Structural determinants of oxidative folding in proteins

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A method for determining the kinetic fate of structured disulfide species (i.e., whether they are preferentially oxidized or reshuffle back to an unstructured disulfide species) is introduced. The method relies on the sensitivity of unstructured disulfide species to low concentrations of reducing agents. Because a structured des species that preferentially reshuffles generally first rearranges to an unstructured species, a small concentration of reduced DTT (e.g., 260 μM) suffices to distinguish on-pathway intermediates from dead-end species. We apply this method to the oxidative folding of bovine pancreatic ribonuclease A (RNase A) and show that des[40–95] and des[65–72] are productive intermediates, whereas des[26–84] and des[58–110] are metastable dead-end species that preferentially reshuffle. The key factor in determining the kinetic fate of these des species is the relative accessibility of both their thiol groups and disulfide bonds. Productive intermediates tend to be *disulfide-secure*, meaning that their structural fluctuations preferentially expose their thiol groups, while keeping their disulfide bonds buried. By contrast, dead-end species tend to be *disulfide-insecure*, in that their structural fluctuations expose their disulfide bonds in concert with their thiol groups. This distinction leads to four generic types of oxidative folding pathways. We combine these results with those of earlier studies to suggest a general three-stage model of oxidative folding of RNase A and other single-domain proteins with multiple disulfide bonds.

Oxidative folding is the composite process by which a protein recovers both its native disulfide bonds (*disulfide-bond regeneration*) and its native structure (*conformational folding*). The course of oxidative folding is affected by three general structural factors that have been identified from *in vitro* oxidative folding studies and model systems, namely, the proximity, reactivity, and accessibility of the disulfide bonds and thiol groups. The *proximity* of two reactive groups (defined as their effective intramolecular concentration) is determined by the propensity of the backbone to bring the two groups into juxtaposition; in unfolded species, this proximity is largely determined by the loop entropy, although enthalpic interactions may contribute significantly as well. The *reactivity* of two reactive groups depends on the fact that most disulfide reactions occur through thiol/disulfide exchange and that only the thiolate (not the thiol) form is reactive; hence, changes in the local electrostatic environment may affect the rate of disulfide-bond reactions. However, the most critical factor seems to be the *accessibility* of the thiol groups and disulfide bonds (1). Thiol/disulfide exchange reactions can occur only when a thiol and a disulfide bond come into contact; hence, burial of the disulfide bond or the thiol prevents their contact and blocks the reaction.

Accordingly, the formation of stable tertiary structure that protects the native disulfide bonds is a key event in the oxidative folding of proteins, because it stabilizes such bonds by making them inaccessible to the redox reagent and protein thiols. Indeed, the rate of intramolecular disulfide reshuffling is sufficiently high so that no native disulfide bond would survive much longer than a few minutes in an unstructured disulfide intermediate with a free thiol group unless it is buried in protective tertiary structure (2). Thus, the rate-determining step in oxidative folding is often the formation of a disulfide intermediate with stable tertiary structure (1–5).

However, the structural protection of the thiol groups is just as important as the protection of the disulfide bonds in reaching the transition state of oxidative folding. The burial of both thiol groups and disulfide bonds hinders any further progress in oxidative folding, because all of the reactive groups have been sequestered from the redox reagent. Disulfide species with buried thiols and disulfide bonds are often metastable (6–8). The kinetic fate of such a metastable species depends on the relative rates of oxidation and reshuffling to unstructured species, which is also true for structured disulfide species in which thiols are exposed for oxidation.

Therefore, an experimental method is needed to determine the kinetic fate of the structured disulfide species; i.e., whether they preferentially oxidize to the native protein or eventually reshuffle back to an unstructured ensemble. Hitherto, this fate has been determined kinetically by examining the rates of regenerating native proteins in the presence of varying amounts of the structured disulfide species and of redox reagents (3–5, 9, 10). However, this method is fundamentally unreliable, because it relies on kinetic fitting and the assumption of a kinetic model.

