Shifting the Competition between the Intramolecular Reshuffling Reaction and the Direct Oxidation Reaction during the Oxidative Folding of Kinetically Trapped Disulfide-Insecure Intermediates†

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ABSTRACT: The oxidative folding pathway(s) of single-domain proteins can be characterized by the existence, stability, and structural nature of the intermediates that populate the regeneration pathway. Structured intermediates can be disulfide-secure in that they are able to protect their existing (native) disulfide bonds from SH/SS reshuffling and reduction reactions, and thereby form the native protein directly, i.e., by oxidation of their exposed (or locally exposable) thiols. Alternatively, they can be disulfide-insecure, usually requiring global unfolding to expose their free thiols. However, such an unfolding event also exposes the existing native disulfide bonds. Thus, the subsequent oxidation reaction to form the native protein in a disulfide-insecure intermediate competes with the intramolecular attack by the thiols of the macromolecule on its own native disulfide bonds, resulting in a large population of intermediates that are reshuffled instead of being oxidized. Under stabilizing conditions, disulfide-insecure species become long-lived kinetically trapped intermediates that slowly and only indirectly convert to the native protein through reshuffling reactions. In this study, *trans*-$[Pt(en)_2Cl_2]^{2+}$, a strong oxidizing agent which has not traditionally been used in oxidative folding, was applied to shift the competition between reshuffling and oxidation reactions in des [58−110] and des [26−84], two long-lived disulfide-insecure intermediates of RNase A, and oxidize them directly under *stable* conditions to form the native protein. Such a successful direct conversion of kinetically trapped intermediates to the native molecule by *trans*-$[Pt(en)_2Cl_2]^{2+}$ may be helpful in facilitating the oxidative folding processes of multi-disulfide-containing proteins in general.

Much effort has been undertaken toward understanding the mechanism of oxidative folding of secreted proteins, a fundamental process in the cell (*1*−*4*).

In vitro studies have provided a framework for such a regeneration process in which native structure and native disulfide bonds are acquired as the fully reduced biopolymer is gradually converted into the biologically active macromolecule (*5*−*10*). In the oxidative folding process of multi-disulfide-containing proteins, structured intermediates play a particularly important role in the mechanism by which the reduced protein is regenerated to the native (disulfide-intact) form (*11*−*16*). During the regeneration process, unstructured intermediates are first populated, except when the reduced form is already folded or the protein has only one disulfide bond (the reduced form of the protein is not considered an intermediate) (*17*).

One important feature of unstructured intermediates in the oxidative folding process is that the remaining thiols and existing disulfides of the intermediate are prone to intramolecular exchange, leading to a continuous rearrangement of the disulfide bonds within a given intermediate (*18*−*20*). These intramolecular thiol−disulfide exchange reactions, also called disulfide reshuffling reactions, take place once every few seconds, depending on the ambient conditions (pH and temperature) and the spatial arrangement of the reacting thiols and disulfides in the intermediate (*21*). In structured intermediates, however, the existing disulfides are sequestered from the thiols of the protein (and from the redox reagent), resulting in the slowing of the thiol−disulfide rearrangement reaction by several orders of magnitude (as compared to thiol−disulfide exchange in the unstructured intermediate). As a result, structured species frequently accumulate during the regeneration process (*19*).

In bovine pancreatic ribonuclease A (RNase A)[1] and in many other single-domain disulfide-containing proteins, the structured species have acquired all but one native disulfide bond and are termed "des species" (*22*−*28*). This condition may not necessarily be true for every protein; for example, there are conditions under which an intermediate in BPTI that lacks *two* disulfide bonds can also fold conformationally (*29*). Nevertheless, the fate of such a structured species is determined by the relative protection of its thiols versus its

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; *n*S, ensemble of disulfide-containing intermediates each having *n* disulfide bonds; N, native RNase A; R, reduced RNase A; des [*x*−*y*], intermediate of RNase A having all native disulfide bonds but lacking the [*x*−*y*] disulfide bond; BPTI, bovine pancreatic trypsin inhibitor; AEMTS, 2-aminoethylmethylthiosulfonate; DTT^{ox} and DTT^{red}, oxidized and reduced dithiothreitol, respectively; GSSG, γ-L-glutamyl-L-cysteinyl-glycine (oxidized form). In $[Pt(en)_2Cl_2]^{2+}$, en is ethylenediamine.

