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Development of a Novel Method To Populate Native Disulfide-Bonded Intermediates for Structural Characterization of Proteins: Implications for the Mechanism of Oxidative Folding of RNase A†

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Abstract: RNase A, a model protein for oxidative folding studies, has four native disulfide bonds. The roles of des [40−95] and des [65−72], the two nativelike structured three-disulfide-bonded intermediates populated between 8 and 25 °C during the oxidative folding of RNase A, are well characterized. Recent work focuses on both the formation of these structured disulfide intermediates from their unstructured precursors and on the subsequent oxidation of the structured species to form the native protein. The major obstacles in this work are the very low concentration of the precursor species and the difficulty of isolating some of the structured intermediates. Here, we demonstrate a novel method that enables the native disulfide-bonded intermediates to be populated and studied regardless of whether they have stable structure and/or are present at low concentrations during the oxidative folding or reductive unfolding process. The application of this method enabled us to populate and, in turn, study the key intermediates with two native disulfide bonds on the oxidative folding pathway of RNase A; it also facilitated the isolation of des [58−110] and des [26−84], the other two nativelike structured des species whose isolation had thus far not been possible.

The oxidative folding of bovine pancreatic ribonuclease A (RNase A), a four disulfide-bond containing protein (see Figure 1), is one of the best-characterized oxidative folding processes.[1−3] Nevertheless, some of the key steps are still not understood. Recent work focuses on both the formation of the structured disulfide intermediates from their unstructured precursors (constituting the rate-determining step) and the subsequent oxidation of the structured species to form the native protein.

For a better understanding of the need for and the difficulties associated with the structural characterization of the precursor species of the nativelike structured des species, we briefly survey the oxidative folding process. The term oxidative folding describes the composite process in which a reduced, denatured protein regenerates its native disulfide bonds with the concomitant formation of its stable structure. This is generally a three-stage process (see Figure 2).[3−5] In the first pre-folding stage, unstructured intermediates are populated and interconvert rapidly among one another by thiol−disulfide exchange reactions. Without a protecting structure, the distribution of these unstructured intermediates is governed by loop entropy and by enthalpic interactions.[6−9] The thermodynamic weight of each unstructured

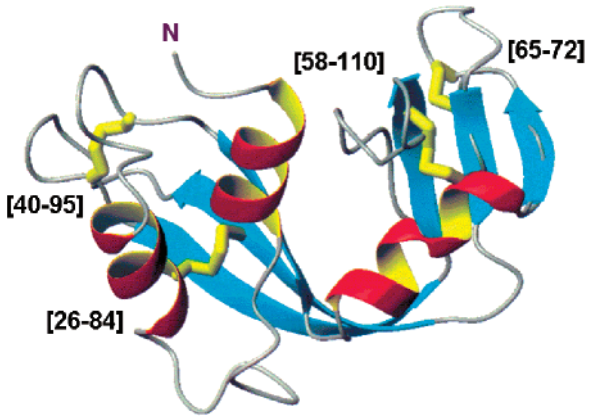

Figure 1. Ribbon diagram of the crystal structure of RNAse A with its four disulfide bonds depicted. Disulfide bonds 65−72 and 40−95 occur near the surface of the protein in turn regions. In contrast, 26−84 and 58−110 are fully buried in the hydrophobic core of the protein. They both connect α-helix domains to β-sheet secondary structure.

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† RNase A, Bovine Pancreatic Ribonuclease A; des species, RNase A with three native disulfide bonds, and missing the fourth native disulfide bond; 1S, 2S, 3S, ensemble of disulfide intermediates of RNase A having one, two, three disulfide bonds, respectively; TCEP, Tris(2-carboxyethyl)phosphine; AEMTS, aminoethyl methylthiosulfonate.

