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Disulfide Bonds and Protein Folding†

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ABSTRACT: The applications of disulfide-bond chemistry to studies of protein folding, structure, and stability are reviewed and illustrated with bovine pancreatic ribonuclease A (RNase A). After surveying the general properties and advantages of disulfide-bond studies, we illustrate the mechanism of reductive unfolding with RNase A, and discuss its application to probing structural fluctuations in folded proteins. The oxidative folding of RNase A is then described, focusing on the role of structure formation in the regeneration of the native disulfide bonds. The development of structure and conformational order in the disulfide intermediates during oxidative folding is characterized. Partially folded disulfide species are not observed, indicating that disulfide-coupled folding is highly cooperative. Contrary to the predictions of "rugged funnel" models of protein folding, misfolded disulfide species are also not observed despite the potentially stabilizing effect of many nonnative disulfide bonds. The mechanism of regenerating the native disulfide bonds suggests an analogous scenario for conformational folding. Finally, engineered covalent cross-links may be used to assay for the association of protein segments in the folding transition state, as illustrated with RNase A.

This article reviews disulfide-bond reactions in proteins and how they may be used to investigate protein structure and stability, as well as the mechanism of protein folding.

The main chemical reaction involved in disulfide-bond studies is thiol/disulfide exchange (*1*, *2*)

$$R_1S^- + R_2SSR_3 \rightarrow R_2S^- + R_1SSR_3$$

in which the thiolate anion R_1S^- displaces one sulfur of the disulfide bond R_2SSR_3. Disulfide bonds are formed and reduced by two such thiol/disulfide exchange reactions with a redox reagent, the first of which involves the formation of a *mixed disulfide bond* between the protein and the redox reagent. Thiol/disulfide exchange reactions can also occur intramolecularly; e.g., a protein thiolate group may attack a disulfide bond of the same protein, leading to *disulfide reshuffling*.

Disulfide-bond reactions may be used to study several conformational properties of proteins. Disulfide-bond reactions are inhibited by the burial of reactive groups (such as protein disulfide bonds, mixed disulfide bonds, and protein thiol groups) in stable tertiary structure. Thus, disulfide-bond reactions can monitor the *accessibility* of the reactive groups and indirectly assay the stability of the protective tertiary structure, similar to H/D exchange (*3*) and proteolysis (*4*, *5*) experiments. The rate of disulfide-bond formation also depends on the *proximity* of the two cysteine residues, defined here as the probability of their sulfur atoms coming within the distance required for thiol/disulfide exchange. This rough assay of interresidue distances can be used to characterize secondary, tertiary, and quaternary structure in

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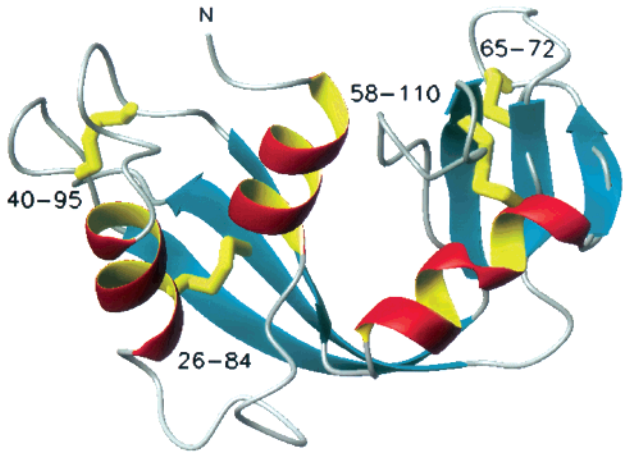

FIGURE 1: Ribbon diagram of a high-resolution X-ray crystal structure (*10*) of RNase A. The four disulfide bonds of the native state are indicated. This figure was prepared with MOLMOL (*106*).

proteins and complements other distance-measuring methods such as fluorescence resonant energy transfer and the nuclear Overhauser effect of NMR spectroscopy. Disulfide-bond reactions are also sensitive to the *reactivities* of the thiolate and disulfide groups, which depend on the pH of the solvent and the pK_a values of the thiol groups involved and, more generally, on the electrostatic environment of the reactive groups.

Disulfide bonds offer several important advantages. First, the formation or reduction of a disulfide bond is a localized, two-state, and structurally well-defined change. Second, disulfide species are stable, covalent intermediates which may be isolated and characterized structurally. Third, the rates of disulfide-bond formation and reduction can be varied without significantly altering other interactions, e.g., by changing the concentrations of the redox couple or by site-directed mutagenesis of the cysteine residues. Fourth, disulfide bonds stabilize the protein in relatively well-understood ways. Their primary effect is to impose distance and angle constraints between the C^β and S^γ atoms of the joined cysteine residues, thus destabilizing the unfolded state by reducing its entropy (*6*−*8*). Disulfide bonds may also stabilize the folded state enthalpically through favorable local interactions, e.g., by stabilizing the packing of a local cluster of hydrophobic residues. These advantages of disulfide-bond studies are illustrated below by experiments on bovine pancreatic ribonuclease A (RNase A)[1].

RNase A (EC 3.1.27.5) has long been a model protein for disulfide studies, having four disulfide bonds (Cys26−Cys84, Cys58−Cys110, Cys40−Cys95, and Cys65−Cys72) in the native state (Figure 1) (*9*, *10*). RNase A is a typical disulfide-bonded protein, in that it generally does not fold to its native structure until nearly all of its native disulfide bonds have been formed (*11*), permitting the study of the mutual effects of disulfide-bond regeneration and conformational folding on each other. The disulfide-intact folding of RNase A has been well studied (*11*−*13*), which aids in interpretations of the disulfide-coupled folding data.

