The Underlying Mechanism for the Diversity of Disulfide Folding Pathways*

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The disulfide folding pathway of bovine pancreatic trypsin inhibitor (BPTI) is characterized by the predominance of folding intermediates with native-like structures. Our laboratory has recently analyzed the folding pathway(s) of four 3-disulfide-containing proteins, including hirudin, potato carboxypeptidase inhibitor, epidermal growth factor, and tick anticoagulant peptide. Their folding mechanism(s) differ from that of BPTI by 1) a higher degree of heterogeneity of 1- and 2-disulfide intermediates and 2) the presence of 3-disulfide scrambled isomers as folding intermediates. To search for the underlying causes of these diversities, we conducted kinetic analyses of the reductive unfolding of these five proteins. The experiment of reductive unfolding was designed to evaluate the relative stability and interdependence of disulfide bonds in the native protein. It is demonstrated here that among these five proteins, there exists a striking correlation between the mechanism(s) of reductive unfolding and that of oxidative folding. Those proteins with their native disulfide bonds reduced in a collective and simultaneous manner exhibit both a high degree of heterogeneity of folding intermediates and the accumulation of scrambled isomers along the folding pathway. A sequential reduction of the native disulfide bonds is associated with the presence of predominant intermediates with native-like structures.

Bovine pancreatic trypsin inhibitor (BPTI) is a compact single-domain protein stabilized by three disulfide bonds (Cys30–Cys51, Cys5–Cys55, Cys14–Cys38) (1). Unfolded and fully reduced BPTI refolds spontaneously to form the native structure. The native disulfide bonds of the native BPTI (Cys30–Cys51, Cys5–Cys55) (N\text{SH}) (3, 5, 6, 8), N\text{SH} is the immediate precursor of the native BPTI. Formation of the third native disulfide, Cys14–Cys38, completes the folding and accounts for the final step of the BPTI folding pathway. Prevalence of the native disulfide bonds along the folding pathway has major implications. It implies that non-covalent specific interactions that stabilize the native BPTI and local structures of BPTI play a crucial role in guiding the folding in its early stages and hence dictate the formation of a limited number of well populated intermediates that admit only native disulfides.

Our laboratory has analyzed the folding pathway(s) of four single-domain, 3-disulfide-containing proteins that have sizes similar to that of BPTI. These four proteins are hirudin (9), potato carboxypeptidase inhibitor (PCI) (10), epidermal growth factor (EGF) (11), and tick anticoagulant peptide (TAP) (12). Their folding mechanism(s) have been shown to differ from that of BPTI in two crucial aspects. Their folding intermediates, including 1- and 2-disulfide species, are far more heterogeneous than those described for BPTI. Aside from EGF, there is also no evidence for the accumulation of predominant intermediates. The most noticeable difference, however, is the presence of scrambled 3-disulfide isomers as folding intermediates, which has not been observed with BPTI. Scrambled isomers are fully oxidized species that contain at least two non-native disulfide bonds (13). They exist in high concentration along the folding pathways of hirudin, PCI, and TAP, as well as EGF. With the exception of EGF, accumulation of scrambled isomers as folding intermediates can be greatly enhanced by allowing the folding in the buffer containing oxidized glutathione or cystine (14). For instance, when folding of PCI was performed in the presence of 0.5 mM cystine, more than 98% of the total protein was trapped as scrambled species before trace amounts of the native PCI even appeared (10).

These discrepancies suggest that the folding pathway of small disulfide-containing proteins is indeed more versatile than what has been learned from the BPTI model alone. To further understand the mechanism of protein folding, it is essential to identify and characterize the underlying cause that generates such diversity of folding pathway(s).

EXPERIMENTAL PROCEDURES

Materials—BPTI was obtained from Roche Molecular Biochemicals. Hirudin core domain (residue 1–49) was derived from the recombinant hirudin variant 1 (HV1) by selective removal of its disordered C-terminal region using \alpha-chymotrypsin (9). Tick anticoagulant peptide (TAP, CYP-5599) is a recombinant protein produced by Novartis (Basel, Switzerland). Potato carboxypeptidase inhibitor was a gift from Dr. F. X. Aviles (University of Barcelona, Spain). Recombinant human EGF was supplied by the Protein Institute Inc. (Broomall, PA). The purity of all five proteins was greater than 96% as judged by HPLC and N-terminal sequence analysis. Dithiothreitol was a product of Sigma with...
RESULTS

The mechanisms of reductive unfolding for five different proteins, including hirudin, PCI, TAP, EGF, and BPTI, were analyzed here. Unfolding experiments were performed at pH 8.4 using various concentrations of dithiothreitol (DTT) as the reducing agent. The unfolding intermediates were trapped in a time course manner by mixing aliquots of samples with an equal volume of aqueous trifluoroacetic acid (4%) and were subsequently analyzed by reverse phase HPLC. The experimental data were analyzed and plotted using MS Excel and Gepasi software (15). The rate constants for the sequential irreversible transformations were calculated using Gepasi software and nonlinear regression. The results show that the mechanism of reductive unfolding of these five proteins can be divided into three groups.