In this article, we introduce a more direct method for determining the kinetic fate of the structured intermediates. The method relies on the sensitivity of unstructured disulfide species to low concentrations of reducing agents such as DTT. Upon reshuffling, structured des species generally first rearrange to an unstructured disulfide species. In the presence of a sufficiently strong reducing agent, such unstructured species will be reduced rather than reshuffled back to a structured disulfide species. By contrast, if the initial structured species is oxidized directly to the native protein, its disulfide bonds remain protected and are not affected by the reducing agent. Thus, a small concentration of reduced DTT may suffice to distinguish on-pathway intermediates from dead-end structured species that preferentially reshuffle.

We applied this method to RNase A, a well characterized model protein for oxidative folding (1, 11). RNase A has 124 residues and four disulfide bonds, specifically Cys26-Cys84, Cys40-Cys95, Cys65-Cys72, and Cys58-Cys110. The disulfide bonds 40–95 and 65–72 occur in relatively flexible loop segments, whereas 26–84 and 58–110 both join an α-helix to a β-sheet and stabilize the surrounding local hydrophobic core. The oxidative folding of RNase A proceeds through a preequilibrium involving ensembles of unstructured *n*S species, followed by rate-determining steps from 3S to des[40–95] and des[65–72], respectively, which then proceed to the native protein, N (Scheme I; refs. 2 and 5).

Abbreviations: RNase A, bovine pancreatic ribonuclease A; des[*x*–*y*], RNase A with three native disulfide bonds but lacking the native disulfide bond between Cys-*x* and Cys-*y*; *n*S, ensemble of unstructured disulfide species with *n* disulfide bonds; AEMTS, aminoethylmethanethiosulfonate; DTT^{red}, reduced dithiothreitol; DTT^{ox}, oxidized dithiothreitol.

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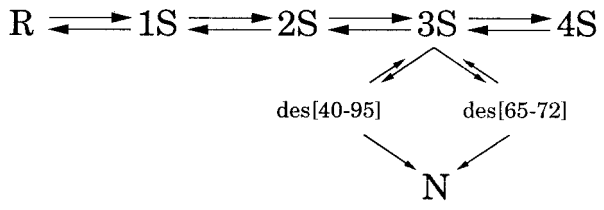

The conformational folding and stability of RNase A have been the subject of many investigations (12, 13), which help in the structural interpretation of kinetic data for oxidative folding.

Materials and Methods

Materials. Bovine pancreatic ribonuclease A (type 1-A) was obtained from Sigma and purified as described previously (14). Reduced and oxidized dithiothreitol (DTT^{red} and DTT^{ox}) were purchased from Sigma, and the DTT^{ox} was purified further by the method of Creighton (15). The thiol-blocking agent aminoethylmethanethiosulfonate (AEMTS) was prepared by the method of Bruice and Kenyon (16). All other reagents were of the highest grade commercially available.

Preparation of the Des Species. Des[40–95] and des[65–72] were isolated following the reductive method of Li and coworkers (5, 12). Des[58–110] and des[26–84] cannot be isolated in the same manner as des[40–95] and des[65–72], because the former two des species are not populated in the reductive unfolding of native RNase A (12). Therefore, a new method is needed to obtain samples with enhanced populations of des[58–110] and des[26–84]. We developed such a method by exploiting the fact that, at low temperatures ($\leq$15°C), all four des species are structured and thermodynamically more stable than the unstructured 3S species. Hence, if the 3S ensemble is isolated and allowed to reshuffle under these conditions, it will equilibrate to a mixture composed almost entirely of the four des species. It was not feasible to fractionate the mixture of the four unblocked structured des species into the individual des species. However, as described below, it was possible to determine the kinetic fate of the individual des species by blocking their interconversion.

Thus, a solution of all four structured des species was prepared from purified des[40–95] and des[65–72] by a two-step procedure. In the first step, an ensemble of 3S species was prepared as follows. Isolated des[40–95] or des[65–72] was incubated for 1 h under unfolding conditions at pH 1.6 in a 20 mM glycine buffer at 4°C, then added to a solution (10× volume) of Tris buffer (pH 8.0, 100 mM Tris·HCl, 1 mM EDTA) that had been equilibrated to 48°C. The jump to pH 8.0 initiates disulfide reshuffling, whereas the jump to high temperature maintains the des species under unfolding conditions (thus preventing the reformation of tertiary structure that might protect their disulfide bonds). The solution was held at 48°C for 2 min to allow the reshuffling to equilibrate fully to the 3S ensemble, and was then rapidly frozen in dry ice. In the second step, the frozen 3S solution was thawed to 8°C (at which the highest yields of des[58–110] and des[26–84] were found), followed by reshuffling at 8°C for 3 hr. The solution was sparged continuously with argon throughout this reshuffling to minimize the effects of air oxidation. The composition of the reshuffling mixture was monitored by removing aliquots at various times, blocking with AEMTS, and analyzing the species with cation-exchange chromatography.

