{47}-Cys^{52}],[78] αLA-IIIA,[130] SLPI-7A[110] and the two native 2SS intermediates of MCoTI-II and EETI-II.[85] *Path (b) Converting to another native intermediate, followed by oxidation of the last native disulfide bond.* These intermediates include BPTI-[N′], BPTI-[N*],[34] LDTI-IIb, LDTI-IIc,[73] IGF-I-[Cys^{18}-Cys^{61},Cys^{6}-Cys^{48}],[79] and αLA-IIIB.[131] *Path (c) Converting to X-isomers, followed by disulfide shuffling to reach the native structure.* These intermediates include proBPTI-[ProN′],[63] EGF-II,[37] LCI-IIIA, LCTI-IIIB,[97] TCI-IIIa and TCI-IIIb.[99] *Path (d) Reverting to lower disulfide species (e.g., from 2SS back to 1SS) in order to reach the native structure.* The native 2SS intermediate of kalata B1[85] goes through this route.

Native intermediates which undergo path (a) preserve their native-like structures en route to form [N]. These isomers are considered as productive species and "disulfide-secure".[113] Their native disulfide bonds are usually protected by or buried in stable subdomain structures, whereas the last two Cys are exposed and ready to oxidize. In contrast, native intermediates which undergo path (b), (c), and (d) require unraveling of an already formed native-like structure in order to reach [N]. These isomers are considered as nonproductive species and "disulfide-insecure".[113,115] Their native disulfide bonds are most likely locked into conformations that impede oxidation of the last two Cys. Consequently, such "disulfide-insecure" isomers often act as kinetic traps and need to undergo substantial structural reorganization in order to carry on the folding.

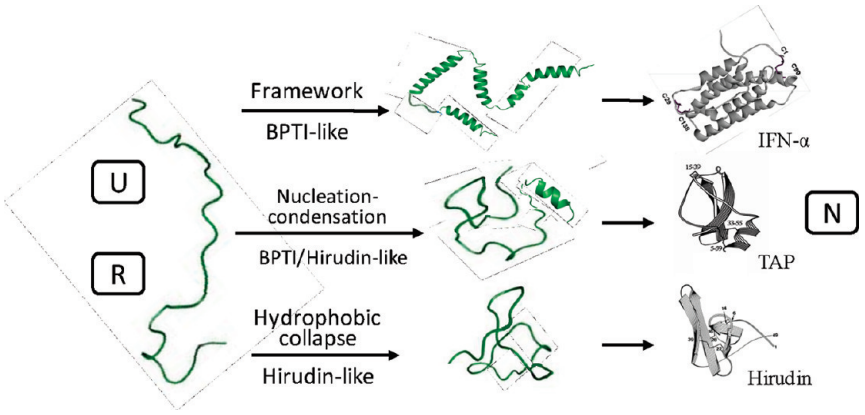

Figure 17. Schematic illustration of three models of conformational folding, the framework model, the nucleation−condensation model, and the hydrophobic collapse model. The two extreme models of oxidative folding, BPTI and hirudin, are consistent with the framework model and hydrophobic collapse model, respectively. The BPTI−hirudin hybrid model is aligned with the nucleation−condensation model. The oxidative folding of IFN-α,[81] TAP[68,69] (Figure 6), and hirudin[58,61] (Figure 3) are used here as examples. [U] stands for an unfolded protein, an abbreviation typically used for the starting material in conformational folding. [R] is a fully reduced protein, an abbreviation routinely used for the starting material in oxidative folding. [N] is a native protein. Note that the major intermediates of framework model are partially structured with preformed secondary structures, whereas those of the hydrophobic collapse model are compact conformation with little native-like structure.

■ EXTREME MODELS OF OXIDATIVE FOLDING ARE COMPATIBLE WITH THE FRAMEWORK MODEL AND HYDROPHOBIC COLLAPSE MODEL

Mechanism of conformational folding has been characterized by three major models.[14] (a) The framework model[8,133,134] proposes that secondary structures (α-helix and 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. (b) The hydrophobic collapse model[7,135−137] stipulates that a rapid hydrophobic collapse accounts for the major driving force of folding, which is followed by searching and fine-tuning of conformation in a compact and confined volume to reach the native structure. (c) The nucleation−condensation model[14,138] represents the middle road between the framework and hydrophobic collapse models. In this model, the formation of secondary structure and compaction of the protein usually occur in parallel during the course of folding.[14,139] This model invokes the importance of the interplay and the interdependence of the secondary and tertiary structures during protein folding.

