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Development of a Novel Method to Study the Rate-Determining Step during Protein Regeneration: Application to the Oxidative Folding of RNase A at Low Temperature Reveals BPTI-like Kinetic Traps

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We describe a novel method to study the rate-determining step in the oxidative folding pathways of disulfide-containing proteins. The rate-determining step frequently involves the formation of a structured intermediate from its unstructured precursor. While structured intermediates can often be separated chromatographically from other components of the regeneration mixture, our method facilitates the isolation of the *unstructured* intermediates (precursors of the structured ones) in an unblocked form, which has not previously been feasible. In the first step, a certain structured intermediate is isolated in an unblocked form. Subsequently, this intermediate is converted quantitatively (by reshuffling) to the unstructured precursor under conditions described herein, without populating other unstructured species.

By applying this method to oxidative folding studies of RNase A,[1] a small protein containing 124 residues and four disulfide bonds (26−84, 40−95, 58−110 and 65−72),[1] we have been able to identify new folding pathways at low temperature (8 °C), which indicates that the oxidative folding pathway(s) of a protein depends strongly upon the oxidative folding conditions. At 8 °C, approximately 40% of the protein regenerates through two newly discovered structured intermediates,[2] des[58−110] and des[26−84] (each containing three native disulfide bonds and lacking the fourth), whereas the remaining fraction regenerates through des-[40−95] and des[65−72], the same species populated at 25 °C.[3] Des[58−110] and des[26−84] are not stable at regeneration temperatures of 25 °C and above and thus do not accumulate.[2]

Consider the oxidative folding of RNase A, at 25 °C[3−6] (Figure 1). The rate-determining step in the oxidative folding process is the formation of the two structured nativelike des species, des-[40−95] and des[65−72].[3] These species are formed largely by reshuffling from the 3S ensemble (their unstructured counterparts) (Figure 1), although a small fraction (no more than 5%) may be formed by oxidation from the 2S ensemble.[7,8] Upon formation and folding of either des species, their three native disulfide bonds become protected from reduction and reshuffling ("locked in"),[6] while the remaining thiol groups are still relatively accessible to the solvent. Hence, these des species accumulate to much higher levels than their unstructured isomers (3S) and can oxidize relatively rapidly, enhancing the regeneration of the native protein (N). These two (independent) pathways (through des[40−95] and des[65−72]) appear to account for nearly all of the protein regenerated with DTT^{ox} at 25 °C.[3]

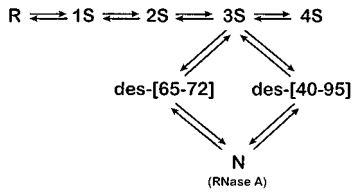

Figure 1. Scheme for the regeneration of RNase A. R is the fully reduced and denatured protein, and N is the native molecule. 1S, 2S, 3S, and 4S refer to ensembles of species having one, two, three, and four disulfide bonds, respectively. The term *des* refers to intermediates having all but one of the native bonds (e.g., des[40−95] is a species that has three native disulfide bonds (26−84, 58−110, and 65−72) but lacks bond 40−95).

Determining the oxidative folding pathway(s) of proteins from regeneration experiments becomes increasingly complicated as the number of disulfide bonds increases and the corresponding number of intermediates (structured and unstructured) increases. Thus, the oxidative folding of only a few disulfide-containing proteins have been examined in detail.[6] These studies suggest that the rate-determining step in oxidative folding of proteins is usually the formation of a structured species from their unstructured isomers (3S→3S* for RNase A, where 3S* is either des-[65−72] or des[40−95]). Alternatively, as in the case of the kinetically trapped structured species of BPTI (des[5−55] and des[30−51]), the rate-determining step may be associated with the loss of this structure.[9]

A major interest in oxidative folding studies is the coupling between disulfide reactions and conformational folding events (which constitute the rate-determining step for many proteins). The effect of alterations of the regeneration conditions (e.g., pH, temperature, denaturing agent, or stabilizing salt) on this structure-forming step can reveal the mechanism for this coupling. However, a study of this key step and the factors affecting it becomes very complicated if one were to conduct an entire regeneration (R→ N) and deduce inferences about the rate-determining step (e.g., nS→nS*) from kinetic fitting.[3] Moreover, such an analysis is still an indirect method and is normally possible only by carrying out several regeneration experiments at different protein concentrations and redox conditions. This is further complicated if there are numerous structured intermediates in a multi-disulfide-containing protein, giving rise to multiple pathways with separate rate-determining steps (one for each pathway).