More than 90% of the 3S species are converted to structured des species during this 3-hr reshuffling at 8°C. Upon blocking with AEMTS, the remaining 3S species (<10%) were coeluted on a cation-exchange column (17) with des[58–110] and des[26–84], slightly skewing the measured initial concentrations of these des species. However, longer reshuffling times are not helpful, because native RNase A appears continuously throughout the reshuffling, presumably because of ambient oxygen and intermolecular disproportionation reactions (3S + des $\rightarrow$ 2S + N).

Determination of the Kinetic Fate of the Four Des Species. The kinetic fate of the individual des species (i.e., whether they are oxidized to native RNase A or reshuffled to the 3S ensemble) can be determined by the addition of low concentrations of a reducing agent such as DTT^{red}. The strategy is to find a DTT^{red} concentration such that an unstructured 3S species is reduced much faster than it reshuffles to a structured des species, whereas a structured des species is not significantly affected. Under such weakly reducing conditions, the disappearance of a structured des species is primarily caused by its reshuffling to a 3S species, which is then rapidly reduced to R before it reshuffles to a structured des species. Thus, once the protective tertiary structure of the des species is lost (by reshuffling), the reducing conditions ensure that it is never regained; the reshuffling of the structured des species is the rate-determining step in its reduction. An appropriate DTT^{red} concentration to implement this strategy for RNase A appears to be 260 μM; at this concentration of DTT^{red} at 25° C, the 3S ensemble is reduced to R about 50 times faster than it reshuffles to des[40–95] (2).

Three experiments were carried out. In the first experiment, the mixture of structured des species was incubated in the presence of 260 μM DTT^{red}; the relative rate of reshuffling of these species is reflected in their relative rate of reduction. In the second experiment, the mixture of structured des species was incubated in the presence of 260 μM DTT^{red} and 100 mM DTT^{ox}. This second experiment determined whether the remaining thiol groups of these des species were available for oxidation to the native protein. Des species with buried thiol groups should be relatively inert in the presence of 100 mM DTT^{ox}, whereas structured des species with exposed thiols should be rapidly oxidized to the native protein. In the third experiment, the mixture of structured des species was allowed to reshuffle in the absence of any redox reagent. These three experiments were repeated individually on the isolated des[40–95] and des[65–72] species (rather than on the mixture of des species) to find more precise estimates of their rate constants.

Finally, in a fourth experiment, the purified (unstructured) 3S ensemble was allowed to reshuffle in the absence of any redox reagent. As shown in *Results*, the reshuffling of the four structured des species in the absence of any redox reagent is slow compared with their formation by reshuffling from the unstructured 3S ensemble. Hence, the relative populations of des species determined by this fourth experiment measure the relative rates of forming these species from the 3S ensemble and, in combination with the first two experiments, indicate the relative contributions of each structured des species to the regeneration of the native protein. All four experiments were carried out at 15°C while continuously sparging the solutions with argon. The relative populations of the disulfide species at various times were determined by removing aliquots, blocking with AEMTS and analyzing the mixture by cation-exchange chromatography, as described previously (9).

Results

Des[40–95] and Des[65–72]. The relative concentrations of des[40–95] and des[65–72] showed no measurable change over the course of 2 days at pH 8.0 and 15°C either in the absence of any redox reagent or in the presence of 260 μM DTT^{red} (data not shown). By contrast, these species disappeared rapidly in the presence of an oxidizing agent (100 mM DTT^{ox}) with a corresponding increase in the native protein (Fig. 1). Hence, these des species were oxidized directly to native RNase A, rather than reshuffling to another des species that is rapidly oxidized.