Interestingly, the two extreme models of oxidative folding, BPTI and hirudin, are generally consistent with the framework model and collapse model, respectively. This is illustrated in Figure 17. (a) For protein folding via BPTI model, the formation of limited numbers of native intermediates along the folding pathway indicates that folding follows the framework model. This is exemplified by the native-like intermediates identified in the folding of BPTI, LDTI, IGF-I, and MCoTI-II. One notable case is oxidative folding of IFN-α.[81] While R-IFN-α is essentially structureless in the presence of 6 M GdmCl, the secondary structures (α-helices) appeared instantly and quantitatively as soon as R-IFN-α was separated from 6 M GdmCl. This was followed by docking and packing of preformed α-helical structures via two native 1SS intermediates, IFN-α-[IA] and IFN-α-[IB], to generate the N-IFN-α. As shown by their CD spectra, R-IFN-α, IFN-α-[IA], IFN-α-[IB], and N-IFN-α exhibit indistinguishable content of α-helical structures (Figure 18A). (b) For protein folding by BPTI/hirudin hybrid model, the mechanism is largely compatible with the nucleation−condensation model; in which formation of native intermediates and compaction of protein occur concurrently during the folding. One example is oxidative folding of TAP.[68,69] During TAP folding, the formation of native 1SS, 2SS isomers, and compact X-3SS isomers occurs in tandem (Figure 6). The early formation of native structures surrounding Cys^{33} and Cys^{55}, including the short segment of α-helix at the C-terminal region, likely accounts for the prevalence of native 1SS intermediate TAP-[Cys^{33}-Cys^{55}]. (c) For protein folding by hirudin model, a rapid hydrophobic collapse at the initial stage of folding leads to the formation of heterogeneous and compact X-isomers as intermediates, which is then followed by reorganization and fine-tuning of X-isomers to reach the native structure. The oxidative folding of hirudin (Figure 3) provides an evident example. As demonstrated in Figure 18B, the structures of major folding intermediates of hirudin (X-hirudin, X-3SS) are largely unordered, whereas N-hirudin exhibits a distinct β-sheet structure.

Spontaneous conformational folding and oxidative folding are both governed by thermodynamic principles and driven by noncovalent interactions. They differ mainly by physicochemical signals utilized to monitor the mechanism of folding, that is, [CD, NMR] vs [disulfide bonding]. The convergence of folding models between the oxidative folding and conformational folding substantiates a unified, albeit diverse protein folding mechanism, independent of distinct signals employed to track the folding. However, the folding landscape of oxidative folding and conformational folding differ. For conformational folding, the energy landscape is commonly represented by the funnel model,[10,23−25] in which the starting material of folding consists of heterogeneous isomers which navigate downhill through rugged V-shape energy slopes to reach the native structure. For oxidative folding, the energy landscape can be properly illustrated by a diamond-shaped model,[22] in which conformational heterogeneity of the protein is characterized by a bell-shaped curve against descending free energy during the spontaneous folding. At the two extreme ends of the energy landscape, the conformational heterogeneity is reduced to minimum, that is, [R] and [N]. In effect, the diamond model is unique in revealing the mechanism of early stage folding. In the case of BPTI, for example, results obtained from both oxidative folding[33−35] and scrambling folding[22,140] have consistently shown

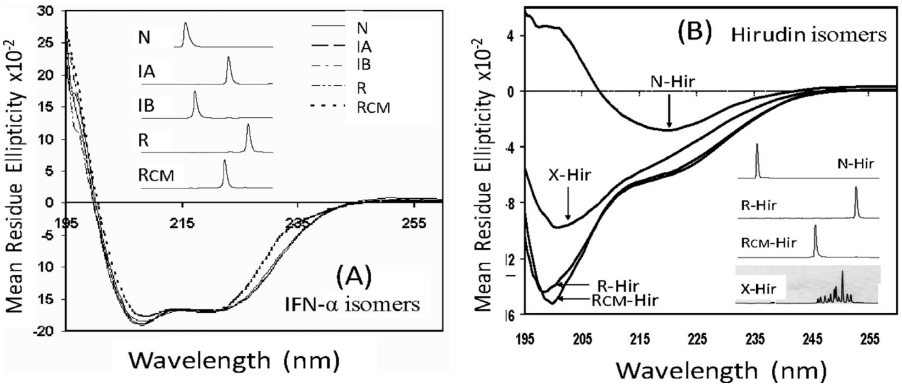

Figure 18. Far UV CD spectra of disulfide isomers isolated from the oxidative folding pathways of INF-α (A) and hirudin (B). They were purified by HPLC, reconstituted in 0.5% aqueous TFA with a final protein concentration of 0.5 mg/mL, and analyzed using a JASCO J-715 spectropolarimeter. Following CD recording, samples were immediately analyzed by HPLC to verify their purity (inset). "N" is native species. "R" is fully reduced species. "RCM" is reduced-carboxymethylated species. "IA" and "IB" are two native 1SS intermediates of INF-α. "X-Hir" is compact folding intermediate of hirudin, which comprises 11 X-3SS isomers.

