A Two-Stage Mechanism for the Reductive Unfolding of Disulfide-containing Proteins*

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Reductive unfolding of disulfide-containing proteins can be experimentally dissected into two distinct stages. In the presence of denaturant and thiol catalyst, native proteins unfold by reshuffling their native disulfides and convert to a mixture of scrambled structures. Subsequent reduction of the disulfide bonds of scrambled proteins requires only mild concentration of reductant (0.2–0.5 mM reduced dithiothreitol) and undergoes intermediates that consist of highly heterogeneous disulfide isomers. These properties have been characterized with three cystine-containing proteins, namely hirudin, tick anticoagulant peptide (TAP), and bovine ribonuclease A. In the cases of hirudin and TAP, most intermediates observed during the oxidative folding were found to exist along the pathway of reductive unfolding as well.

Elucidation of the pathway of protein folding and unfolding has remained one of the most demanding tasks in protein chemistry (1–7). The challenge stems primarily from the difficulty of analyzing the transient intermediates involved in these pathways. For proteins containing disulfide bonds, unfolding and refolding are generally followed by reduction and oxidation of the native disulfides (8, 9). Since breaking and formation of disulfide bonds can be chemically trapped and characterized (10), the disulfide unfolding and folding pathways can thus be constructed on the basis of the heterogeneity and structures of the trapped intermediates (11–15).

Unfolding of a disulfide-containing protein can be achieved conventionally by either reduction of disulfide bonds in the absence of denaturant (reductive unfolding) (11, 15–17) or by denaturation (e.g., GdmCl) in the absence of reductant (disulfide-intact unfolding) (18, 19). In the latter case, the unfolded protein retains intact native disulfides. So far, the pathway of reductive unfolding has been investigated with limited numbers of proteins (11, 15, 20–23). BPTI and RNase A are two most notable examples. These data, however, were largely obtained from the reductive unfolding performed in the absence of denaturant (11, 15).

During our recent analysis of the properties of scrambled hirudins (24), we have observed that denaturant and reductant can actually be applied in a two-step manner to unfold native protein. In an alkaline solution including strong denaturant and thiol catalyst, the native protein unfolds to form a mixture of scrambled species that admit mostly non-native disulfides but still retain the intact number of disulfide bonds. This is followed by reduction of the disulfides of scrambled proteins. This approach is distinguished from the conventional tech-

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The abbreviations used are: TAP, tick anticoagulant peptide; RNase A, ribonuclease A; GdmCl, guanidine hydrochloride; DTT, dithiothreitol; HPLC, high performance liquid chromatography.

"in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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HPLC. Reduction of scrambled proteins was performed at 23 °C in the Tris-HCl buffer (0.1 M, pH 8.4) containing varying concentrations of DTT (0.2–4 mM). The protein concentration was 1 mg/ml. The reaction was carried out both in the absence and presence of 6 M GdmCl. Reduced samples were trapped in a time course manner by mixing aliquots of the sample with 2 volumes of 4% trifluoroacetic acid.

Reduction of the Native Protein in the Absence of Denaturant—The native protein (1 mg/ml) was treated with different concentrations of DTT (5–200 mM) in the Tris-HCl buffer (0.1 M, pH 8.4). Reduction was carried out at 23 °C. Unfolding intermediates were trapped by mixing aliquots of the reaction sample with 2 volumes of 4% trifluoroacetic acid, followed by HPLC analysis.

Protein Analytical Methods—The disulfide content of scrambled proteins was determined by the dabsyl chloride pre-column derivatization method (29), which permits direct quantification of the disulfide bonds of proteins. The MALDI mass spectrometer was a home-built time of flight (TOF) instrument with a nitrogen laser of 337 nm wavelength and 3 ns pulse width. The apparatus has been described in detail elsewhere (30). The calibration was performed either externally or internally, by using standard proteins (hypertensin, M.W. 10311.19; synacthen, 2934.50, and calcitonin, 3418.91). The unfolding/folding intermediates were analyzed by HPLC using the conditions described in the legend of Fig. 1.