For hirudin, PCI, and TAP, reduction leads to the direct conversion of the native structure (N) to the fully reduced species (R) without accumulation of 1- and 2-disulfide intermediates (Fig. 1). This phenomenon of concurrent reduction was observed with the concentration of the reducing agent ranging from 0.5 to 100 mM. The rate constant (kN→R) displays a linear dependence upon the concentration of DTT in all three cases (Fig. 2). Based on the observed rate constants of reduction, hirudin is about 11-fold more stable than PCI and TAP.

The mechanism of EGF unfolding is different from that of hirudin, PCI, and TAP. Reduction of the native EGF undergoes a stable 2-disulfide intermediate (II). This 2-disulfide intermediate subsequently converts to the fully reduced EGF (R) with no significant buildup of a 1-disulfide intermediate along the pathway (Fig. 1). The calculated rate constants of N to II (kN→II) and II to R (kII→R) are plotted in Fig. 3. kN→II also exhibits a linear dependence on the DTT concentration, but kII→R reaches a plateau at DTT concentration of more than 10 mM. Therefore, accumulation of the 2-disulfide intermediate was most evident when a high concentration of the reducing agent was applied. The structure of this stable 2-disulfide intermediate was characterized through analysis of the thermolysin peptides by both Edman sequencing and MALDI mass spectrometry (results not shown). The data revealed that this intermediate contains two free cysteines (Cys6 and Cys20) and two native disulfide bonds of EGF (Cys14-Cys31, Cys33-Cys42).

The unfolding mechanism of BPTI bears resemblance to that of EGF. Reduction of the native BPTI (N) also goes through a 2-disulfide intermediate (II) that eventually converts to the fully reduced BPTI (R) without an accumulation of 1-disulfide intermediates (Fig. 1). The structure of this 2-disulfide intermediate has been analyzed by us as well, and it was found to

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**FIG. 1.** Mechanism(s) of the reductive unfolding of hirudin, PCI, TAP, EGF, and BPTI. The native protein (0.5 mg/ml) of hirudin, TAP, and EGF were treated with indicated concentration of DTT. Time course intermediates were trapped with acid (4% trifluoroacetic acid) and analyzed by HPLC. The native proteins of PCI and BPTI were treated with various concentrations of DTT for the indicated time intervals. The end products were similarly trapped with acid prior to HPLC analysis. N, II, and R indicate the elution positions of the 3-disulfide native species, the 2-disulfide species, and the fully reduced species, respectively. Solvent A for the HPLC was water containing 0.05% trifluoroacetic acid. Solvent B was acetonitrile/water (9:1, by volume) containing 0.042% trifluoroacetic acid. The flow rate was 0.3 ml/min. Column was Zorbax C-18 for peptides and proteins, 4.6 mm, 10 cm. Column temperature was 23 °C. The gradient varied for each protein: for hirudin, the gradient was 14–36% B in 60 min; for TAP, 28–45% solvent B linear in 40 min; PCI, 14–42% solvent B linear in 50 min; EGF, 14–56% B in 50 min; BPTI, 10–60% B in 60 min.

**FIG. 2.** Rate constant for the direct conversion of the native species to the fully reduced species. The three disulfide bonds of hirudin, TAP, and PCI were shown to be reduced simultaneously and collectively by DTT. For all three proteins, the observed rate constants (kN→R) display a linear dependence upon the concentration of DTT. Quantitative analysis was based on the peak integration of HPLC data.

**FIG. 3.** Rate constant for the sequential conversion of N to II (kN→II) and II to R (kII→R). The disulfide bonds of EGF and BPTI were shown to undergo sequential reduction. The HPLC-generated data were analyzed and plotted by MS Excel. The rate constants for the sequential irreversible transformations were calculated using Gepasi software.

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a purity grade of more than 99%.

**Reductive Unfolding**—The native protein (0.5 mg/ml) was dissolved in Tris-HCl buffer (0.1 M, pH 8.4) containing varying concentrations of dithiothreitol (0.5–100 mM). Reduction was carried out at 23 °C. To monitor the kinetics of unfolding, aliquots of the sample were removed at various time intervals, quenched with an equal volume of 4% aqueous trifluoroacetic acid, and analyzed by HPLC. The samples were stored at −20 °C.

**Structural Analysis of Partially Reduced EGF and BPTI**—The unfolding intermediates of EGF and BPTI (EGF-II and BPTI-II, both 2-disulfide species) were purified from HPLC and freeze-dried. The samples (20 μg) were derivatized with 50 μl of vinylpyridine (0.1 M) in Tris-HCl buffer (0.1 M, pH 7.5) at 23 °C for 45 min. Vinylpyridine-derivatized EGF-II and BPTI-II (~20 μg) were further purified by HPLC and treated with 2 μg of thermolysin (Sigma, P-1512) in 65 μl of N-ethylmorpholine/acetate buffer (50 mM, pH 6.4). Digestion was carried out at 37 °C for 16 h. Peptides were then purified by HPLC and analyzed by amino acid sequencing and mass spectrometry to identify the disulfide-containing peptides.