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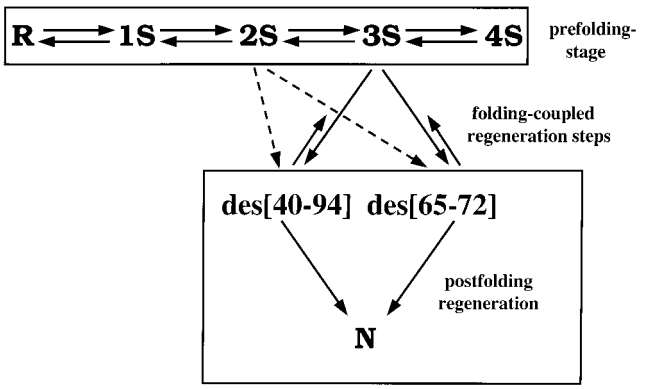

Figure 2. Kinetic model of oxidative folding of RNase A. The rate-determining step is the formation of the two structured intermediates from the unstructured ensembles. The des [40−95] pathway is the major pathway (~80%) under typical conditions. Not more than 5% of native is regenerated by direct oxidation from the 2S ensemble (dashed lines).

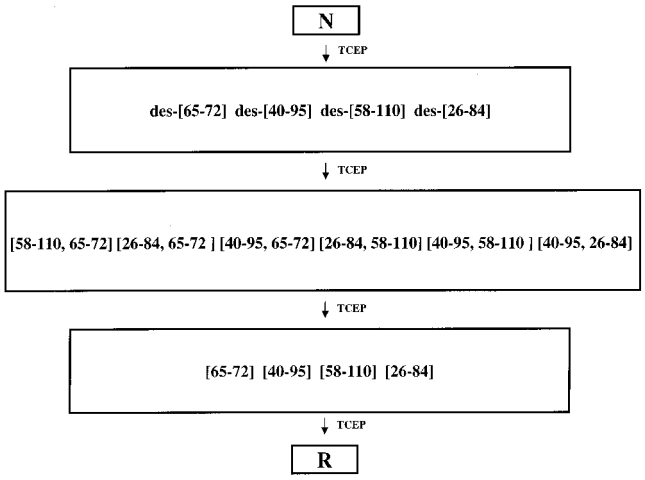

Figure 3. Native bonded intermediates in the TCEP reduction pathway, at low pH. The successive stages after N are the des species, the native two-disulfide species (in brackets), the native one-disulfide species (in brackets), and the fully reduced protein.

species of a four-disulfide-containing protein such as RNase A is usually small because of the large number of possible intermediates, e.g., the native one-disulfide-bonded species of RNase A having the (40−95) or (26−84) disulfide bond contribute 1.2 and 1.3%, respectively, to the 1S ensemble.[6] The native two-disulfide-bonded intermediate (containing the 40−95 and 58−110 disulfide bonds) constitutes less than 0.5% of the whole regeneration mixture.[7] In the second stage of the process—the folding-coupled regeneration steps—a set of native disulfide bonds is formed, which stabilizes the protein enough to fold conformationally.[8] In the third stage, the post-folding regeneration takes place, in which terminal disulfide reactions occur in the folded intermediates resulting in the formation of the native molecule.[3]

In ribonuclease A, as frequently occurs in oxidative folding of other proteins, the rate-determining steps are the formation of the structured des intermediates.[1,3,4] The des species can also be formed on minor pathways (less than 5%) by the oxidation of native two-disulfide-bonded precursor intermediates that are members of the 2S ensemble.[10,11] Reshuffling reactions from precursor intermediates having two native and one non-native disulfide bonds (that are members of the 3S ensemble) constitute the major pathways for formation of the des species.[2−4] The structural characterization of these precursor species would greatly facilitate the elucidation of the mechanism of this folding-coupled regeneration step.[5] Heretofore, however, it has not been possible to carry out these studies because these precursor species were not populated enough to allow their detection and their selection from numerous other intermediates.

In this study, these difficulties were overcome by using a new reduction procedure: (i) Tris (2-carboxyethyl)phosphine (TCEP) can reduce disulfide bonds at low pH,[12] where the intramolecular disulfide reactions that cause scrambling of disulfide bonds are extremely slow. Therefore, the TCEP reduction can produce only native-disulfide-bonded intermediates (see Figure 3). (ii) To obtain a more even distribution of the intermediates, the reduction was carried out in 5 M GdnHCl. Under these conditions, the disulfide-intact protein and all subsequent intermediates are unfolded. Hence all disulfide bonds are equally exposed to the reducing agent, thereby providing a comparable reduction rate for all four native disulfide bonds and, thus, comparable populations of the intermediates with the same number of disulfide bonds. (iii) AEMTS blocking of the reaction, as described previously,[13] facilitates the grouping and separation of intermediates with the same number of disulfide bonds on a cation-exchange column. As a consequence, the native two-disulfide-bonded intermediates, which can be populated in TCEP reduction, are separated from other intermediates on the chromatogram (such as the native one- and three-disulfide-bonded ones) facilitating this structural study.