RESULTS

Unfolding of the Native Protein to the State of Scrambled Structures (Stage I)—In the presence of denaturant and trace concentration of thioli reagent (0.25 mM β-mercaptoethanol), unfolding of the native protein was accompanied by reshuffling of the native disulfides, which led to the formation of scrambled structures. Under these conditions, β-mercaptoethanol functioned as a thiol catalyst instead of reducing agent, and the unfolded proteins retained negligible residues of free cysteine. An example of time course unfolding of hirudin in the alkaline solution containing 8 M GdmCl and β-mercaptoethanol (0.25 mM) is shown at the left column of Fig. 1. The unfolding reaction reached a plateau within about 4–5 h, and in this case, 85% of the native hirudin was unfolded and denatured. Prolonged incubation of up to 24 h or further replenishment of the catalyst neither altered the composition nor improved the yield of scrambled hirudins, suggesting that the native and scrambled hirudins had reached a state of equilibrium. Using this method, hirudin, TAP, and RNase A were unfolded in the presence of varying concentrations of GdmCl. The results are presented at the middle and right columns of Fig. 1.

The extent of unfolding, and hence the equilibrium constant (K∞) between scrambled species and the native protein, was shown to be dependent upon the strength of denaturant (Table 1). In addition, there exist narrow ranges of the concentration of denaturant in which native proteins precipitously unfold. For instance, in between 1 and 3 M GdmCl, the equilibrium constant of unfolding of RNase A increased from <0.05 to >100. The same phenomenon was observed with TAP at 3–5 M GdmCl. These critical concentrations of denaturant appear to resemble “melting point” at which forces of non-covalent interactions that stabilize the native proteins become abruptly nullified.

All scrambled proteins contain intact numbers of disulfide bonds. Free cysteines or mixed disulfides with β-mercaptoethanol have not been detected. These were confirmed by amino acid composition analysis and MALDI mass spectroscopic analysis of unfolded species that were treated with 0.1 M iodoacetic acid. The composition of scrambled proteins are heterogeneous. For hirudin and TAP that contain 3 disulfides, there exists 14 possible scrambled isomers. Eleven species of scrambled hirudins have been isolated and structurally characterized (27). Similar numbers of scrambled TAP were identified as well (26). In the case of RNase A, there are 104 possible scrambled isomers. Complete separation of these isomers will be a daunting task. It is not possible to estimate how many isomers of scrambled RNase A do exist since they are poorly separated by the HPLC conditions employed here. However, if the cases of hirudin and TAP are any indication, scrambled RNase A could be very heterogeneous as well.

It is relevant to mention that the selection of 0.25 mM β-mercaptoethanol has been concluded from a systematic study of unfolding performed at 6 M GdmCl and varied concentrations of β-mercaptoethanol. The end products (after 16 h of unfolding) were carboxymethylated, followed by amino acid composition analysis and molecular mass analysis. The results reveal that the optimized concentration of β-mercaptoethanol ranges from 0.2 to 0.3 mM. At higher concentration of β-mercaptoethanol, the disulfide bonds of proteins may become partially reduced (data not shown).

Reduction of Scrambled Proteins (Stage II)—The disulfide bonds of scrambled proteins were reduced with varying concentrations of DTT (0.2–4 mM). The reactions were trapped in a time-course manner, and intermediates that appeared along
Intermediates are largely unaffected by the presence of 6 M GdmCl. Furthermore, from the HPLC pattern, the composition of these unfolding intermediates also comprises highly heterogeneous species (Fig. 4). Thus, the native disulfides of hirudin are approximately 500-fold more stable against reduction than the non-native disulfides of scrambled hirudins. However, unlike their scrambled counterparts, the stability of native disulfides varies substantially among these three proteins. Judging from the concentration of DTT required to achieve the same kinetics of full reduction, the native disulfides of RNase A were about 8-fold and 64-fold more stable than that of hirudin and TAP, respectively (Fig. 5). These differences apparently reflect the extent of contribution of non-covalent interactions of individual proteins. When the factors of non-covalent forces are largely abrogated, as in the state of scrambled structures, the stability of disulfide bonds (against reduction) become more or less indistinguishable.

The most striking outcome is that disulfide bonds of three different sets of scrambled proteins exhibit very similar stability against DTT reduction. All of them could be fully reduced by 0.5–1 mM DTT within 10–20 min at room temperature (23 °C). The stability of scrambled disulfides is also comparable with the inter-disulfide bond that cross-links two intact hirudin monomers (32).