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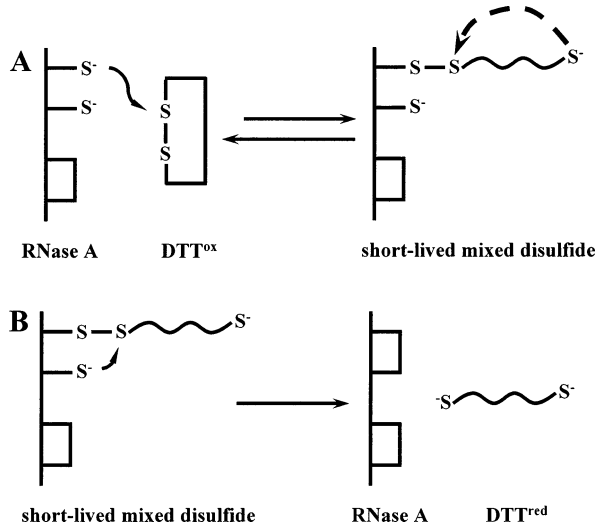

FIGURE 1: Schematic representation of the oxidation of protein thiols using the redox reagent DTT^{ox}. (A) First step of the reaction in which a mixed disulfide is formed between a protein thiol and DTT^{ox}, but the mixed disulfide is short-lived because of the possible attack by the terminal thiol, indicated by the dashed arrow, or because of step B. (B) Forward reaction showing the attack of a free protein thiol on the mixed disulfide to form the corresponding disulfide bond and the liberation of DTT^{red}.

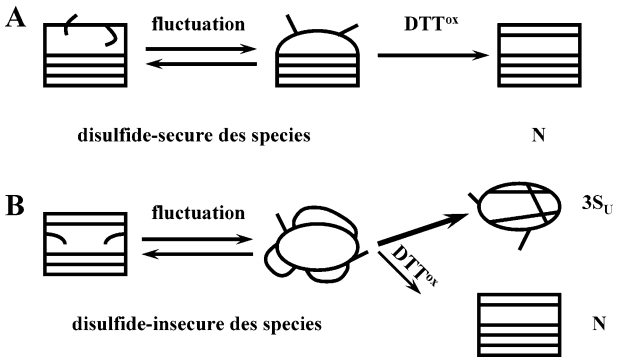

FIGURE 2: Schematic representation of the reaction between DTT^{ox} and a structured intermediate. (A) A disulfide-secure des species is able to expose its thiols by a local fluctuation unfolding process while keeping its existing disulfide bonds securely buried within the native-like structure. The redox reagent is then easily able to oxidize the free thiols to form the final disulfide bond. (B) A disulfide-insecure des species has buried thiols and can expose them for oxidation usually by a global unfolding process which also exposes the native disulfide bonds. In the second step, a competition ensues between the oxidation process (bimolecular reaction) and intramolecular reshuffling with most of the species being reshuffled.

disulfide bonds by stable native-like structure. To understand the effect of stable structure on the subsequent disulfide reactions, we have to consider the individual steps in the oxidation reaction. For simplicity, we limit our discussion to traditional redox reagents such as glutathione and dithiothreitol. In practice, the cyclic redox reagent, dithiothreitol, is often used instead of linear reagents because DTT does not form long-lived mixed disulfides with protein thiols, thereby making oxidative folding studies easier to interpret because of the reduced number of possible intermediates in the regeneration system (*30*).

The first step of the oxidation reaction, i.e., the formation of a mixed disulfide bond between the protein and the redox reagent, is a bimolecular step (Figure 1A) (*30*). The second step involves the attack by the (appropriate) free thiol of the protein on the mixed disulfide in formation of a new native disulfide bond, liberating the reduced form of the redox reagent in the process (Figure 1B). The rate of the first step can be accelerated by increasing the concentration of the oxidized form of the redox couple (e.g., GSSG). However, with an increase in the concentration of the oxidizing agent, mixed disulfides can also form readily with other thiol(s) of the protein and thereby interfere with the second reshuffling-like step of the oxidation. Therefore, there are optimum concentrations of the oxidized form of the redox reagents which result in maximal oxidation rates (*56*, *57*).

