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Assessing the Magnitude of Folding Forces along the Oxidative Folding Pathway of Multi-Disulfide-Containing Proteins

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Oxidative folding is a process by which a reduced polypeptide acquires both its native disulfide bonds and its three-dimensional structure through thiol−disulfide exchange reactions and a conformational folding event, respectively.[1] Two major factors that influence the distribution of disulfide bonds (over their entropically favored probability of formation[2]) during the regeneration, eventually favoring and sustaining the native set of disulfide bonds, are enthalpic interactions that lead to conformational ordering[3] among intermediates, and conformational folding, resulting in a native (or nativelike) structure which protects the native disulfide bonds from reduction and intramolecular thiol−disulfide exchange reactions.[2,3] Here, we report a novel technique to provide a quantitative description of the enthalpic and conformational folding forces present at any stage of the oxidative folding process of multi-disulfide-containing proteins by assessing their ability to form "structures" which are able to protect disulfide bonds from reduction. Application of this technique to an unstructured 3S[4] ensemble of bovine pancreatic ribonuclease A (RNase A)[4] reveals that the enthalpic interactions that lead to conformational ordering of the ensemble are unable to protect the formed disulfides from reduction in this protein. Furthermore, by using a structured intermediate (des [40−95]) of RNase A as a model, we have obtained a quantitative estimate of the magnitude of the conformational folding event in terms of the impact that the resulting structure has on the susceptibility of the disulfide bonds to reduction, and have demonstrated the key role of a native (or nativelike) structure in the regeneration process. Finally, we discuss the applicability of this technique to assess the magnitude and impact of various folding events in the regeneration of multi-disulfide-containing proteins.

Multi-disulfide-containing proteins generally regenerate from their fully reduced forms (R) by first forming unstructured intermediates.[5] The distribution of disulfide bonds within any ensemble of isomers of an unstructured intermediate (e.g., 1S or 2S ensembles of the four-disulfide-containing RNase A) is governed by entropic and enthalpic factors.[2] Since the least loss of entropy is desired, disulfide bonds that form smaller loops are preferred over those that link distant cysteines in the primary sequence. However, due to favorable enthalpic interactions, the formation of disulfides that link distant cysteines may become more likely than those predicted by the entropic probability of formation.[2] Such enthalpic interactions give rise to a "conformational ordering" of the molecules within the unstructured ensemble.[3,6] While favorable entropic and enthalpic factors can accelerate the regeneration, the formation of stable structure through a conformational folding process has the greatest impact on the regeneration process[3] [a kinetically trapped intermediate exerts a negative impact on the regeneration process and can easily be distinguished from a productive intermediate[1]].

The "locking-in" of native disulfide bonds takes place upon conformational folding of the polypeptide (leading to their burial), and effectively inhibits them from reduction and reshuffling reactions (in the case of thiol-containing structured intermediates) which would otherwise reverse or stall the regeneration process.[3] The formation of the parent molecule from a structured intermediate requires oxidation to form the final disulfide bond, which lends additional stability and complete biological activity to the biopolymer.[1,3,7] An estimate of the protection of disulfide bonds in unstructured (albeit, conformationally ordered) and structured species is a useful quantity with which to monitor and provide a quantitative description of folding interactions that are present during various steps of the regeneration. We have accomplished this using RNase A as a model.

Des [40−95] (a stable three-disulfide-containing nativelike intermediate[8] of RNase A lacking the [40−95] disulfide bond), obtained from previous studies,[9] was used to generate the unstructured 3S ensemble (by intramolecular reshuffling reactions). This was carried out by placing unblocked des [40−95] (0.1 mg/mL) in an argon-sparged pH 8 buffer (100 mM Tris-HCl, 1 mM EDTA) at 50 °C for a period of 5 min, which is sufficient to reshuffle the structured species to its unstructured 3S isomers.[9] After 5 min, the quantitative conversion of des [40−95] to 3S was confirmed by blocking a small aliquot of the above solution with excess AEMTS,[10] and eluting the AEMTS-blocked mixture on a cation-exchange column, a procedure in which the elution positions of des [40−95] and 3S are known.[9] The pH of the remaining solution was reduced to 3 with glacial acetic acid to inhibit any air oxidation of the unstructured species, and the 3S ensemble was desalted on a reversed-phase column and lyophilized.

Lyophilized 3S was then divided into two parts. In part (i), 3S was reduced under strongly denaturing conditions and, in part (ii), the 3S was reduced under folding conditions (see legend of Figure 1 for details of the reduction procedure). Figure 1A shows a typical HPLC chromatogram of the reduction of the unstructured 3S ensemble (under strongly denaturing conditions) to R through the 2S and 1S ensembles. Figure 1B shows the kinetics of reduction of the 3S ensemble under strongly denaturing (○) and folding conditions (□), and also includes a simulation of the reduction of des [40−95] (- - -) using the rate constant for its reduction [carried out under conditions similar to the reduction of the 3S ensemble (see Figure caption)] from previously published data.[11]

We define k_d and k_f as the rate constants for reduction of the 3S species under strongly denaturing and folding conditions, respectively, and k_s as the rate constant for the reduction of the structured intermediate, des [40−95]. Under strongly denaturing conditions, enthalpic interactions in a protein can be considered to be negligible, and therefore, the disulfide bond population within any ensemble can be well-approximated by an entropic probability distribution.[2] Therefore, k_d is the rate constant for the reduction of disulfides whose distribution is influenced only by entropic probability. Under folding conditions, but in the absence of structure, both entropic factors and enthalpic interactions (the latter giving rise to a conformational ordering within the ensemble) dictate the formation of disulfide bonds.[3] Therefore, k_f represents the rate constant for