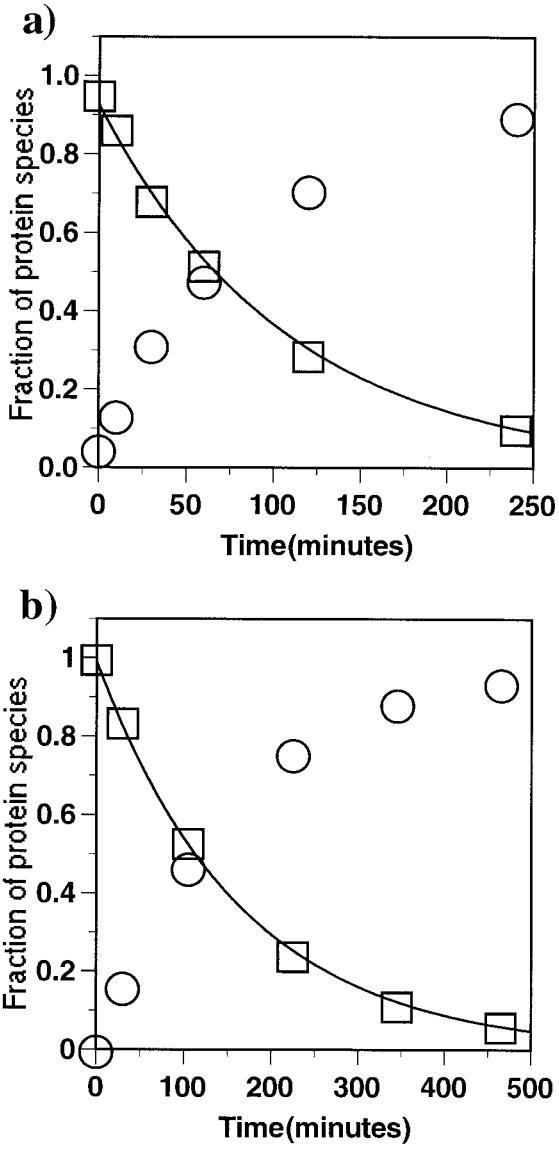

Fig. 1. (*a*) Decrease in the concentration of des[40–95] at pH 8.0, 15°C in the presence of DTT^{ox} and DTT^{red} (100 mM and 260 μM, respectively). □ represents des[40–95] whereas ○ denotes the native protein. (*b*) Decrease in the concentration of des[65–72] at pH 8.0, 15°C in the presence of DTT^{ox} and DTT^{red} (100 mM and 260 μM, respectively). □ represents des[65–72], whereas ○ denotes the native protein. At 15°C and pH 8, the oxidation rates of des[40–95] and des[65–72] are 6.0 and 9.2 $\times$ 10^2 $min^{-1} \cdot M^{-1}$, respectively.