For clarity, we introduce the following terminology, which represents a consensus of the literature. *Oxidative folding* refers to the composite process by which a protein recovers both its native disulfide bonds (*disulfide-bond regeneration*) and its native structure (*conformational folding*); the reverse process is termed *reductive unfolding*. A *disulfide species* refers to a protein with a particular pairing of cysteines in disulfide bonds, while a *disulfide ensemble* is any collection of disulfide species. Disulfide species can be specified by enclosing their disulfide bonds in parentheses; e.g., the native disulfide species of RNase A is (26−84, 40−95, 58−110, 65−72). A *des species* is a disulfide species with all but one of the native disulfide bonds; e.g., the des[65−72] species of RNase A has three native disulfide bonds but lacks the 65−72 disulfide bond. The term *des* signifies only a covalent property (the absence of one native disulfide bond) and does not specify whether the species is folded. Other terms are introduced and defined in the text.

This perspective is organized as follows. The reductive unfolding and oxidative folding of RNase A are reviewed in the first and second sections, respectively. The third section addresses the development of conformational structure in the disulfide intermediates, while the final sections consider the cooperativity of oxidative folding and the implications of disulfide-bond studies for conformational folding. Two appendices are included as Supporting Information; the first surveys the experimental methods used in disulfide-bond studies, while the second discusses recent experiments suggesting that the *stable* conformational order observed in fully reduced RNase A may resemble the *transient* conformational order formed in the "burst-phase" folding of the disulfide-intact protein.

Complementary reviews of disulfide-coupled folding have appeared in recent years (*14*, *15*), which may be consulted to compare the oxidative folding of RNase A with that of other proteins. (Space limitations preclude a general survey here.) Several older references also offer valuable insights (*16*−*18*). Some alternative methodologies and explanations of disulfide-coupled folding (both general and specific to RNase A) should also be considered (*19*−*21*).

Reductive Unfolding of RNase A

In reductive unfolding experiments, a native protein is placed under strongly reducing conditions and the loss of its disulfide bonds and conformational structure is monitored. Since disulfide bonds are generally buried in native proteins (*22*), the reduction of such a disulfide bond must be preceded by a local or global unfolding step that exposes the bond to the redox reagent. The $\Delta G°$ for these unfolding steps can be estimated from the rate at which the corresponding disulfide bonds are reduced (*23*), and measures the stability

[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; BPTI, bovine pancreatic trypsin inhibitor; des[*x*−*y*], disulfide species having all the native disulfide bonds except that between Cys*x* and Cys*y*, e.g., des[65−72] in RNase A; des_U and des_N, unfolded and folded forms of a des species, respectively; R, fully reduced disulfide species; *n*S, ensemble of unstructured disulfide species with *n* disulfide bonds, e.g., the 2S ensemble; 3S*, ensemble of structured disulfide species of RNase A (with three native disulfide bonds) that are not in quasi-equilibrium with the 3S ensemble, but rather oxidize preferentially to the native protein; N′, covalent adduct of RNase A in which a DTT molecule cross-links cysteines 65 and 72; [C65A,C72A] and [C65S,C72S], three-disulfide mutants of RNase A in which cysteines 65 and 72 have been replaced with alanines and serines, respectively; [C40A,C95A], three-disulfide mutant of RNase A in which cysteines 40 and 95 have been replaced with alanines; DTT^{ox}, oxidized dithiothreitol; DTT^{red}, DL-dithiothreitol; HPLC, high-performance liquid chromatography; CFIS, chain-folding initiation site.

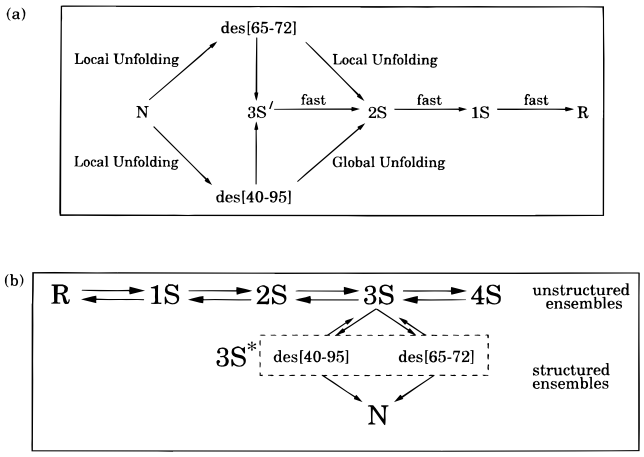

FIGURE 2: (a) Kinetic model of the reductive unfolding of RNase A with DTT^{red} (*23*). Two kinetic pathways are observed, corresponding to two independent, local unfolding events that expose the 40−95 and 65−72 disulfide bonds, respectively. The resulting species, des[40−95] and des[65−72], are themselves reduced by an unfolding mechanism; however, the reductions of des[65−72] and des[40−95] involve local and global unfolding, respectively. (b) Kinetic model of the oxidative folding of RNase A (*47*). In the first stage, a pre-equilibrium is established between the R, 1S, 2S, 3S, and 4S ensembles. These ensembles are unstructured, and the disulfide species within each ensemble relax quickly to a quasi-equilibrium distribution. In the second stage, species of the structured 3S* ensemble are formed through reshuffling from the 3S ensemble (major pathways) and oxidation from the 2S ensemble (minor pathways; not shown). In this diagram, the horizontal direction indicates the regeneration of disulfide bonds, while the vertical direction indicates conformational folding.

of the protective tertiary structure. The reduction of only one native disulfide bond often produces a (des) species with native-like structure. However, when enough disulfide bonds (usually two) become reduced or reshuffled, most disulfide-bonded proteins unfold globally because the native fold is no longer thermodynamically stable relative to the unfolded state. After global unfolding, the protein is rapidly reduced to its fully reduced state R, because all disulfide bonds are accessible to the redox reagent.