**Amino Acid Sequencing and Mass Spectrometry**—Amino acid sequences of disulfide-containing peptides were analyzed by automatic Edman degradation using a Perkin-Elmer Procise sequencer (model 494) equipped with an on-line PTH-amino acid analyzer. The molecular mass values of disulfide-containing peptides were determined by MALDI time-of-flight mass spectrometry (Perkin-Elmer Voyager-DE STR).
contain two free cysteines (Cys\textsuperscript{14} and Cys\textsuperscript{38}) and two native disulfide bonds of BPTI (Cys\textsuperscript{30}–Cys\textsuperscript{51}, Cys\textsuperscript{5}–Cys\textsuperscript{55}) (data not shown). These results are consistent with those reported earlier (8, 16). Unlike with EGF, there is a huge disparity between the two rate constants that characterize the conversion of N to II (k\textsubscript{N→II}) and II to R (k\textsubscript{II→R}) (Fig. 3). The transformation of N to II is extremely rapid and requires only mild concentration of DTT. For instance, in the presence of 2 mM DTT, the conversion is completed within 1.5 min. However, the subsequent conversion of II to R is exceedingly slow (8, 17). In the presence of 100 mM DTT, only 7% of the fully reduced BPTI was recovered after 90 min of incubation, and the observed rate constant (k\textsubscript{II→R}) was found to be 0.75 × 10\textsuperscript{−3} min\textsuperscript{−1}. By comparison, the rate constant k\textsubscript{N→II} is greater than k\textsubscript{II→R} by a factor of 250,000. The vast difference of these two rate constants reflects both the weak nature of the Cys\textsuperscript{14}–Cys\textsuperscript{38} bond and the outstanding stability of the structures surrounding Cys\textsuperscript{30}–Cys\textsuperscript{51} and Cys\textsuperscript{5}–Cys\textsuperscript{55}. It is relevant to point out that the native disulfide of BPTI, Cys\textsuperscript{14}–Cys\textsuperscript{38}, is about 10-fold more susceptible to DTT reduction than non-native disulfides of scrambled hirudin and TAP.

**DISCUSSION**

Our data demonstrate the distinctive unfolding mechanisms of BPTI, EGF, TAP, PCI, and hirudin. These differences in the mechanisms reflect how their native disulfide bonds are stabilized and may well account for the diversity of their folding pathways (2–7, 9–12). The correlation is elaborated as follows. 1) Hirudin, PCI, and TAP share the same all-or-none mechanism of reductive unfolding, which suggests that their native disulfide bonds are stabilized in a concerted and interdependent manner. These three proteins also display common characteristics in their folding pathways that include high heterogeneity of 1- and 2-disulfide intermediates and the presence of scrambled 3-disulfide isomers as folding intermediates (9, 10, 12). 2) BPTI unfolds and refolds through a unique mechanism. Unlike in hirudin, PCI, and TAP, the three native disulfide bonds of BPTI are not stabilized in a concerted fashion. Native-like stable structures containing 2- and 1-disulfide bonds exist along the folding and unfolding pathways of BPTI (18–21). These properties govern the formation of limited numbers of predominant intermediates that admit mainly native disulfide bonds and preclude the formation of scrambled 3-disulfide species, such as those observed in the cases of PCI and hirudin. 3) The unfolding and refolding mechanisms of EGF appear to rest in between those of BPTI and hirudin and exhibit characteristics of both BPTI and hirudin. Similar to BPTI, there is a stable unfolding intermediate that contains two native disulfides (Cys\textsuperscript{14}–Cys\textsuperscript{31}, Cys\textsuperscript{33}–Cys\textsuperscript{42}). This native-like 2-disulfide species was shown to accumulate rapidly along the folding pathway of EGF (11). But unlike BPTI, the major 1-disulfide intermediates of EGF were shown to contain both non-native and native disulfide bonds (11, 22). Moreover, scrambled 3-disulfide species were found along the folding pathway of EGF (11), which is characteristic of the folding of hirudin and PCI.

Among these diversities, the one observed between TAP and BPTI is most intriguing. Both TAP and BPTI belong to the Kunitz-type inhibitor and share close structural homology in terms of disulfide pattern and three-dimensional structure (Fig. 4) (23). Despite the structural similarity, their native disulfide bonds are apparently stabilized in very different ways (Fig. 1). Our results also demonstrate that the folding intermediates of TAP are more heterogeneous than those of BPTI (12). At least 18 fractions of the 1- and 2-disulfide intermediates of TAP were detected. In addition, scrambled (3-disulfide) TAP were shown to exist along the folding pathway and act as folding intermediates (12). However, among the heterogeneous folding intermediates of TAP, some major species have been shown to contain the native disulfide bonds, as was observed in the case of BPTI.

All these data, taken together, clearly show that the folding mechanism of small 3-disulfide-containing proteins is more complex than what has been understood previously. The major characteristics of the disulfide folding pathway, including the extent of heterogeneity of folding intermediates, their disulfide structures, and the accumulation of scrambled species, are closely associated with the manner in which native disulfide bonds are stabilized and vary among different proteins.

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