The characterization of these species with two native disulfide bonds, as we argue later, provides information on the mechanism of the structure-forming step on both the major and minor pathways. Because of the lack of a feasible method for the separation of these (six) 2S species from the other intermediates in the TCEP reduction mixture (viz., the four des species and the four 1S intermediates), various methods such as D/H exchange or calorimetry are not applicable for the structural characterization of these species. Therefore, the well-known dependence of disulfide reactions on the accessibility of the disulfide bonds in the protein was used to probe structure.[14] To obtain structural information on these precursor intermediates, a mild reduction under folding conditions was applied to the native-bonded 2S intermediates. The rate of reduction of native-disulfide-bond-containing species was compared to the reduction rate of their unstructured, non-native disulfide-bond-containing isomers. The reduction strength was carefully chosen to be as mild as possible but strong enough to reduce any unstructured species. Thus, even a weak structural protection, which is manifested by a decreased rate of reduction, would readily be revealed in these experiments.

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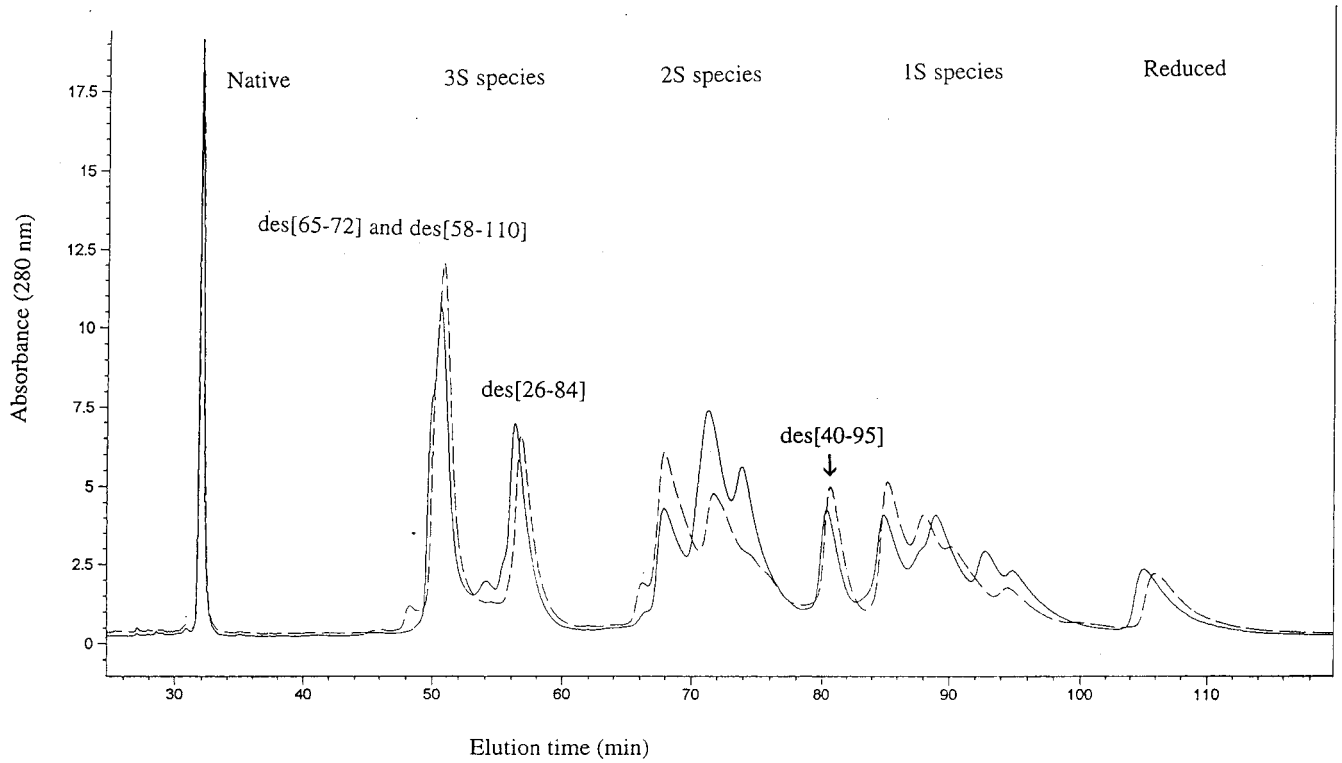