This general scenario is illustrated by the reductive unfolding of RNase A. Under typical conditions (pH 8, 15−25 °C, with DTT^{red} as the reducing agent), the reductive unfolding of RNase A appears to proceed entirely through two parallel pathways, corresponding to two independent, local unfolding events exposing the 65−72 and 40−95 disulfide bonds, respectively (Figure 2a) (*23*). Reduction of these disulfide bonds results in two des species, des[65−72] and des[40−95], both with native-like structure (*24*−*27*). The nature of these unfolding steps is currently being investigated; one key question is whether proline cis/trans isomerization contributes to the structural fluctuations exposing these disulfide bonds (*28*).

The two remaining des species, des[26−84] and des[58−110], have not been observed in the reductive unfolding of native RNase A, presumably because the activation free energies for the unfolding steps exposing these disulfide bonds are much higher than those for the unfolding steps exposing the 40−95 and 65−72 disulfide bonds. This explanation is structurally plausible, because the 26−84 and 58−110 disulfide bonds are buried in the core of the native conformation (*10*) and, thus, may require global unfolding to expose them; by contrast, the 40−95 and 65−72 disulfide bonds are only partially buried and may be exposed with local unfolding. Des[26−84] and des[58−110] also have a low conformational stability (*29*−*32*) and, hence, are reduced much more rapidly than des[40−95] and des[65−72]. In principle, this could also account for their apparent absence in reductive unfolding; kinetic data (*23*) indicate that little of the protein is reduced through such pathways, however.

The native-like species, des[40−95] and des[65−72], also bury their disulfide bonds in protective tertiary structure and are likewise reduced through unfolding events (Figure 2a) (*23*). The more stable des[65−72] seems to be reduced through the *same* unfolding event that precedes the reduction of the 40−95 disulfide bond in the native protein (*23*); i.e., the 40−95 disulfide bond is preferentially reduced in the des[65−72] species, while the 26−84 and 58−110 disulfide bonds are largely untouched. As in the native protein, this preference probably results from a relatively low free energy for exposing the 40−95 disulfide bond compared to that for exposing the two other disulfide bonds. By contrast, the des-[40−95] species seems to be reduced through a global unfolding step (*28*) that exposes all three of its disulfide bonds roughly equally (*33*). The reductive unfolding of isolated des[26−84] and des[58−110] has not been studied, but these species are even less stable than des[40−95] and are likely reduced through global unfolding as well (unpublished results). Following either the reduction or reshuffling of a disulfide bond in any of these des species, the protein is unfolded conformationally and is reduced rapidly to the fully reduced state R, because its disulfide bonds are accessible to the redox reagent (Figure 2a) (*23*).

Under strongly reducing conditions, the reshuffling pathways of the des species are relatively minor, presumably because the same unfolding step exposes the disulfide bond both to the redox reagent and to the protein thiolate groups and, under such conditions, the redox reagent competes effectively with the protein thiolate groups to reduce (not reshuffle) the protein disulfide bond.

Oxidative Folding of RNase A

In oxidative folding experiments, the reduced protein is placed under oxidizing conditions and the regeneration of its disulfide bonds and conformational structure is monitored. Under typical conditions (pH 8.0, 25−37 °C, with DTT^{ox} as the redox reagent), the oxidative folding of RNase A is well-fit by the kinetic model of Figure 2b (*34*−*47*).

According to this model, the process of oxidative folding of RNase A can be divided into two stages. In the first stage, R is successively oxidized to populate the unstructured 1S−4S ensembles, which establish a pre-equilibrium (steady-state-like condition) among themselves (*36*, *43*). However, the native protein regenerates slowly from these unstructured ensembles (*46*, *47*).

The disulfide species within each unstructured ensemble likewise reach a quasi-equilibrium distribution, because the disulfide reshuffling *within* the ensembles is generally more rapid than the redox reactions *between* the ensembles. (The fully oxidized 4S ensemble is a special case and is discussed in the section on conformational structure in disulfide intermediates.) Hence, the distribution of disulfide species within each ensemble is relatively insensitive to the redox conditions and the relative concentrations of the other

unstructured ensembles. This *quasi-equilibrium condition* may be observed qualitatively in the HPLC elution profiles of the unstructured ensembles, which are relatively unchanging throughout the regeneration and even when the redox reagent is removed (*46*). Moreover, the quasi-equilibrium condition has been confirmed directly for the 1S ensemble by determining the distribution of disulfide bonds at different regeneration times (*48*). Therefore, it is reasonable to treat each unstructured disulfide ensemble as a single kinetic species and, in particular, to model their interconversions with effective rate constants (*39*), e.g., 1S → 2S. Such a grouping of disulfide species is essential for practical kinetic modeling of the oxidative folding of RNase A and other proteins with many cysteines, because the unstructured ensembles can contain hundreds of disulfide species (*49*, *50*) and an accurate kinetic fitting of their individual interconversions would likely be impossible.