In a *disulfide-secure* intermediate, the thiols are exposed or can be exposed for oxidation by a local fluctuation unfolding event that does not expose the pre-existing native disulfide bonds (Figure 2A) (*19*, *31*). Thus, the attack by the protein thiols (reshuffling) and/or by the thiols of the redox reagent (reduction) on the formed disulfide bond(s) does not occur during the first and second steps of the oxidation. The oxidized form of the redox reagent (GSSG or DTT^{ox}) can oxidize the disulfide-secure species directly to form the native protein (Figure 2A). Examples of disulfide-secure intermediates are found in BPTI, lysozyme, and RNase T1 (*19*). In RNase A, des [40−95] and des [65−72] are two examples of three-disulfide-bond-containing native-like intermediates that were shown to be disulfide-secure (*24*, *25*).

In *disulfide-insecure* intermediates, the thiols and disulfides can only be exposed simultaneously, usually, by global unfolding (Figure 2B) (*19*, *31*). Thus, the fast one-step reshuffling reaction competes with both the first and second step of the oxidation reaction with the resulting unimolecular reshuffling reaction being favored over the two-step oxidation process (Figure 2B). Examples of disulfide-insecure intermediates are des [58−110] and des [26−84] in RNase A (*31*), and des [30−51] and des [5−55] in BPTI (*19*).

Thus, the stability and dynamics of the protein (global vs local fluctuation unfolding) and the molecular mechanism of the thiol−disulfide exchange reactions determine the nature of the structured intermediates *in vitro* (disulfide-secure or disulfide-insecure). *In vitro* techniques applied to consume the disulfide-insecure des species generally involve the addition of denaturants or detergents to disrupt the stable three-dimensional structure of the des species (*5*, *25*, *32*, *33*). While the consumption of the kinetically trapped intermediates may be rapid, the formation of N can still be slow because the destabilized species can only reshuffle to their unstructured isomers which may then form N only by reshuffling to other species, which is an indirect process. [In RNase A, the unstructured isomers (3S) formed by reshuffling from des [58−110] and des [26−84] can easily be oxidized to form the scrambled species (4S), and the 4S ensemble would need to be reduced back to the 3S isomers before being able to form N.]

While it has yet to be established whether folding catalysts (*34*−*40*) can oxidize these disulfide-insecure species directly *in vivo*, there are several possible ways in which these enzymes may accelerate the oxidation reaction selectively over the reshuffling reactions. In situations in which a mixed disulfide bond is formed between the catalyst and the protein thiol, there may be steric interference with the formation of a second mixed disulfide. Thus, the rate of the first step of

the oxidation can be increased by increasing the catalyst concentration without much restriction, in contrast to the uncatalyzed reaction; e.g., in an uncatalyzed reaction, using GSSG, this relatively small oxidizing agent can bond to two thiol groups and thereby prevent formation of a protein disulfide bond. A second way in which catalysts may accelerate the oxidation reaction selectively over reshuffling reactions is if the mixed disulfide bond between the catalyst and the protein thiol may be more reactive than the protein disulfide bond(s); therefore, the second step of the oxidation (Figure 1B), i.e., the thiolate attack on the mixed disulfide (between the second thiol of the protein and the mixed disulfide with the catalyst), is favored over the competing reshuffling reaction (between the second thiol of the protein and the nearby protein disulfide).

Several nontraditional linear and cyclic redox reagents have been described and used for oxidative folding (*41*−*43*). However, the conditions for the direct and efficient oxidation of kinetically trapped species have not been reported.

Here, we have attempted to shift the competition between the oxidation reaction and the thiol−disulfide reshuffling reaction in disulfide-insecure species which are in a stable but fluctuating local environment. For this purpose, we have used *trans*-$[Pt(en)_2Cl_2]^{2+}$, an oxidizing agent that specifically and rapidly oxidizes protein thiols, to form the corresponding disulfides (*44*, *45*).

EXPERIMENTAL PROCEDURES

Materials. RNase A (type 1-A, Sigma) was purified by cation exchange HPLC (SP-Sepharose, Amersham) according to the procedure of Rothwarf and Scheraga (*30*). Reduced RNase A (R) was prepared from the purified native protein as described previously (*30*). DTT^{ox} was obtained from Sigma and used without further purification. The thiol-blocking reagent AEMTS was synthesized by the procedure described by Bruice and Kenyon (*46*). *trans*-$[Pt(en)_2Cl_2]^{2+}$ was prepared by oxidation of *trans*-$[Pt(en)_2Cl_2]$ (*44*) and used directly in solution without further purification. The final concentration of *trans*-$[Pt(en)_2Cl_2]^{2+}$ was estimated from the absorbance at 332 nm (*44*) and, as a check, by reacting it with known concentrations of DTT^{red} at pH 5.