Figure 4. Cation exchange HPLC profiles of populated species during reduction of both reshuffled (dashed line) and nonreshuffled (solid line) batches with 5 mM TCEP on ice at pH 6.5, as described in the text. AEMTS blocking was carried out at 25 s reduction time, and samples were injected, after desalting, onto an analytical cation-exchange HPLC column, as described previously.[2]

Materials and Methods

Preparation of the Nonreshuffled Samples. Native RNase A (735 μM) was incubated in 5 M GdnHCl and 1.4 mM TCEP at pH 4.8 for 2.5 h. At the end of the incubation period, the pH was dropped to 2 and the reduction mixture was desalted on a G-25 column at 4 °C using a 100 mM acetic acid buffer. This step also diluted the protein to about 30 μM. The low pH inhibits further disulfide reactions during the removal of both GdnHCl and TCEP. Subsequently, with the protein on ice, the pH was raised to 5, which allows the intermediates that are stable enough under these conditions to fold without reshuffling. After 15 min on ice at pH 5, the pH was raised further to 6.5. At this point, the solution was divided into two batches.

Reduction of the Nonreshuffled samples. To one batch, a mild reduction (5 mM TCEP) was applied directly. The disappearance of the two-disulfide-bonded intermediates was monitored by taking aliquots of the sample and blocking them with AEMTS, followed by cation-exchange separation.

Preparation and Reduction of the Reshuffled Samples. The temperature of the second batch was raised to 13 °C at pH 6.5 for 20 min, which enabled the one- and two-disulfide-bonded intermediates to reshuffle, but the structured three-disulfide-bonded species, which had been folded during the previous pH 5 incubation, did not undergo reshuffling (2,3, as well as evidence in Figure 4 from the peak profiles of each family of intermediates). After 20 min at 13 °C, the temperature was dropped back to 4 °C and the same mild reduction was applied as for the first batch. This second reshuffled mixture served as a control for the reduction experiments of the first nonreshuffled batch.

Results and Discussion

Figure 5 shows schematically how the 2S intermediates containing only native disulfide bonds are populated, and subsequently characterized in reduction experiments. In the first step, the protein was reduced by TCEP in 5 M GdnHCl at pH 4.8. Under these conditions, no significant reshuffling to form

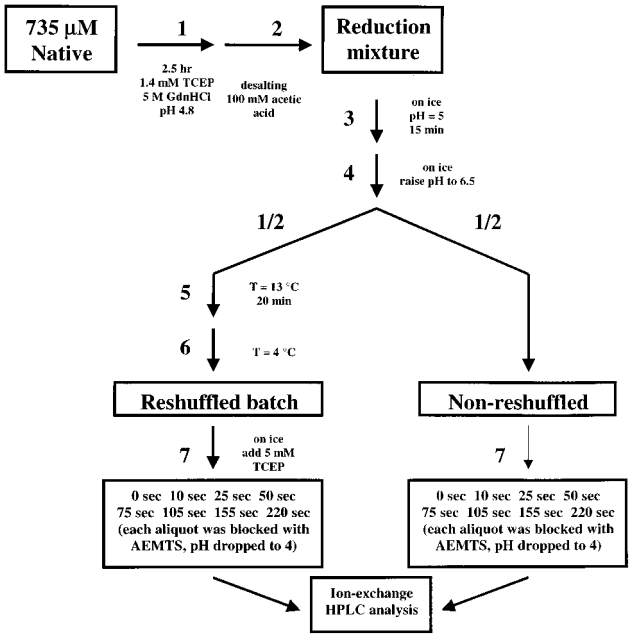

Figure 5. Summary of the experimental steps for the population and characterization of the 2S species with native disulfide bonds.

non-native disulfide bonds was observed in control experiments (data not shown). The TCEP concentration and reduction time were optimized to obtain higher populations of 2S intermediates (constituting about 35% of the total protein). The 5 M GdnHCl ensured that all disulfide bonds of the protein are exposed to the solvent and thus equally to TCEP. Therefore, exclusively, all the possible native disulfide-bonded intermediates were populated in the experiment, as demonstrated by the approximately identical population of all four des species (Figure