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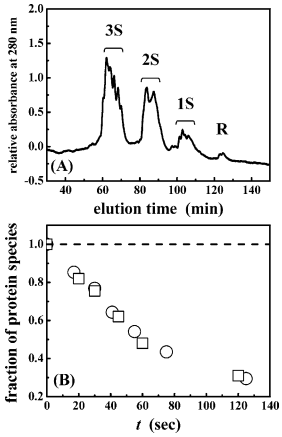

Figure 1. (A) Typical cation-exchange HPLC chromatogram showing the reduction of the 3S ensemble of RNase A by 1.2 mM DTT^{red} (pH 8, 6 M GdnHCl, 100 mM Tris-HCl, 1 mM EDTA, 15 °C). The concentration of DTT^{red} (1.2 mM) used for reduction allows the sequential reduction of 3S to be captured. (B) Plots showing the loss of 3S isomers of RNase A by reduction under strongly denaturing conditions (○) (6 M GdnHCl, pH 8, 100 mM Tris-HCl, 1 mM EDTA, 15 °C) and under folding conditions (□) (no GdnHCl, pH 8, 100 mM Tris-HCl, 1 mM EDTA, 15 °C). The superposition of the data constituting the reduction of the 3S isomers under strongly denaturing and folding conditions, respectively, indicate that the enthalpic interactions that are present in the ensemble under folding conditions[3,6] are unable to protect its disulfide bonds from reduction. The kinetics of the reduction processes was monitored by periodically withdrawing aliquots of the reaction mixture, blocking all free thiols with AEMTS, and then separating the products on a cation-exchange column.[9] The reduction of des [40−95] (- - -) (simulated from the rate constant at pH 8, 100 mM Tris-HCl, 1 mM EDTA, 15 °C, reported previously[11]) is also shown.

Table 1. Rate Constants for the Reduction of the 3S Ensemble of RNase A under Strongly Denaturing and Folding Conditions, Respectively, and for the Reduction of des [40−95] RNase A (pH 8, 15 °C)

rate constant	k_d	k_f	k_s[a]
$min^{-1}\ M^{-1}$	585 ± 15	600 ± 35	$(68 \pm 5) \times 10^{-4}$

[a] From ref 11.

the reduction of disulfide bonds (in unstructured intermediates) that may be "protected" by conformational ordering. Hence, by comparing k_f and k_d we can infer whether the conformational ordering that results from enthalpic interactions is able to protect the disulfide bonds from reduction. k_s, the rate constant for the reduction of des [40−95] is an indicator of the magnitude of protection (from reduction) offered to the disulfide bonds by nativelike structure, and therefore, a comparison of k_s and k_d enables us to draw inferences about the impact of structure on the protection of disulfide bonds using the reduction of an entropic distribution of disulfide bonds as a reference.

Table 1 lists the rate constants for the reduction of disulfide bonds obtained under the aforementioned experimental conditions. $k_d/k_f \approx 1$, whereas $k_d/k_s = 8.6 \times 10^4$, indicating an $\sim 10^5$-fold greater impact of the conformational folding event on the protection of disulfide bonds in des [40−95] compared to the negligible effect of conformational ordering on the protection of disulfide bonds in the 3S ensemble. Therefore, once the disulfide bonds are locked in within the protein, they become immune to reduction (and to reshuffling), and the regeneration of the native protein is thereby accelerated. The next step usually involves the oxidation of any free thiols (such as Cys 40 and Cys 95 in des [40−95]) to form the native protein, and such a productive intermediate can easily be differentiated from a kinetically trapped one as previously described.[1]

In oxidative folding studies, it is not always convenient to determine the exact structure of the stable disulfide-containing intermediates. Instead, stability measurements[12] can serve as a more convenient indicator of the presence of structure and also provide important thermodynamic information about the intermediate and the contribution of specific disulfide bonds toward maintaining stable structure.[7] Characterization of unstructured intermediates is more difficult and is often reported subjectively (such as "poor dispersion", "no transition", "weak interactions")[3]. In this study, we have obtained a quantitative estimate of the susceptibility of protein disulfides to reduction in both unstructured and structured intermediates using their reduction under strongly denaturing conditions as a reference point, thereby enabling us to obtain a direct and objective comparison of the effects of various folding forces that are present during the regeneration of the native protein.

Our technique can easily be extended toward understanding the interactions that are present in other oxidative folding processes. For example, in the oxidative folding of multi-disulfide-containing proteins having cofactors, the effect of the ligand on protein structure can be determined along any stage in the folding pathway (such as the effect of introducing the calcium atom during any stage of the oxidative folding of α-lactalbumin); the structural impact of a molten-globule conformation in α-lactalbumin can be estimated quantitatively, and the structural contributions of individual disulfide bonds in its molten-globule state can be assessed; the influence of buried thiols on the structure of any intermediate (such as the des [58−110] or des [26−84] intermediates of RNase A) can be determined and compared with isomers containing exposed thiols (such as des [40−95]).

In conclusion, we have demonstrated that it is easily possible to sample "structure-inducing" events and gain information about their magnitude and impact on the regeneration of multi-disulfide-containing proteins. When applied to RNase A, our results indicate that the conformational folding event in des [40−95] imparts an $\sim 10^5$-fold protection to the thiols in this intermediate compared to the protection imparted by conformational ordering (arising as a result of enthalpic interactions) in the 3S ensemble of RNase A, reinforcing the key role of structure in oxidative folding.

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(4) Abbreviations: RNase A, bovine pancreatic ribonuclease A; R, fully reduced RNase A; *n*S is an ensemble of disulfide-containing intermediates each having "*n*" disulfide bonds; AEMTS, 2-aminoethyl methanethiosulfonate; DTT^{red}, reduced dithiothreitol; des [*x*−*y*], species containing all native disulfide bonds, except the [*x*−*y*] disulfide bond.
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