Conditions for Preferentially Populating Des [58−*110] and Des [26*−*84] (31, 47).* Fully reduced RNase A (10 mg/mL in a 50 mM acetic acid solution) was dissolved (final concentration of 30 μM) in a pH 8.3 buffer (100 mM Tris-HCl and 1 mM EDTA) containing 50 mM DTT^{ox} that had previously been equilibrated at 10 °C. The resulting pH of the solution was 8.0. Oxidative folding was allowed to proceed at 10 °C for a period of 20 h, after which the temperature of the mixture was dropped to 4 °C for an additional 10 h. The reaction mixture was sampled periodically by drawing aliquots and blocking the unreacted cysteines with AEMTS. The samples were desalted on a Sephadex G25 column before injection onto a cation exchange column (Rainin Hydropore SCX) for HPLC separation. During the HPLC runs, the salt gradient was increased linearly from 50 to 150 mM over a period of 130 min. AEMTS blocking introduces a positive charge for each blocked thiol and facilitates the separation of the *n*S intermediates of RNase A (*30*).

When the HPLC chromatogram indicated the exclusive presence of des [58−110] and des [26−84] along with the native protein (*31*, *47*), the remaining reaction mixture was frozen at −80 °C.

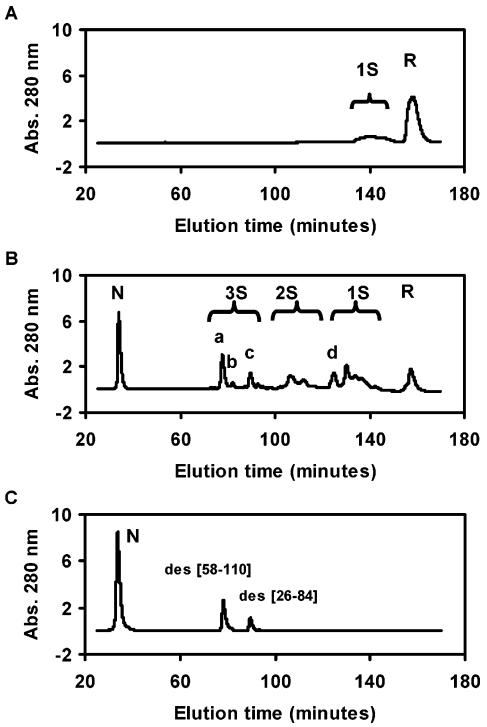

FIGURE 3: HPLC chromatograms showing the sequential steps involving oxidation of fully reduced RNase A (30 μM) at pH 8 (10 °C, 100 mM Tris-HCl, and 1 mM EDTA) using 50 mM DTT^{ox}. (A) The mixture was blocked with AEMTS 10 min after the start of the reaction, resulting in the formation of a small amount of 1S. (B) Formation of all intermediates and the native protein 4 h after the start of the reaction. Peaks labeled a−d are des [58−110], des [65−72], des [26−84], and des [40−95], respectively. (C) Presence of kinetically trapped des [58−110] and des [26−84] along with N, 30 h after the start of the regeneration.

Procedure for Determining the Kinetic Fate of Des [58−*110] and Des [26*−*84] under Strongly Oxidizing Conditions*. The previously frozen sample, containing a mixture of des [58−110], des [26−84], and N, was allowed to thaw in a water bath set at 10 °C. The sample was then divided into five equal parts (i−v). Sufficient AEMTS (from a 1 M stock solution) was added to part i to achieve a final AEMTS concentration of 40 mM. Part ii was introduced into a solution containing 6 M GdnHCl (pH 8) and DTT^{ox} (saturating concentration) such that the final concentration of the denaturant was 3 M. Unreacted thiols, if any, were blocked with AEMTS after 5 min. Excess *trans*-$[Pt(en)_2Cl_2]^{2+}$ was added to part iii, and the mixture was blocked after 10 s with AEMTS. A mixture of 6 M GdnHCl (pH 8) containing *trans*-$[Pt(en)_2Cl_2]^{2+}$ was added to part iv to achieve a final denaturant concentration of 3 M {with the *trans*-$[Pt(en)_2Cl_2]^{2+}$ present in a 10-fold excess over the protein concentration}, and the mixture was blocked with AEMTS after 1 min. GdnHCl was added (pH 8, 6 M) to part v to achieve a final denaturant concentration of 3 M. After 10 min, excess *trans*-$[Pt(en)_2Cl_2]^{2+}$ was added to this mixture, and then any free thiols were blocked with AEMTS.