The second stage of oxidative folding consists of the formation of structured disulfide intermediates and their subsequent disulfide reactions. Such intermediates can significantly alter the subsequent regeneration by burying reactive groups in stable tertiary structure, inhibiting their rearrangement by thiol/disulfide exchange. In RNase A, the native protein regenerates predominantly through two pathways associated with two des species, des[65−72] and des[40−95], which are generated from the unstructured 3S ensemble by reshuffling (Figure 2b) and have native-like structure under most conditions that have been studied (*25*, *26*). These species bury their three native disulfide bonds, protecting them from reduction and reshuffling, but leave the two remaining thiol groups largely accessible to the redox reagent, allowing them to be oxidized relatively rapidly to the native protein.

Stable conformational structure in these des species seems to be the critical factor accelerating the regeneration of the native protein. This regeneration becomes drastically slower if it is carried out under conditions that destabilize the tertiary stucture of these intermediates (*45*, *47*). The rapid regeneration can be restored if other agents (e.g., phosphate) are added to restabilize the conformational structure (*31*). The slow regeneration of the native protein from unstructured des species may result from two factors. First, under conditions that favor their unfolding, the des species may constitute a small fraction of the 3S ensemble, because there are many competing disulfide species with similar thermodynamic weights; when their disulfide bonds are protected, however, the des species may accumulate and thereby constitute significant fractions of the total population of three-disulfide intermediates. Second, the final disulfide bond may form more slowly in the unstructured des species because a higher entropic penalty must be paid to bring the final pair of cysteines together.

Des[65−72] and des[40−95] can also be formed by oxidation from the 2S ensemble (*51*, *52*). This oxidation pathway is relatively minor under typical conditions; studies of site-directed mutants in which the reshuffling reaction from 3S is blocked suggest that, in the wild-type protein, ≤5% of the des[65−72] and des[40−95] populations result from oxidation. In contrast, des species cannot be formed directly from the 4S ensemble, because the species of the 4S ensemble have at most two native disulfide bonds.

The two other des species, des[26−84] and des[58−110], have stable tertiary structure at lower temperatures [e.g., 15 °C (*30*)] or in the presence of stabilizing salts [e.g., 400 mM phosphate (*31*)]. However, these des species appear to be *kinetic traps* since, under typical conditions, they do not undergo oxidation, reduction, or reshuffling on a time scale characteristic of other species (unpublished data). Hence, these des species presumably bury not only their disulfide bonds but also their thiols, inhibiting their oxidation to the native protein as well as their reduction and reshuffling. The burial of thiols in des[26−84] and des[58−110] and their relative accessibility in des[40−95] and des[65−72] are consistent with the native structure (*10*), in which the 26−84 and 58−110 disulfide bonds are buried in the core of the protein but the 40−95 and 65−72 disulfide bonds are only partially buried.

Mixed disulfide bonds can also become buried in stable tertiary structure, as demonstrated by the covalent adduct N′ observed in the oxidative folding of RNase A, in which a DTT molecule cross-links cysteines 65 and 72 (*53*). Kinetic data suggest that this adduct is formed through the burial of a mixed disulfide bond between the DTT molecule and one cysteine, probably Cys72 (*24*). The remaining thiolate of the bound DTT molecule forms a disulfide bond with a second DTT molecule, which is attacked by the remaining protein thiolate for adduct formation. Such buried mixed disulfide bonds are likely to be more prevalent with linear redox reagents (*54*) than with rapidly recyclizing reagents (such as DTT).

Conformational Structure in Disulfide Intermediates

This section describes the development of structure during the oxidative folding of RNase A and assesses the structural contributions of specific disulfide bonds. However, three technical terms (structure, conformational order, and tertiary topology) must first be defined to make these descriptions more precise.

Structure is defined here as a specific spatial arrangement of chemical groups. More precisely, residues are said to be *structured* in an ensemble of conformations if the rms deviations in corresponding interatomic distances are small (e.g., <3 Å). For example, secondary structure describes a specific spatial arrangement of hydrogen-bonded peptide groups, while tertiary structure describes a specific spatial arrangement of all types of atoms, e.g., the precise packing of side chains in the hydrophobic core. Conversely, residues are said to be *unstructured* in an ensemble of conformations when there is significant variation in corresponding interatomic distances among the conformations of the ensemble.

The more general term *conformational order* signifies only a nonrandom distribution of conformational variables without requiring that the spatial arrangement of atoms be similar in all conformations of the ensemble. Thus, a hydrophobically collapsed homopolymer may be conformationally ordered without being structured; its equilibrium ensemble of conformations is characterized by a low radius of gyration (the nonrandom conformational variable) but may include many dissimilar conformations, with large variations in corresponding interatomic distances.

Tertiary topology is defined here as the rough spatial arrangement of the protein backbone (side chains are not

considered). Hence, it can be considered as a low-resolution analogue of structure; an ensemble of conformations has a characteristic tertiary topology if the relative distances between backbone atoms agree to low resolution. For example, homologous proteins often share a common tertiary topology. [The tertiary topology of a protein is also called its *tertiary fold* (*55*), its *backbone topology* (*56*), or its *topomer* (*57*).] Tertiary topology should be distinguished from the related concept of *β-sheet topology*, which describes the relative disposition of β-strands in a β-sheet. The resolution of tertiary topology is usually so low that it rarely makes sense to speak of the tertiary topology of a residue or even of an oligopeptide, but only of domains and subdomains.

Conformational Structure in the Pre-Equilibrium Ensembles of RNase A. Ensembles R and 1S−4S appear to have little native structure. They show very low enzymatic activity (*58*) and poor NMR dispersion (*59*) and are rapidly reduced to R under weakly reducing conditions (5 mM DTT^{red}) (*47*). Their optical properties (such as tyrosine absorbance and CD spectra) resemble those of the conformationally unfolded state (*60*−*62*). In the 1S ensemble, nearly every possible disulfide species is represented [albeit in different percentages (*48*)], and the same appears to be true for the 2S ensemble (*63*).