We demonstrate a new method here by which one can simplify such a study by restarting the regeneration from the isolated ensemble of the unstructured (unblocked) precursor species of the structured intermediate and thus study the rate-determining step directly (for RNase A, the unstructured precursors are the ensemble of 3S species that have three disulfide bonds, native and non-native). The chief difficulty impeding such a study has heretofore been the separation (and isolation) of the unstructured isomers (3S) involved in the rate-determining step from other

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(1) Wlodawer, A.; Svensson, L. A.; Sjolin, L.; Gilliland, G. L. *Biochemistry* **1988**, *27*, 2705−2717. Abbreviations: RNase A, bovine pancreatic ribonuclease A.; AEMTS, aminoethylthiosulfonate; BPTI, bovine pancreatic trypsin inhibitor.

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(4) A typical oxidative folding study may be carried out as follows: Fully reduced protein is prepared by incubating purified native protein with a strong reducing reagent (e.g., 100 mM DTT^{red}) and a strong denaturant (e.g., 6 M GdnHCl) that unfolds the protein, ensuring that its disulfide bonds are exposed to the reducing reagent. Oxidative folding is initiated by introducing the fully reduced protein to conditions that favor conformational folding and the formation of disulfide bonds (e.g., 100 mM DTT^{ox} at pH 8, 25 °C). Aliquots are then withdrawn at various times, and thiol−disulfide exchange is quenched by AEMTS-blocking.[3] The mixture of disulfide species is then fractionated by cation-exchange HPLC. The cysteamine-blocking group allows the disulfide species to be separated by ion-exchange HPLC according to their number of free thiol groups. Kinetic fitting[3] is used to model the variations in the concentrations of the disulfide-protected species and unstructured *n*S ensembles with time (Figure 1).

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unstructured species (R, 1S, 2S, and 4S) in the regeneration mixture, primarily because all unstructured species (when unblocked) have similar chromatographic properties. [Grouping and separation of these unstructured species on a cation-exchange column is achieved only by AEMTS-blocking of the thiol groups, which introduces additional charges (two positive charges for 3S, four for 2S, and so on).] We have overcome this difficulty by obtaining one of the *structured* intermediates that is isolatable,[3] namely either des[40−95] or des[65−72], and converting this *pure* structured species quantitatively to its unstructured precursor(s) (the 3S ensemble), as described below. We note that this approach is also applicable to other proteins.[9,10]

The key point in generating pure unstructured counterparts (3S) is to destabilize the structure of one of the structured intermediates (e.g., des[40−95]) that can be isolated unblocked. The structure of these intermediates (des species) is destroyed, first by low pH and further by application of high temperature, and subsequently, the remaining thiol groups are deprotonated by a pH jump (to pH 7−9), so that they can easily attack the native set of disulfide bonds in the now *unstructured* des intermediate to form nonnative disulfide-bonded (unstructured) isomers (3S)[11] (it should be noted that the number of disulfide bonds does not change in this intramolecular reshuffling step).

Using this procedure,[11] we have been able to isolate the precursor ensemble of des[40−95] and des[65−72] of RNase A, that is, the 3S species, in unblocked form, thereby enabling us to carry out a direct study of the rate-determining reaction (3S→3S*) and the factors affecting it. We have also applied this method to produce two other structured intermediates of RNase A (des[58−110] and des[26−84]) in unblocked form (albeit as a mixture with the other two des species) which are otherwise impossible to isolate from the overall regeneration reaction because they coelute with N (when unblocked).

This process can be applied to any protein that has a structured intermediate that can be isolated and whose rate-determining step involves reshuffling between unstructured intermediates (it will not work for a 2S→3S* type rate-determining step which involves oxidation instead of reshuffling).[6] Nevertheless, it can be applied to improve the yield of any structured intermediate which forms through a reshuffling reaction (*n*S→*n*S*) and which does not accumulate to a significant extent and cannot be isolated easily.[6] Thus, this process not only facilitates the study of the formation of the structured intermediates (rate-determining steps) but also of the subsequent disulfide reactions that occur in the structured intermediate, without resorting to entire regeneration procedures.