RESULTS

Preferentially Populating Des [58−*110] and Des [26*−*84].* Figure 3A shows the HPLC elution profile of

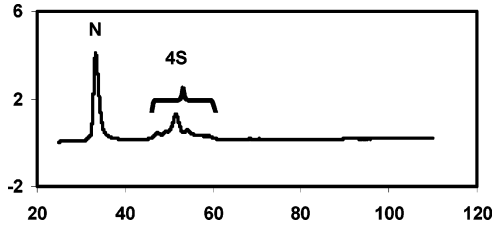

FIGURE 4: HPLC chromatogram showing the formation of 4S when 6 M GdnHCl and DTT^{ox} (saturating conditions) were added simultaneously to the solution containing des [58−110], des [26−84], and N (i.e., solution ii in Experimental Procedures).

reduced RNase A that was incubated with 50 mM DTT^{ox} (pH 8, 100 mM Tris-HCl, 10 °C) and blocked after 10 min of the start of the regeneration process with AEMTS. Figure 3B shows an aliquot of the same mixture that was blocked after 4 h. The presence of other intermediates along with des [58−110] and des [26−84] can quite clearly be identified from the chromatogram after comparison with previous studies from our lab (*31*, *47*). Figure 3C is an HPLC chromatogram of an aliquot of the solution that was blocked 30 h after the oxidative folding had started. Only native protein along with des [58−110] and des [26−84] is present. This is because all other intermediates have been converted to N (via des [40−95] and des [65−72]) (*31*, *47*). Thus, even under conditions in which 50 mM DTT^{ox} is present, des [58−110] and des [26−84] are long-lived kinetic traps (*31*, *47*, *48*).

Competition between Oxidation (using DTT^{ox}) and Reshuffling Reactions in Disulfide-Insecure Intermediates. At 10 °C, des [58−110] and des [26−84] are stable kinetic traps that are unable to be oxidized by 50 mM DTT^{ox} during the time course of the experiment (Figure 3C). As demonstrated previously, they can only reshuffle slowly to form the unstructured isomers (*31*). Figure 4 shows the result 5 min after the simultaneous addition of GdnHCl (3 M) and a saturated solution of DTT^{ox} to the mixture containing des [58−110], des [26−84], and N (solution ii). Des [58−110] and des [26−84] are no longer present in the chromatogram, but a quantitative amount of the 4S ensemble is now visible.

The addition of the denaturant to the mixture containing the des species results in the unfolding of des [58−110] and des [26−84]. Since the oxidant (saturating concentration of DTT^{ox}) was added along with the denaturant, a competition exists between thiols 58 and 110 of unfolded des [58−110], and thiols 26 and 84 of unfolded des [26−84], to undergo either intramolecular reshuffling reactions or become oxidized to form N (the native RNase A that was originally present in the mixture is unaffected in this experiment).

Kinetic Fate of Des [58−110] and Des [26−84] in the Presence of trans-$[Pt(en)_2Cl_2]^{2+}$. Figure 5A shows the region of the HPLC chromatogram where the native protein, des [58−110], and des [26−84] elute (solution i). When *trans*-$[Pt(en)_2Cl_2]^{2+}$ is added to the solution containing N, des [58−110], and des [26−84] (solution iii), the latter two species vanish and only N remains as seen in Figure 5B. Area analysis of the peaks under des [58−110] and des [26−84] and the difference between the areas under the native protein peaks in panels A and B of Figure 5 indicate

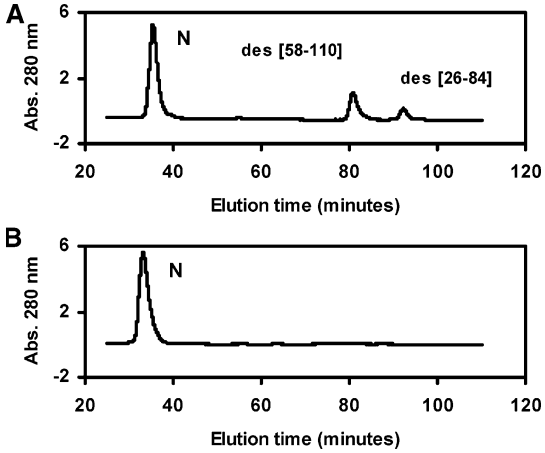

FIGURE 5: (A) HPLC chromatogram (same as in 3C) focusing on the area where N, des [58−110], and des [26−84] elute. (B) Addition of excess *trans*-$[Pt(en)_2Cl_2]^{2+}$ to the mixture used for panel A, resulting in the quantitative conversion of des [58−110] and des [26−84] to N.