Reduction of scrambled TAP also proceeds via heterogeneous 2- and 1-disulfide isomers. However, the patterns of 2- and 1-disulfide unfolding intermediates are visibly influenced by denaturant (Fig. 3), an indication that some of the 2- and 1-disulfide species of TAP must be stabilized by non-covalent interactions. Those stabilizing factors, however marginal, are also reflected by a roughly 2-fold increase of the rate of reduction when the reaction was carried out in the presence of 6 M GdmCl. Again, the composition of unfolding intermediates of scrambled TAP cannot be distinguished from that observed along the course of oxidative folding of fully reduced TAP, both with or without the participation of denaturant (26). Scrambled RNase A could be similarly reduced and the unfolding intermediates also comprise highly heterogeneous species (Fig. 4).

It is important to point out that the order of stability of native proteins exhibited by their resistance against reduction (RNase A > hirudin > TAP) (Fig. 5) differs from that measured against denaturant (hirudin > TAP > RNase A) (Table I). For instance, the native disulfide bonds of RNase A are about 64-fold more stable than those of TAP. On the other hand,

### Table I

| GdmCl | 1 M | 2 M | 3 M | 4 M | 5 M | 6 M | 7 M | 8 M |
|-------|-----|-----|-----|-----|-----|-----|-----|-----|
| Hirudin | <0.01 | ND | 0.11 | 0.30 | 0.67 | 1.43 | 3.0 | 5.25 |
| TAP | ND | <0.05 | <0.10 | 0.90 | 4.75 | 16.0 | 25.0 | ND |
| RNase | <0.01 | 1.0 | >100 | >100 | >100 | >100 | ND | ND |

* ND, not determined.
complete denaturation of TAP requires double concentration of GdmCl (5–6 M) as compared with that needed for RNase A (3 M). This discrepancy is probably related to the nature of non-covalent interactions (e.g. the number of hydrogen bonds, etc.). While non-covalent interactions can be added up collectively to enforce the stability of a disulfide bond against reduction, their own stability in the presence of denaturant may not behave in the same fashion.

Examination of time course-trapped samples shows that reduction undergoes an apparent all or none mechanism in which only trace amounts of partially reduced intermediates accumulate. This phenomenon was observed with all three proteins at DTT concentrations ranging from 10 to 200 mM. With hirudin and TAP, about 1–4% of 1- and 2-disulfide intermediates was indeed detectable (Fig. 5). In the case of RNase A, two fractions of intermediates comprising approximately 5–15% of the total protein were observed (Fig. 5). Molecular mass analysis of carboxymethylated derivative has revealed that these two minor fractions contain mostly 3-disulfide RNase A. These two intermediates may correspond to those observed by Scheraga and colleagues (15). It has been suggested that the concentration of these intermediates is related to the efficiency of the trapping agent (15). Notwithstanding, the level of their accumulation has not exceeded 15% of the total protein in repeated experiments despite the fact that acid trapping represents one of the most efficient methods in quenching the disulfide formation and rearrangement (16).

An “all or none” mechanism suggests that the reduction of the first disulfide bond exists as a rate-limiting step. Following breakdown of the first disulfide, the remaining cystines become precipitously reduced. This phenomenon thus implies that disulfides of a protein are stabilized in a cooperative and concerted manner by the non-covalent forces of the folded structure. However, it must be emphasized that this mechanism does not represent a general property for disulfide-containing proteins. For large proteins or those consisting of multiple domains, it is most likely that stability of disulfides will reflect their local structures that are strengthened by varied degrees of non-covalent interactions. Many disulfide-containing proteins, even small proteins, are known to be reduced in a sequential manner (21, 33–35). The reality is that the pathway of disulfide reduction for most proteins probably lies in between the strict mode of all or none and sequential. The reduction of human anti-thrombin III is a typical example (36). Even hirudin, demonstrated here, does not follow an absolute all or none mechanism because of the presence of approximately 2% of intermediates.