Nevertheless, some conformational order is apparent in these ensembles. The fully reduced species R has been studied by several methods, including antigenic response (*64*), fluorescence resonant energy transfer (*65, 66*), small-angle X-ray scattering (*67*), dynamic light scattering (*68*), and ^{17}O nuclear magnetic relaxation dispersion (*69*). The evidence suggests that, under folding conditions, R is not a statistical coil but partially collapsed, with a significant fraction of the native tertiary topology (A. Navon, V. Ittah, H. A. Scheraga, and E. Haas, manuscript to be published), contradicting earlier suggestions that fully reduced RNase A is a random coil (*21*).

The 1S and 2S ensembles likewise appear to have some conformational order. The relative concentrations of disulfide bonds in these ensembles show significant deviations from random probabilities (*48*, *63*), indicating significant enthalpic contributions to the free energy in addition to the loop entropy. In particular, the 65−72 disulfide bond is strongly favored, comprising fully 40% of the 1S ensemble (*48*), and exists in an even larger fraction of the 2S ensemble (*63*). However, these enthalpic contributions appear to be dominated by local interactions, i.e., interactions between residues near the cysteines of the disulfide bonds, since nearly identical enthalpic contributions are observed in homologous oligopeptide model systems (*70*−*72*). Moreover, the enthalpic contributions observed in the 1S ensemble appear to account for those observed in the 2S ensemble (W. J. Wedemeyer and H. A. Scheraga, unpublished results). An NMR study of two-disulfide mutant analogues of RNase A (*73*) also indicated that a disulfide species with two native disulfide bonds may exhibit no more structure than those with nonnative disulfide bonds. These results suggest that the conformational order does not increase strongly with the number of disulfide bonds, prior to the appearance of native-like structure in the 3S* species.

The 3S and 4S ensembles probably have conformational order equal to or greater than that of the 2S ensemble, but this remains to be studied experimentally. As noted above, the 4S ensemble is fully oxidized and, hence, cannot reshuffle. Nevertheless, the distribution of species within the 4S ensemble can satisfy the quasi-equilibrium condition, insofar as the distribution is relatively insensitive to the concentrations of the redox reagent and the other disulfide ensembles. Such a quasi-equilibrium condition may arise because the 4S ensemble is produced by the oxidation of disulfide species in the 3S ensemble, which by assumption has reached a quasi-equilibrium state; thus, the constant relative concentrations of disulfide species of the 4S ensemble may arise from the constant relative concentrations of the 4S-precursor species in the 3S ensemble. However, the 4S ensemble has a low population under typical conditions, and generally does not affect the regeneration of the native protein significantly.

Conformational Structure in the Des Species of RNase A. The des species des[65−72] and des[40−95] have stable, native-like structures, which have been investigated by X-ray crystallographic and NMR studies of their blocked form (*24*) and of the mutant analogues [C65A, C72A], [C65S, C72S], and [C40A, C95A] (*25*−*27*). The two des species have significant enzymatic activities (*23*, *29*), and their prolines appear to be in their native isomeric state (*25*−*28*). Both species exhibit cooperative, two-state transitions under chemical (*28*, *74*) and thermal (*25*, *26*, *74*) denaturation, albeit with reduced stabilities relative to those of native RNase A. The 65−72 and 40−95 disulfide bonds stabilize the native state by destabilizing the unfolded state (by lowering its conformational entropy) and by stabilizing the native fold both locally and globally (*25*, *26*, *28*, *74*).

The other two des species, des[26−84] and des[58−110], have not yet been investigated as thoroughly, but regeneration studies indicate that they have stable native structure at low temperatures [≤15 °C (*30*)] or in the presence of high phosphate concentrations [>100 mM (*31*)]. Mutant analogues of these species exhibit significant enzymatic activity (*29*, *32*) and cooperative thermal transitions with a T_m of ≈25 °C for both species (*32*).

Cooperativity of Oxidative Folding in RNase A

As shown above, the early disulfide intermediates have weak conformational order, whereas the late intermediates have nearly native structure. A key question is whether the native structure develops from the weak conformational order gradually (noncooperatively) or suddenly (cooperatively) as disulfide bonds are added.

The present evidence suggests that the conformational structure develops cooperatively, i.e., in a two-state fashion, in RNase A. The average intrinsic rates of reduction and reshuffling appear to be constant in the 1S−3S ensembles (*43*, *47*), contrary to the gradual-structure hypothesis, which would predict a monotonic decrease in the rate constants. Moreover, no disulfide species with partial protection against reduction have been observed; a reduction pulse of DTT^{red} (5 mM) reduces all disulfide species to R except those with fully native-like structure, e.g., des[40−95] and des[65−72] (*46*). Thus, the process of oxidative folding resembles an ordinary equilibrium folding transition (e.g., by temperature), in that the native structure appears cooperatively in an "all-or-none" fashion as the protein is gradually stabilized (by adding native disulfide bonds).

This "all-or-none" character implies that the structured des species are formed from the unstructured 3S ensemble in two sequential reactions. In the first reaction, the disulfide bonds of the des species form by reshuffling from the unstructured ensemble 3S, but the protein has little structure initially; we denote this state as des_U. In the second reaction, the protein folds conformationally to its native-like structure, denoted as des_N. Schematically, these reactions can be written as $3S \rightarrow des_U \rightarrow des_N$, where the first and second reactions are the reshuffling reaction and conformational folding, respectively. The back-reactions are also possible, i.e., conformational unfolding ($des_N \rightarrow des_U$) and back-reshuffling ($des_U \rightarrow 3S$). Experiments designed to test the validity of this model and to characterize des_U are underway.