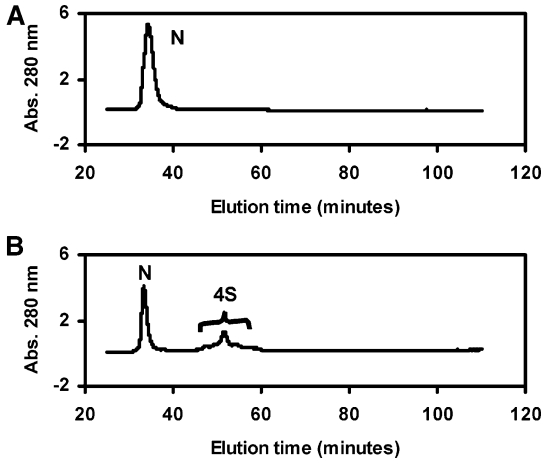

FIGURE 6: (A) HPLC chromatogram resulting from the simultaneous addition of 6 M GdnHCl and excess *trans*-$[Pt(en)_2Cl_2]^{2+}$ to the mixture used to generate Figure 5 (A) (same as 3C). (B) Results of the addition of 6 M GdnHCl to the mixture used to generate Figure 5 (A) (same as 3C) followed by the addition of *trans*-$[Pt(en)_2Cl_2]^{2+}$.

the complete conversion of the disulfide-insecure intermediates to the native protein.

Shifting of the Competition between Oxidation and Reshuffling Reactions in Disulfide-Insecure Intermediates Using trans-$[Pt(en)_2Cl_2]^{2+}$. Figure 6A shows the results of the simultaneous addition of *trans*-$[Pt(en)_2Cl_2]^{2+}$ and GdnHCl to solution iv containing des [58−110], des [26−84], and N (as in Figure 5A). The disappearance of the disulfide-insecure des species and the quantitative increase in the peak area of the native protein indicate that the oxidation of the free thiols of the two des species (to form the fourth and final native disulfide bond in each of the species) was much faster than intramolecular thiol−disulfide exchange reactions even under conditions in which the disulfide bonds were prone to attack.

Figure 6B shows the results of a control experiment in which the denaturant was added to the solution containing the disulfide-insecure intermediates prior to the addition of *trans*-$[Pt(en)_2Cl_2]^{2+}$ (solution v). The disappearance of des [58−110] and des [26−84] and the (quantitative) appearance of the 4S ensemble are evident.

DISCUSSION

In the oxidative folding of multi-disulfide-containing proteins under stabilizing conditions, disulfide-insecure species become long-lived kinetically trapped intermediates (*19*, *31*, *48*). A global unfolding event is usually necessary to expose the buried thiols in such intermediates, at which time there exists a *competition* between *intramolecular reshuffling reactions (SH/SS)* and the *two-step oxidation reaction* with an external oxidizing agent (e.g., GSSG or DTT^{ox}) (*19*, *31*). A large percentage of such an intermediate is then reshuffled to its unstructured isomer because intramolecular reshuffling is much faster than oxidation. In this work, we have shifted the competition between the two competing processes by using a powerful oxidizing agent {*trans*-$[Pt(en)_2Cl_2]^{2+}$} and, as a result, have been able to oxidize disulfide-insecure species rapidly to form the native protein directly.

Nature of the Structured Intermediates and the Role of Traditional Redox Reagents in Oxidative Folding. During the oxidative folding of RNase A with DTT^{ox} ($\leq$25 °C), a sequential distribution of unstructured ensembles in various states of oxidation is formed from R which then reaches a steady state (*30*). The critical and rate-determining step (25 °C) involves the formation of two structured three-disulfide-containing species (des [40−95] and des [65−72]) by thiol−disulfide exchange reactions from their unstructured isomers (3S species) (*24*, *25*). On the other hand, all four des species of RNase A are stable when oxidative folding is carried out at the lower temperature (10 °C) and, as a result, accumulate during the regeneration process (*47*).