Furthermore, the mode of reduction, either proceeding via a sequential manner or undergoing an all or none mechanism, bears crucial implications in interpreting the structure and function relationship of disulfide bonds. In searching for the functional role of disulfide bonds, proteins are generally treated with reducing agent in the absence of denaturant. Partially reduced proteins are irreversibly trapped by alkylation (e.g. carboxymethylation). The remaining biological activity of the modified protein is then correlated to the residual number of disulfides, conventionally determined by amino acid composition analysis. If the surviving numbers of disulfides and biological activity display a close to linear relationship, interpretation of the data will be dependent upon the mode of disulfide reduction. Take TAP as an example. It has been shown that reduction of one-third of the disulfide bonds of TAP results in a loss of approximately one-third of its biological activity (37). In case that disulfide reduction proceeds sequentially, this data would suggest that the intactness of one specific disulfide bond accounts for one-third of the biological function of the protein. In case that reduction undergoes an all or none pathway, then it is obvious that the modified protein contains two-thirds of the intact species and one-third of the fully reduced species that lack inhibitory activity. The data obtained here together with those observed by Sardana et al. (37) clearly indicate that TAP belongs to the latter case.

**Fully Reduced Hirudin Refolds via the Same Pathway Regardless of Whether Reduction Is Carried Out in the Presence or Absence of Denaturant—**Since the native protein can be fully reduced both in the absence and presence of denaturant, one important question is whether these two types of fully reduced proteins adopt the same extent of unfolding. Here, we have attempted to partly answer this question by comparing their refolding behaviors. These experiments were designed to examine whether the sample reduced without denaturant retains residual native-like, non-covalent structures that are not abrogated simply by the breakdown of disulfide linkages. If this is the case, one may expect it to refold with increased efficiency and decreased heterogeneity of intermediates, as compared with that unfolded in the presence of denaturant.

Two types of fully reduced hirudins, that unfolded either by reduction alone (R*) or by reduction in the presence of 6 M GdmCl (R), were allowed to refold in the same alkaline buffer both in the presence and absence of thiol catalyst. The mechanism of their folding was compared by characterizing acid-trapped intermediates. Analysis of the time course-quenched intermediates shows that folding of both reduced species (R*)
and R) undergoes an initial stage of nonspecific disulfide formation that leads to the formation of scrambled 3-disulfide species as essential folding intermediates (Figs. 6 and 7). When folding was carried out in the buffer alone (Fig. 6), about 70% of the sample became permanently trapped at scrambled structures and unable to convert to the native structure due to the lack of free thiol to catalyze the reshuffling of non-native disulfides. As folding was performed in the presence of thiol catalyst, the extent of accumulation of scrambled species significantly reduced along the pathway and the native hirudin was quantitatively recovered (Fig. 7). The data clearly demonstrate that both reduced hirudins refold with comparable efficiency and via the same complexity of intermediates. Indeed, the sample reduced in the absence of denaturant refolds at a slightly slower rate. This difference of kinetics was consistently observed with three repeated experiments. These results thus strongly indicate that the sample unfolded without denaturant adopts no unique element of structure that would facilitate its refolding. However, one can not rule out the possibility that some native-like interactions may be retained, but they need to be broken and remade during the process of refolding. This can well explain why it refolds slightly slower than that unfolded in the presence of denaturant.

DISCUSSION

Correlation of the Pathways of Unfolding and Folding—Oxidative folding of hirudin and TAP has been shown to undergo an initial stage of nonspecific 2-disulfide pairing that leads to the formation of scrambled species as essential folding intermediates. This is then followed by disulfide reshuffling of scrambled species in the presence of thiol catalyst to attain the native structure (25, 26, 31). This two-stage mechanism of oxidative folding can be experimentally reversed during the process of reductive unfolding, as demonstrated here. In the presence of denaturant and thiol catalyst, the native protein unfolds back to the state of scrambled structures. Subsequent reduction of scrambled protein generates the fully reduced species. Thus, both unfolding and folding are accomplished by a sequential abrogation (unfolding) or formation (folding) of the two major structures that stabilize the native protein, namely the non-covalent specific interactions and the disulfide bonds. The flow chart of folding and unfolding pathways of hirudin and TAP, and conditions that influence kinetics of these pathways are outlined in Fig. 8.