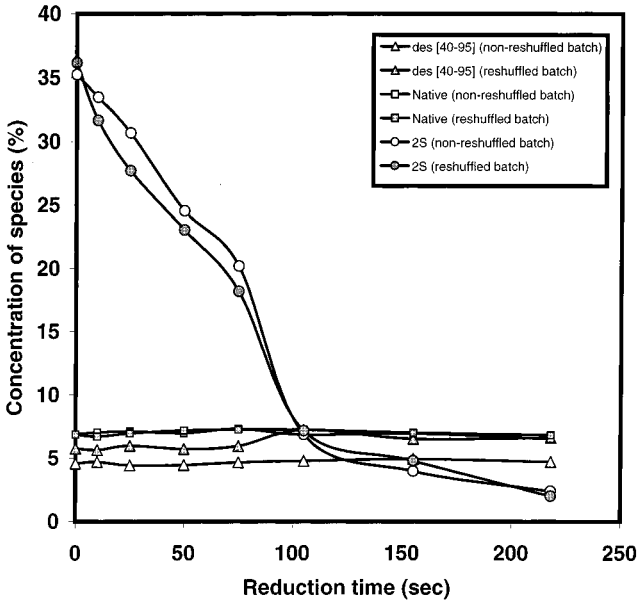

Figure 6. Changes of concentration of intermediates of RNase A in reshuffled or nonreshuffled samples during reduction with 5 mM TCEP on ice at pH 6.5 as a function of reduction time. Aliquots of the reduction solution were blocked with AEMTS and, after desalting, injected onto an analytical cation-exchange HPLC column.

4). The progress of the reduction was followed by aliquots blocked with AEMTS and injected to a cation-exchange column. A few TCEP concentrations, pH values, and reduction times were tested to obtain higher concentrations of 2S in the mixture without intramolecular disulfide reshuffling reaction taking place. After the 2.5 h TCEP reduction under the chosen conditions, the pH of the mixture was dropped to slow the reduction and the mixture (without AEMTS blocking) was desalted on a G 25 column. The low pH of the desalting buffer ensured that no reshuffling of the intermediate occurred during the removal of GdnHCl and TCEP.

Subsequently the pH of the solution was adjusted to 5 (step 3 on Figure 5), which allowed for the folding of the intermediates that were stable enough to fold under these conditions. Then, in step 4, the pH was raised to 6.5. At this point the mixture was divided into two portions. To one-half, a mild reduction was applied to distinguish between folded and unfolded intermediates (step 7). For technical convenience, TCEP was used rather than DTT, for which significantly higher concentrations are necessary to reach the same reduction rate. This in turn would require a much higher amount of AEMTS for blocking if DTT was used as the reducing agent. Several pH values and TCEP concentrations were tested. At pH 6.5, 5 mM TCEP provided a very sensitive detection of marginally stable structured intermediates because the reduction of the two-disulfide-bonded intermediates is relatively slow, requiring more than 3 min for complete reduction, as depicted in Figure 6.

The temperature of the second half was raised to 13 °C. At this temperature, scrambling of the disulfide bonds occurs for all intermediates except for the des species, which have a stable tertiary structure (step 5). This reshuffling of the intermediates resulted in an altered elution profile for the 1S and 2S intermediates. The 3S region that contains the des species, however, has an unaltered elution profile. This reshuffled sample served as a control for further reduction experiments involving native disulfide-bonded isomers. The conditions (relatively low protein concentration, pH, and temperature and short incubation time) of reshuffling were chosen to minimize possible intermolecular reactions (such as $1S + 2S \leftrightharpoons 3S + R$). Hence, the amount of the 1S and 2S intermediates did not change during this reshuffling step, making it easier to compare the disappearance rate of the nonreshuffled 2S species to that of the reshuffled, control 2S species. The four des species, whose reduction would produce 2S species, are folded and thus resistant toward reduction under the applied conditions and, as a result, have little influence on the amount of measured 2S species during the reduction of both (reshuffled or nonreshuffled) samples, as can be seen in Figure 4.