As described in the introductory section, des [40−95] and des [65−72] are disulfide-secure species that are converted relatively rapidly to the native protein and eventually disappear from the HPLC chromatogram (Figure 3C). On the other hand, des [58−110] and des [26−84] are disulfide-insecure species, which are long-lived kinetically trapped intermediates (*31*, *47*) under the conditions that were applied (10 °C and 50 mM DTT^{ox}). Thus, they are still present even 30 h after commencement of the regeneration process, during which time all other intermediates have been converted to the native protein (Figure 3C). With a linear reagent such as glutathione, which forms a stable mixed disulfide with the protein thiol, the effective blocking of every protein thiol, at high concentrations of the oxidizing agent, to form the corresponding mixed disulfides prevents us from increasing the oxidation rate. However, DTT^{ox}, which was used in this experiment, is unable to form stable mixed disulfides under ordinary circumstances (*49*) and, as a result, is a weak oxidizing agent. Thus, it is only the low solubility of DTT^{ox} that places an upper limit on the oxidation rate.

Competition between Oxidation (using DTT^{ox}) and Reshuffling Reactions in Des [58−110] and Des [26−84]. Previous studies on the kinetic fate of des [58−110] and des [26−84] indicate that they are able to convert to the native protein only indirectly by first reshuffling to their unstructured isomers, viz., 3S species (*31*). Such an indirect conversion arises because reshuffling reactions are much faster than oxidation reactions; to demonstrate this, des [58−110] and des [26−84] were subjected to a saturated concentration of DTT^{ox} in conjunction with denaturing concentrations of GdnHCl (solution ii). The denaturant is required to enhance the unfolding ("breathing") of the protein, during which it has a chance to reshuffle to its 3S isomers before oxidation can take place. From the quantitative conversion of these two intermediates to the 4S ensemble, it is evident that, even at a saturating concentration of DTT^{ox}, the oxidation of the protein thiols is slower than the reshuffling reactions in the guanidinium-unfolded protein (Figure 4). This result demonstrates how reshuffling occurs much faster than oxidation during the real regeneration process when these disulfide-insecure intermediates occasionally unfold globally.

Kinetic Fate of Des [58−110] and Des [26−84] in the Presence of the Strong Oxidant trans-$[Pt(en)_2Cl_2]^{2+}$ and the Underlying Basis for the Results. In contrast to DTT^{ox}, *trans*-$[Pt(en)_2Cl_2]^{2+}$ is able to oxidize these disulfide-insecure intermediates directly to the native protein as judged by the disappearance of des [58−110] and des [26−84] without the appearance of any scrambled species (4S) (Figure 5A,B). To support this conclusion, in a control experiment, disulfide-insecure des [58−110] and des [26−84] were first denatured and allowed to reshuffle to their unstructured isomers (viz., the 3S ensemble) before *trans*-$[Pt(en)_2Cl_2]^{2+}$ was added (solution v). The resulting 4S species, formed by the oxidation of the unstructured 3S isomers, were easily detected on the chromatogram (Figure 6B).

To demonstrate that the oxidation by *trans*-$[Pt(en)_2Cl_2]^{2+}$ was indeed faster than reshuffling under our experimental conditions, GdnHCl and the platinum reagent were added simultaneously to des [58−110] and des [26−84] (solution iv) (Figure 6A). This resulted in the conversion of these two disulfide-insecure des species directly to the native protein (without the appearance of any 4S intermediates on the same time scale as the previous experiment). Thus, our data indicate that *trans*-$[Pt(en)_2Cl_2]^{2+}$ is capable of oxidizing the thiols faster than any intramolecular thiol−disulfide reshuffling that could otherwise occur upon the fluctuation global unfolding of a disulfide-insecure species.