Most intermediates observed during the oxidative folding exist along the pathway of reductive unfolding as well. Indeed, the composition of 1- and 2-disulfide species that appeared during the first stage of folding (f of Fig. 8) is indistinguishable from those found at the second stage of unfolding (g of Fig. 8). This holds for both hirudin and TAP. However, the composition of 3-disulfide scrambled intermediates is distinguishable along the two opposite pathways. These differences are shown in Fig. 9. Some scrambled species that are well-populated along the direction of folding become diminished along the route of unfolding. This is because unfolding that leads to the scrambled intermediates has to be performed in the presence of denaturant. Scrambled species that are denaturant-sensitive presumably adopt structural elements that are stabilized by non-covalent interactions. These structures still remain to be elucidated. Even if they are native-like, their existence appears to bear no direct link to the native disulfides since most denaturant-sensitive scrambled species do not contain any native disulfide (24, 26). Species f (Fig. 9) of scrambled TAP is a vivid example. The concentration of species f decreases from 30% of the total scrambled TAP in the absence of denaturant (folding) to less than 3% in the presence of 6 M GdmCl (unfolding). Yet,
The force of non-covalent interactions alone is apparently in favor of the native structure. For proteins like hirudin and TAP, and thus help shift the unfolding/folding equilibrium to decrease and limit the entropy allowed to the unfolded structure. The hypothesis (40, 45, 46) that asserts that the primary role of non-covalent forces may be incapable of maintaining their native structure when disulfide bonds are ruptured by reducing agents, then non-covalent interactions will be nullified during the process of unfolding, 3) nullifying the non-covalent interactions that leads to the formation of scrambled species as folding intermediates (△). This process is uniquely accelerated by oxidized glutathione and cystine. In the case of hirudin, the presence of denaturant exerts no apparent influence on the compositions of I and II, but about half of species III are sensitive to the denaturant. In the case of TAP, the compositions of all three classes of intermediates are affected by the presence of denaturant. The final stage of folding is characterized as scrambled species reshuffle their non-native disulfides in the presence of thiol catalyst and consolidate to form the native structure (△). This process is driven by the non-covalent specific interactions and, therefore, is sensitive to denaturant in both cases of hirudin and TAP. The first stage of unfolding, performed in the presence of strong denaturant and thiol catalyst, leads to a reversible conversion of the native structure to the scrambled species again (△). Reduction of scrambled structures converts the protein back to the fully reduced species (△). This process undergoes the same 1- and 2-disulfide intermediates observed in the first stage of folding.

The Intertwining Dependence of the Disulfide Bonds and Non-covalent Forces—Disulfides are known to enforce the stability of native proteins (16, 38–40). Most disulfide-containing proteins lose essentially all of their biological activity as well as their tertiary structures when disulfide bonds are ruptured by reduction (41). Even with exceptional cases (42) in which partial activity remains after complete disulfide reduction, the stability of their native conformation usually becomes drastically diminished (43). Less certain is how disulfide bonds interact with non-covalent structures to stabilize the folded native conformation (40, 44). The data of reductive unfolding, together with those obtained from the oxidative folding of hirudin and TAP (25, 26), may have provided an important insight into the intertwining dependence of the disulfide bonds and non-covalent forces. These crossing dependences, based on those observed in the cases of hirudin and TAP, are summarized in the following. During the process of folding, 1) the non-covalent interactions do not actively drive or direct the folding until non-native scrambled disulfides are formed; and 2) on the other hand, conversion of scrambled disulfides to the native disulfides is driven and guided by the non-covalent interactions. Under conditions in which non-covalent interactions are abrogated (e.g. in the presence of strong denaturant), scrambled structures accumulate as the end products because they are unable to convert to the native structure. While during the process of unfolding, 3) nullifying the non-covalent interactions by denaturant, the native disulfides will collapse into a mixture of scrambled disulfides; and 4) breaking the native disulfides directly by reducing agent, the non-covalent interactions will be incapable of maintaining their native structure and will eventually disintegrate. These findings are fundamentally consistent with the hypothesis (40, 45, 46) that asserts that the primary role of disulfide bonds is to cross-link the protein at its unfolded state, to decrease and limit the entropy allowed to the unfolded structure, and thus help shift the unfolding/folding equilibrium to the favor of the native structure. For proteins like hirudin and TAP, the force of non-covalent interactions alone is apparently insufficient to drive the unfolded protein to cross the energy barrier and guide the way to attain the native conformation. Furthermore, once it assumes the native structure, it still needs the constant support of disulfide bonds.

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