Non-native disulfide bonds that are protected by stable three-dimensional structure against reduction have not been reported in the literature. Thus, the reshuffled intermediates were assumed to be unfolded. This assumption is supported by several previous studies.[6−9] Therefore, if the 2S intermediates containing only native disulfide bonds have any structure that is stable enough to affect the accessibility of its disulfide bonds, their disappearance rate would be slower than that of the 2S intermediates in the reshuffled sample.

The result of the reduction experiment indicated stable global structure in all four des species under the applied condition. The disappearance rate of the 2S species was found to be the same for the reshuffled and nonreshuffled samples, indicating that these species have no detectable structural protection for their disulfide bonds, even under highly stabilizing (4° C) conditions. These data strongly suggest that, under less stabilizing conditions (25° C), the precursor species of des [40−95] or des [65−72] do not even have marginally stable global structure that can influence disulfide reactions.

In a previous different approach, mutant analogues of only one of these native two-disulfide-bonded intermediates were prepared.[15] By contrast, in our study, all six possible native two-disulfide-bonded species were examined without replacing the four cysteines by alanines or serines.

The precursor species on the major reshuffling pathways have one non-native disulfide bond in addition to the two native ones that the 2S intermediates, which are the subject of this investigation, have. Although an additional disulfide bond (either native or non-native) destabilizes the unfolded form of the intermediates by lowering their entropy, a non-native disulfide bond also destabilizes the folded conformation by diminishing native interactions that stabilize the fold. Most of the non-native disulfide bonds would cause a huge distortion of the folded protein and hence are not compatible with the native conformation. Therefore, the precursor species having an additional non-native disulfide bond conceivably have no more structure than these native two-disulfide-bonded intermediates. Thus, our results provide information not only for the precursors of the des species on the oxidation pathways (minor pathways) but also for the ones on the reshuffling pathways (major pathways). Consequently, these experiments provide strong support for an oxidative folding mechanism at 25 °C that postulates that global structure forms during the regeneration of RNase A only in the des species.[8] By contrast, our results do not support an oxidative folding model in which these native two-disulfide-bonded

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species undergo conformational folding to an extent that affects disulfide reactions.[16]

Isolation of the des Species. Structured intermediates, especially the des species, have drawn great attention in oxidative folding.[2,17] Two types of structured disulfide intermediates, disulfide-secure and disulfide-insecure, were discerned from oxidative folding studies.[3,4] RNase A has both disulfide-secure (des [40−95] and des [65−72]) and disulfide-insecure (des [26−84] and des [58−110]) intermediates and, hence, is a good model protein for studying oxidative folding of structured species. However, isolation of the structured des species (such as des [26−84] and des [58−110] in RNase A) that are not populated enough on either reductive or oxidative folding pathways of a multiple-disulfide bonded protein has been extremely difficult. Our method, as demonstrated here, enables these des species of RNase A to be populated, and facilitated their subsequent isolation unblocked on a reversed phase column by HPLC with a reasonable 10% yield for each.

This method is not specific to RNase A, but likely is generally applicable to many other disulfide-containing proteins. Although the exact conditions had to be carefully chosen for each step and specifically apply to RNase A, most of the reactions were carried out on unstructured species. Hence, similar conditions should work for many other proteins as well. In our experimental setup, we adjusted the conditions to facilitate the detection of the most sensitive structure in the 2S ensemble. At higher temperatures such as 25 °C, stable global structure would be detected only in two intermediates: des 65−72 and des 40−95. However, even at that temperature, one can still adjust the pH and TCEP concentration to facilitate study of those structures.

In summary, our laboratory has an ongoing commitment to unravel the detailed mechanism of oxidative folding of proteins. The population and isolation of the key intermediates, especially those that are involved in the critical structure-forming step, is one of the most fruitful ways for accomplishing this aim. In a previous study, we have demonstrated a method to populate an unstructured ensemble provided an isolatable structured isomer species of this ensemble exists.[18] In this study, a method was established that enables all intermediates containing only native disulfide bonds to be populated during the regeneration, regardless of whether they are structured or not. This method facilitates the structural characterization of the precursors of the des species which is of critical importance in the regeneration process, and can provide information about the mechanism of the last step before the structured des species forms on the folding pathway. In conjunction with the previous method,[18] we now have powerful tools to study the rate-determining steps in the oxidative folding of most disulfide-containing proteins.

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