This method was applied to the oxidative folding of RNase A at low temperature (8 °C) in order to elucidate the kinetic role of des[58−110] and des[26−84] that accumulate under these conditions. Unlike des[40−95] and des[65−72], these des species coelute with N when unblocked, making a study of their kinetic fate (under isolated conditions) difficult. Only when blocked, do des[58−110] and des[26−84] elute distinctly from each other, and from N.[2]

Des[26−84] and des[58−110] were obtained (in addition to des[40−95] and des[65−72]) by allowing the frozen 3S solution (obtained as described above) to thaw to 8 °C, followed by reshuffling at the same temperature for a period of 3 h. Monitoring

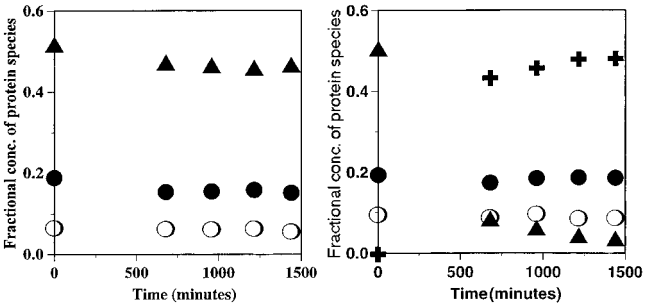

Figure 2. Fraction of des[40−95] (▲), des[26−84] (○), and des[58−110] (●) incubated in 100 mM Tris buffer, pH 8, 8 °C without DTT^{ox} (A) and with 100 mM DTT^{ox} (B) as a function of time, revealed by cation-exchange chromatographic analysis of the AEMTS-blocked samples. Des-[65−72] is not shown because of its very low population. Native protein generated by oxidation of both des[40−95] and des[65−72] is also shown (+). During formation of the des species from the isolated 3S ensemble, native protein and a small amount of 4S and 2S species were observed due to stray oxygen and intermolecular redox reactions resulting in a lower fraction of the des species.

of the reshuffling process and all further quantitative analyses of the four des species were carried out by withdrawing aliquots of the reaction mixture at various times, followed by blocking with AEMTS and fractionation by HPLC. When the thermodynamically more stable des species accumulated and the 3S species vanished from the chromatogram, we applied 100 mM DTT^{ox} to the mixture containing the four des species to follow the conversion of these structured intermediates to N. Des[40−95] and des[65−72] were consumed relatively rapidly with a concomitant increase in N. However, the concentration of both des-[58−110] and des[26−84] did not change during the time course of the experiment (Figure 2). Neither oxidation nor reshuffling occurred, indicating that both of these species are kinetically inert.

Since all four des species are stable under the oxidative folding conditions (neither reduction nor reshuffling occur on the time scale of the experiment), it is possible to determine the flux of the protein regenerating through each species (i.e., through each pathway; since these des species cannot convert directly to one another, they constitute separate kinetic entities). The relative ratio of each des species is the percentage of flow through each pathway. Analysis of the relative ratios indicates that ∼40% of the protein forms the kinetically trapped des[58−110] (∼25%) and des[26−84] (∼13%) at 8 °C. Of course, the regeneration of the native protein through these two des species may eventually take place, albeit very slowly, by direct oxidation or by reshuffling (through 3S) to the other two productive des species.

In conclusion, our method successfully facilitates a study of the rate-determining step (in case of reshuffling-related processes) in protein regeneration.

For some time now, the oxidative folding of RNase A has been considered as a counter-example to that of BPTI.[12,13] The results presented here indicate that, at low temperature, RNase A adopts BPTI-like pathways. Similarly a long-lived kinetically trapped species was found recently in the oxidative folding of lysozyme.[10] Our study demonstrates that this phenomenon (long-lived kinetically trapped species on the oxidative folding pathways adopted by a protein) appears to be more general than was previously considered, and suggests that oxidative folding pathways adopted by proteins are not primarily a function of the particular protein but rather of the applied regeneration conditions.

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(11) Unstructured isomers of the des species of RNase A, i.e., the 3S ensemble, were prepared by incubating either isolated and unblocked des-[40−95] or des[65−72] in glycine buffer (20 mM) at pH 1.6 for 1 h. This mixture was then added to a solution (10 × volume) of Tris buffer (pH 8, 100 mM Tris-HCl, 1 mM EDTA) that was previously equilibrated at 48 °C. The resulting mixture (pH 8) was incubated at 48 °C for a period of 2 min before rapid freezing on dry ice. The pre-denaturation of the des species (by low pH) accelerates the subsequent disulfide reaction (at pH 8) and makes it possible to shorten the incubation time at 48 °C, which is critical for avoiding unwanted side reactions such as intermolecular disulfide exchange. Care must be exercised to prevent spurious oxidations/reductions, especially air oxidation, since, at elevated temperatures and longer incubation times, stray oxygen can cause significant oxidation of the intermediates despite argon sparging.

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