Not all single-domain proteins exhibit cooperative formation of structure upon formation of native disulfide bonds. A counterexample is BPTI, in which the formation of the 30−51 disulfide bond causes the formation of local secondary and tertiary structure around the disulfide bond without causing global structure formation (*75*). Subsequent experiments have shown that, despite its small size, BPTI is composed of two sequentially folding subdomains (*76*) corresponding to its two hydrophobic cores (*77*, *78*).

Implications for Conformational Folding

Two features of the oxidative folding are remarkable. First, only native disulfide bonds appear to cause the formation of stable tertiary structure; in no case studied thus far has such structure developed in response to the formation of a nonnative disulfide bond. Second, only native tertiary structure develops in oxidative folding; misfolded proteins have not been observed, despite the fact that the pre-equilibrium ensembles sample most or all of the possible disulfide species (*48*, *63*). (Misfolded proteins are defined here as proteins with stable nonnative tertiary structure.)

These two observations cast doubt on "rugged funnel" models of protein folding (*79*), and lead one to hypothesize that misfolded states are common in the energy landscape, and serve as kinetic traps for folding proteins. If such misfolded states did populate the energy landscape densely, then one or more such misfolded states should occur among the conformations populated by the nonnative disulfide species; these misfolded states should be stabilized considerably by the disulfide bonds and, hence, should be observable as kinetic traps. It has been predicted explicitly that proteins will collapse into a glassy (i.e., slowly rearranging), misfolded state if they are kept from entering the "native folding funnel" (e.g., by nonnative disulfide bonds) (*80*). However, no such misfolded kinetic traps have been observed in the oxidative folding of any protein; the disulfide species of the pre-equilibrium ensembles reshuffle quickly and appear to have no protective tertiary structure, despite having many nonnative disulfide bonds. A few kinetic traps have been observed in oxidative folding (e.g., des[26−84] and des[58−110] in RNase A), but these result from the burial of thiol groups in disulfide species with *native* disulfide bonds and *native* structure, and do not correspond to species with nonnative disulfide bonds and misfolded structures. Misfolded states have indeed been observed in the folding of a few proteins, e.g., in disulfide-intact hen egg-white lysozyme (*81*−*83*); however, the present evidence suggests that such states are relatively rare in the energy landscape of most proteins under typical folding conditions.[2]

Another remarkable feature of oxidative folding is that a spatially localized change (the addition of a single disulfide bond) can cause cooperative, global folding of the entire protein. For example, the des[65−72] and des[40−95] species of RNase A have native-like structure, whereas the (26−84, 58−110) species with two native disulfide bonds exhibits no more conformational order than nonnative 2S species (*73*). This observation suggests a surprisingly strong cooperativity between local and global folding, namely, that the strengthening of native interactions in a localized region of a single-domain protein (e.g., by forming a native disulfide bond) generates local structure only in concert with formation of the global native structure. This coupling is consistent with a multi-nucleation mechanism of conformational folding. [BPTI provides a counter example to this coincidence of local and global structure formation (*75*), because it is effectively composed of two sequentially folding domains (*76*).]

The formation of a disulfide bond does have a global effect on the interactions between protein residues, by altering the loop entropy penalty to bring those residues together and, hence, changing the effective concentrations of the protein residues for each other throughout the entire protein. In particular, a native disulfide bond may promote the formation of a productive folding nucleus by juxtaposing its residues and lowering the entropic penalty for structuring those residues. This is plausible, since disulfide bonds are often buried in local hydrophobic regions, i.e., regions composed of hydrophobic residues separated by relatively few residues along the backbone or across a disulfide bond. Such local hydrophobic regions have also been proposed as the nucleation sites for conformational folding, e.g., in the chain-folding initiation site (CFIS) model (*84*−*89*).

Parallels between Disulfide-Bond Regeneration and Conformational Folding

As noted in the introductory section, oxidative folding is a composite process, consisting of two processes: the recovery of the native disulfide bonds (disulfide-bond regeneration) and the recovery of the native tertiary structure (conformational folding). Despite the obvious differences between these two processes, they share several important features. The fundamental difficulty of both searches (that for the native disulfide pairing and that for the native tertiary contacts) is the large entropy associated with alternative disulfide pairings and tertiary contacts. Moreover, the individual interactions stabilizing the native disulfide pairing and tertiary contacts are not grossly stronger than nonnative interactions; native and nonnative disulfide bonds presumably have similar strengths, as do native and nonnative hydrogen bonds or hydrophobic interactions. Given these two factors, both searches are likely to sample nonnative disulfide pairings and nonnative tertiary contacts, respectively. The qualitative similarities of these two searches suggest that the strategies adopted by the protein to find the native disulfide pairing may also pertain to the search for the native tertiary structure. In this section, the general mechanism of disulfide-bond regeneration is reviewed and an analogous scenario for conformational folding is suggested.

[2] We do not consider aggregation equivalent to misfolding.