One must consider an alternative (indirect) mechanism for the formation of N from des [58−110] and des [26−84]. In such a mechanism, the −SCl adduct formed by the platinum reagent with the protein thiol unfolds the disulfide-insecure des species. Once unfolded, des [58−110] and des [26−84] can easily reshuffle to the disulfide-secure species (via their unstructured 3S isomers) and then become oxidized to N (disulfide-insecure species → unstructured 3S isomers → disulfide-secure species → N). Such an indirect method for forming N can easily be ruled out by considering previous kinetic data on the oxidative folding of RNase A (*25*). Kinetic studies of the regeneration of reduced RNase A have shown that the formation of the disulfide-secure des species (i.e., des [40−95] and des [65−72]) from their 3S isomers constitutes the rate-determining step (requiring hours to go to completion) (*25*). Considering that the mixture containing des [58−110] and des [26−84] (solution iv in Experimental Procedures) was blocked with AEMTS 1 min after the simultaneous addition of GdnHCl and *trans*-$[Pt(en)_2Cl_2]^{2+}$, and the resulting HPLC chromatogram (Figure 6A) showed the presence of N alone, the alternative (indirect) mechanism can be ruled out unequivocally because there was no time for the disulfide-insecure species to form N via disulfide-secure species.

A scenario that would explain the inability of a traditional oxidizing agent such as DTT^{ox} to oxidize the disulfide-insecure species to N might be envisaged when considering

that des [58−110] and des [26−84] are stable for 1 day at 8 °C in the presence of 100 mM DTT^{ox} (*48*), suggesting that global unfolding of these disulfide-insecure species is not very frequent. However, it was demonstrated in this study that both species were oxidized in a few seconds by *trans*-$[Pt(en)_2Cl_2]^{2+}$, indicating that global unfolding does take place within relatively short time intervals. The fact that the platinum reagent can reach the thiols is also consistent with the fact that AEMTS blocking of the thiols in des [58−110] and des [26−84], a process that requires the exposure of their thiols, takes place in a few seconds (*31*, *47*, *48*, *50*). Taken together, the evidence presented above suggests that, although global unfolding may take place frequently in the disulfide-insecure intermediates, the time spent in the unfolded state is not sufficiently long for DTT^{ox} to oxidize the thiols or for reshuffling. In contrast, the much more potent platinum reagent is successfully able to oxidize the disulfide-insecure species efficiently during the brief time that the disulfide-insecure species is globally unfolded.

Our experiments have demonstrated that it is possible to alter the outcome of the competition between reshuffling and oxidation that is inherently present in a disulfide-insecure species. Reshuffling is favored over oxidation when "traditional" redox reagents are used and can convert these disulfide-insecure species to the native protein only indirectly.

The first step in the oxidation with the platinum reagent is the transfer of Cl^+ from *trans*-$[Pt(en)_2Cl_2]^{2+}$ to the protein thiolate, resulting in the formation of an −SCl adduct (*44*), a procedure somewhat akin to the formation of a mixed disulfide with a traditional redox reagent. Nevertheless, unlike a reaction with GSSG (an increase in the concentration of which would lead to blocking of both free thiols of the protein and prevent the formation of N), such blocking of a *pair* of thiols does not seem to occur with the platinum reagent. Thus, the rate of formation of the −SCl adduct does not seem to limit the oxidation rate of the disulfide-insecure species to N. This is perhaps because of the instability of the −SCl adduct. In the second step of the oxidation process, the formation of the protein disulfide bond (by an attack of the second protein thiol on the −SCl adduct) is evidently much faster than any reshuffling reaction (by an attack of the second protein thiol on neighboring protein disulfide bonds), suggesting that the −SCl adduct is much more reactive (or perhaps attractive) than any protein disulfide bond in the neighborhood, thereby effectively shifting the competition.

Long-lived kinetic traps, which are still present when all other intermediates have been transformed to the native protein, were reported to be present in the oxidative folding studies of several proteins (*51*−*53*). In BPTI, the des [30−51] species is stable for weeks, and the des [5−55] species is stable for several hours (*32*, *54*). In lysozyme, at the end of the regeneration, 60% of the protein accumulates as a des [76−94] kinetically trapped intermediate when all other species have been converted to N (*28*). In the case of BPTI, which is expressed *in vivo* as a precursor (proBPTI) containing an extra cysteine in the pro region, nature seems to have successfully accelerated the oxidative folding of this trypsin inhibitor by employing the extra cysteine as an intramolecular thiol−disulfide reagent, thereby eliminating the need for global unfolding of the kinetically trapped intermediate (*55*). Here, we show that the delivery of an oxidative pulse using *trans*-$[Pt(en)_2Cl_2]^{2+}$ at the end of the regeneration process of proteins with long-lived kinetically trapped species can convert these species quantitatively to N, thereby accelerating the oxidative folding process. Thus, this compound may find its way to large-scale production of recombinant proteins in biotechnology applications.

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