that early events of BPTI folding include formation of native [Cys^{30}-Cys^{51}] bond as well as conformational restructuring at the C-terminal region.

LIMITATIONS AND TRENDS OF PROTEIN OXIDATIVE FOLDING

Despite the distinct advantage of being able to trap and isolate folding intermediates, the method of oxidative folding has inherent limitations. The technique monitors solely the structure of disulfide bonds. It is certain that, aside from the native protein, each disulfide isomer (specifically those with non-native disulfide bonds) further consists of heterogeneous conformational structures that can only be identified with additional techniques. The convergence of folding models between oxidative folding and conformational folding indicates that future studies of protein oxidative folding should involve concurrent applications of the expanding arsenal of analytical techniques used in studying conformational folding.[4] For instance, application of far and near UV CD to evaluate the secondary structure content and packing of aromatic residues,[144] or 1-anilino-8-naphthaline sulfonic acid (ANS) binding to identify exposure of aromatic surface area.[145] The ANS binding analysis will be particularly useful in characterizing isolated X-isomers which are formed via hydrophobic collapse. Ultimately, all native and non-native intermediates ought to be characterized at the molecular level by NMR. These data, together with the structure of disulfide bonding, should provide further insight into the molecular events of protein folding pathway. Also, protein models that have been elucidated so far are largely models of small disulfide-rich proteins; most of them are protease inhibitors with a molecular mass of less than 10 000 Da. It is imperative to further analyze folding pathways of larger proteins (MW of 20−40 K_d), which comprise significantly more structural elements to direct oxidative folding. Data of structural and kinetic properties of their folding intermediates may shed additional light on folding diversity.

Finally, the mechanism of in vitro oxidative folding reviewed here should provide reference and basis for the elucidation of in vivo oxidative folding pathways. In endoplasmic reticulum (ER), protein oxidative folding occurs in a crowded milieu and is facilitated by complex redox machinery,[146−149] including mM concentration of PDI which is 100−1000 fold higher than that demonstrated in the hirudin folding (Figure 4). It remains to be shown whether the pathway of in vivo oxidative folding would resemble that observed in vitro. It is also not known whether fully reduced polypeptides are a common starting structure of in vivo oxidative folding. If the rate of disulfide oxidation exceeds that of polypeptide synthesis, non-native disulfide bonds would most likely form before the polypeptide detaches from the ribosome. In this scenario, disulfide bonds shuffling and scrambling[22] would account for the major folding activity.

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ABBREVIATIONS USED

AAI, *Amaranthus* α-amylase inhibitor; ACI, ascaris carboxypeptidase inhibitor; BPTI, bovine pancreatic trypsin inhibitor; CTX-III, cardiotoxin-III; DTT, dithiothreitol; EETI-II, *Ecballium elaterium* trypsin inhibitor II; EGF, human epidermal growth factor; GdmCl, guanidine hydrochloride; GSH/GSSG, reduced/oxidized glutathione; Hirudin, leech derived thrombin inhibitor; HPLC, high performance liquid chromatography; IGF-I, insulin-like growth factor-I; IFN-α, bovine α-interferon; αLA, bovine α-lactalbumin; LA5, ligand binding module 5 of low density lipoprotein receptor; LCI, leech carboxypeptidase inhibitor; LDTI, leech derived tryptase inhibitor; MCoTI-II, *Momordica cochinchinensis* trypsin

inhibitor II; N, native protein; NTX-α62, neurotoxin-α62; PCI, potato carboxypeptidase inhibitor; PDI, protein disulfide isomerase; R, fully reduced protein; SLPI, secretory leucocyte protease inhibitor; TAP, tick anticoagulant peptide; TCI, tick carboxypeptidase inhibitor; TFA, trifluoroacetic acid; 1SS, 1-disulfide isomer; 2SS, 2-disulfide isomer; 3SS, 3-disulfide isomer.

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