As illustrated above for RNase A, proteins generally adopt a "lock-in" strategy in the regeneration of their native disulfide bonds. After the initiation of oxidizing conditions, the protein equilibrates rapidly to a distribution of disulfide species that is biased in favor of native disulfide pairing but which generally contains many nonnative disulfide species, e.g., the pre-equilibrium of *n*S ensembles in RNase A. The disulfide species of the pre-equilibrium ensembles rearrange rapidly until a minimal set (or sets) of native disulfide bonds are formed that induce conformational folding, e.g., des[40−95] and des[65−72] in RNase A. The resulting tertiary structure protects these disulfide bonds from further rearrangement by sequestering them away from the redox reagent and the thiolate groups of the protein itself. (No nonnative set of disulfide bonds appears to become protected in this way.) This "locking in" of the native disulfide bonds causes such intermediates to accumulate, which favors the subsequent formation of the remaining native disulfide bonds (presuming that no kinetic traps are encountered).[3] In effect, the formation of protective tertiary structure stabilizes the buried native disulfide bonds by drastically lowering the rates of their reduction and reshuffling; in the absence of such tertiary structure, these species do not accumulate above the level determined by the bias in pre-equilibrium ensembles. Thus, such a minimal set of native disulfide bonds is populated preferentially not because its formation is strongly favored (e.g., 3S $\rightarrow$ des_U in RNase A) but because its rearrangement is strongly disfavored (e.g., $des_N \rightarrow des_U \rightarrow$ 3S in RNase A).

Proteins may adopt a similar strategy in conformational folding. In such a scenario, the protein would pre-equilibrate upon initiation of refolding conditions to an unstructured ensemble of conformations that is biased toward the native structure but includes many nonnative conformations. The conformations of this ensemble would rapidly rearrange until they formed a minimal subset (or subsets) of native interactions that are relatively protected from further rearrangement by being sequestered in local structure away from competing interactions with other groups in the protein and the solvent. (By assumption, no nonnative structure forms such a core of protected interactions.) Moreover, the formation of such a core promotes the formation of structure in adjacent residues, since much of the entropic penalty has been paid (*87*); adjacent residues have a higher effective concentration due to the initiating structure. [Such an effect is seen in the "zippering" between β-strands (*88*, *89*) and in the cooperative formation of α-helices (*90*).] Thus, as in disulfide-bond regeneration, the formation and protection of local native structure drives the protein toward the fully folded state by increasing the forward rate of native structure formation and decreasing the backward rate of its rearrangement.

A clear example of this scenario is provided by the formation of the native backbone hydrogen bonds (secondary structure). These hydrogen bonds rearrange by the attack of water molecules or of other main-chain and side-chain hydrogen-bonding groups; water is particularly effective at such rearrangements, since it forms strong hydrogen bonds and competes very effectively with the protein peptide groups for hydrogen bonding (*91*). Thus, stable native secondary structure requires that its hydrogen bonds be protected from such attack, e.g., by the association of nonpolar groups near the hydrogen bond to lower the local activity of water and other attacking groups. [In effect, nonpolar residues modulate the strength of hydrogen bonds in their vicinity (*92*).] This scenario is supported by experiments and computer simulations suggesting that the destruction of secondary structure is principally mediated by the attack of water on the backbone hydrogen bonds (*93*−*96*). Clearly, an analogous mechanism could be proposed for hydrophobic interactions between nonpolar residues.

A key element of this scenario is that the native substructure is significantly more protected from rearrangement than nonnative substructures of the pre-equilibrium ensemble. However, the mechanism for discriminating native from nonnative substructures seems obscure, since both are stabilized by the same physical interactions (hydrogen bonding, burial of hydrophobic area, etc.) which should have comparable strengths. One plausible discriminating factor is the unusually high density of the native packing (*97*, *98*). A dense packing should protect a core of local structure more effectively than a loose packing, both because there are intrinsically fewer groups exposed to attack and because the unfolding of such densely packed substructures would likely require concerted changes in several residues. By contrast, a loosely packed core would presumably expose more groups to attack and could be unfolded in an uncooperative fashion, by small changes in individual residues. Nonnative structures would be very likely to adopt loose packings, because such packings are favored by entropy, requiring no specific spatial arrangement of the residues. Thus, in such a scenario, the native structure is distinguished from nonnative structures by an unusually dense packing that offers better protection against rearrangement.

Engineered Covalent Cross-Links as Probes of Tertiary Structure in the Folding Transition-State Ensemble

To assess this and other folding scenarios, experimental methods of characterizing the development of structure during conformational folding, which may be rapid and complex, are needed. One such method involves the addition of covalent cross-links for determining whether protein segments are associated in the folding transition state, as follows.

If the stabilizing effect of an added covalent cross-link is primarily entropic (i.e., if the added cross-link does not affect the enthalpy), then the effect of such an added cross-link on the folding and unfolding rates determines whether the linked segments of the protein are associated in the folding transition state of the wild-type protein (Figure 3). Specifically, if the added cross-link accelerates the folding but does not affect the unfolding, then the two segments are associated in the folding transition state (Figure 3a); the added cross-link destabilizes the unfolded state, but not the native and transition states. Conversely, if the added cross-link decelerates the unfolding but does not affect the folding, the two segments are not associated in the transition state (Figure 3b); the added cross-link destabilizes both the unfolded and transition states, but not the native state. Such a study has

[3] For simplicitly, we are considering here only situations in which this structure forms in a disulfide intermediate (e.g., des[40−95]). It should be noted, however, that some proteins fold only when all the native disulfide bonds are formed.

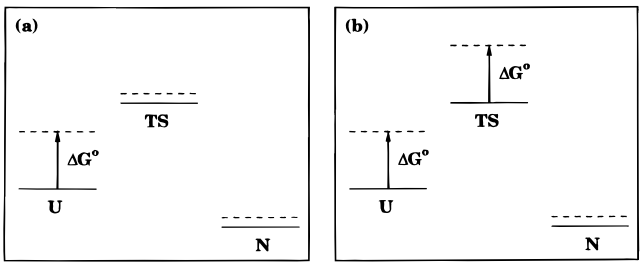

FIGURE 3: Addition of a covalent cross-link with primarily entropic effects can determine whether the two segments of the protein are associated in the transition state of folding (*100*). The relative free energies of the unfolded (U), folded (N), and transition (TS) states before and after adding such a covalent cross-link are indicated by the solid and dashed lines, respectively. By assumption, the addition of the covalent cross-link causes a large change in the free energy $\Delta G°$ of U and a relatively small change in the free energy of N. (a) If the two segments are associated in the wild-type transition-state ensemble, the TS free energy is not changed much by adding the covalent cross-link; the folding should become more rapid, but the unfolding rate should remain relatively constant. (b) If the two segments are not associated in the wild-type transition-state ensemble, the addition of the covalent cross-link changes the TS free energy by approximately the same amount $\Delta G°$ as in the unfolded state U. In this case, the folding rate should remain relatively constant, whereas the unfolding rate should become much slower.

been carried out for RNase A by linking two exposed lysine residues (Lys7 and Lys41) in the folded state (*7*, *99*, *100*); the results suggest that the N-terminal α-helix of RNase A is not associated with the major *β*-sheet in the folding transition state, as in Figure 3b.

This approach requires that the added covalent cross-link primarily change the entropy and not the enthalpy of the native and transition states. This requirement can be fulfilled most easily by linking solvent-exposed residues of the protein (particularly in flexible loop segments), since fewer native interactions are likely to be disrupted. Disulfide bonds can be introduced into such loop segments, but it may be preferable to engineer lysine cross-links, since lysines often are present in solvent-exposed, flexible loops whereas disulfide bonds tend to become buried in hydrophobic regions.

This approach is complementary to the more recent Φ-analysis approach (*101*) in that it alters the entropy, not the enthalpy, of the transition state, rendering its structural interpretation less ambiguous (*101*).

Summary

Disulfide bonds are useful tools for studying protein structure, thermodynamics, and folding. In particular, disulfide bonds may be used to assess the accessibility, proximity, and reactivity of cysteine residues. For example, the reductive unfolding of the protein reports on structural fluctuations in the native state because the protein must undergo local or global unfolding to render buried disulfide bonds accessible to the redox reagent. The proximity assay of disulfide bonds has been used to study *β*-hairpin propensities (*102*−*104*), the tertiary fold of the molten globule (*55*), and the rotation of molecular motors composed of many proteins (*105*). More generally, the distribution of disulfide-bonded species and the rates of their individual formation provide information about the conformational order in the protein; a disordered protein should show a random distribution of disulfide species (determined primarily by the entropy loss upon forming the disulfide bonds), whereas a highly ordered or structured protein should exhibit a strongly nonrandom distribution of disulfide species and bonds. As another example, the contribution of individual disulfide bonds to the conformational stability of the native state can often be assessed by reducing a single disulfide bond; provided that the resulting des species remains folded, its thermodynamic stability can be examined by equilibrium unfolding transitions and by the H/D exchange properties of its residues (*25*, *26*, *74*).

The oxidative folding of RNase A can be divided into two stages. In the first stage, the reduced protein is successively oxidized to populate the 1S−4S ensembles. These ensembles have no stable tertiary structure, although they exhibit conformational ordering, e.g., a nonrandom distribution of disulfide species. The second stage of oxidative folding consists of the disulfide reactions in the structured disulfide-containing intermediates that are stable enough to maintain their folded conformation (des species). The resulting tertiary structure stabilizes these folded species by protecting their native disulfide bonds from reduction and reshuffling; these structured species do not interconvert readily among themselves or with their unstructured ensemble. Under typical oxidative folding conditions, only des[40−95] and des[65−72] are stable enough to fold; these species are productive intermediates, since their remaining thiol groups are accessible, allowing these species to oxidize preferentially to the native protein. However, under unusually stabilizing conditions, des[26−84] and des[58−110] species can also acquire stable tertiary structure. When folded, these latter species, accounting for only a minor percentage of the total protein, appear to be kinetic traps, and presumably bury both their disulfide bonds and thiol groups, rendering all the reactive groups inaccessible to the redox reagent and to each other. Such traps may preferentially oxidize, reshuffle, or be reduced, depending on the redox conditions and the relative free energies of the unfolding reactions that must precede each reaction.

The processes of reductive unfolding and oxidative folding and the conformational structure of disulfide intermediates have several implications for conformational folding. The evidence seems to support multinucleation models of protein folding such as the CFIS model and to contradict the recently proposed "rugged funnel" scenarios of protein folding. By analogy with the regeneration of the native disulfide bonds, it is suggested that conformational folding proceeds through a biased random search of an initially unstructured ensemble, leading to the formation of a densely packed core of native structure that is protected from attack by the solvent and competing groups of the protein; this core then promotes the formation of the remaining native structure. The central idea of this scenario is that certain native structures become favored, not because they form much more quickly than non-native structures, but chiefly because they rearrange much more slowly.

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SUPPORTING INFORMATION AVAILABLE

Basic introduction to the procedures, such as blocking and fractionation methods, used in disulfide-bond studies (Appendix A) and an introduction to recent experiments which suggest that, under folding conditions, fully reduced RNase A may serve as a stable analogue of an early intermediate in the conformational folding of disulfide-intact RNase A (Appendix B). This material is available free of charge via the Internet at http://pubs.acs.org.

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