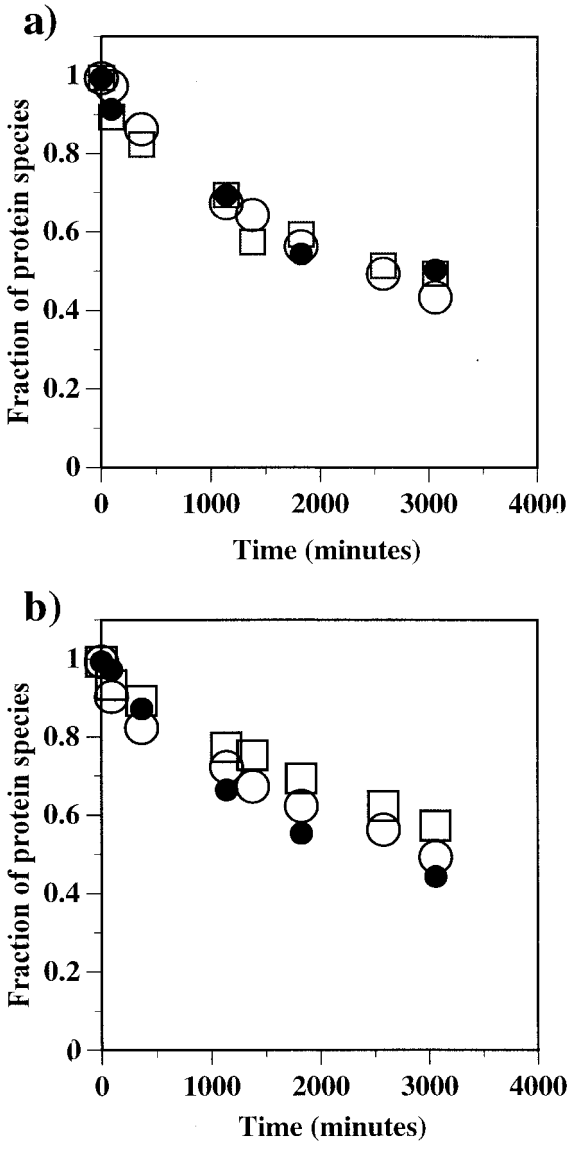

Fig. 2. (*a*) Decrease in the concentration of des[58–110] at pH 8.0, 15°C under three solution conditions: in the presence of DTT^{red} alone (260 μM) (○), in the presence of DTT^{ox} and DTT^{red} (100 mM and 260 μM, respectively) (□), and in the absence of any redox reagent (●). (*b*) Decrease in the concentration of des[26–84] at pH 8.0, 15°C under three solution conditions: in the presence of DTT^{red} (260 μM) (○), in the presence of DTT^{ox} and DTT^{red} (100 mM and 260 μM, respectively) (□), and in the absence of any redox reagent (●).

Des[58–110] and Des[26–84]. By contrast, the relative concentrations of des[58–110] and des[26–84] decreased significantly over the course of 2 days in the presence of 260 μM DTT^{red} (Fig. 2). Moreover, these two des species disappeared at nearly the same rate under all three conditions, i.e., in the presence of 260 μM DTT^{red}, in the presence of 260 μM DTT^{red} and 100 mM DTT^{ox}, and in the absence of any redox reagent (Fig. 2). The relative insensitivity of these des species to the external redox conditions indicates that these des species preferentially reshuffle and are metastable dead-end species, rather than direct precursors of native RNase A.

In principle, the decrease in the concentrations of the four des species over time should be slightly different in the presence of 260 μM DTT^{red} than in the absence of any redox reagent. Upon the reshuffling of the structured des species to the 3S ensemble, the resulting 3S species are reduced in the presence of 260 μM DTT^{red} and, hence, do not reshuffle back to the structured des species. By contrast, in the absence of any redox reagent, the 3S species are *not* reduced but partly reshuffle back to the structured des species. However, such second-order effects are too small to be detected reliably by our experiments, being of the same magnitude as the effects from stray oxidation and from the initial uncertainties in the concentration of the des species.

Relative Contributions of the Various Des Species to Regenerating Native RNase A. At 15°C and pH 8.0, the rates of reduction and reshuffling of the des species are much slower than the rate of forming these des species by reshuffling of the 3S ensemble (18). Therefore, in the absence of a redox reagent, the relative populations of the four structured des species reflects their relative rate of formation from the 3S ensemble. [This conclusion assumes that the rate of forming these des species by

oxidation from the 2S ensemble is negligible compared with their rate of formation by reshuffling from the 3S ensemble, which has been shown to be valid for RNase A under the conditions cited above (2).] The measured values for these relative populations (from the fourth experiment) are 65%, 20%, 10%, and 5% for des[40–95], des[58–110], des[26–84], and des[65–72], respectively. Because des[58–110] and des[26–84] apparently do not oxidize to native, the corresponding 30% of the structured des species eventually reshuffle to 3S and regenerate to the native structure via des[40–95] and des[65–72]. Thus, given the roughly 13:1 (65%:5%) ratio of forming des[40–95] rather than des[65–72] by reshuffling from the 3S ensemble, more than 90% of native RNase A regenerates through des[40–95] at 15°C and pH 8.0.

Structural Determinants of Oxidative Folding. The kinetic fate of the various structured des species seems to be determined by the relative protection of their thiols and disulfide bonds. On the one hand, des[40–95] and des[65–72] do not reshuffle significantly either in the presence of 260 μM DTT^{red} or in the absence of a redox reagent. Hence, global unfolding occurs at a negligible rate in these des species at 15°C and pH 8.0. This conclusion is consistent with other structural data, including thermal and chemical unfolding transitions, x-ray crystal *B*-factors, and hydrogen/deuterium exchange data (13, 19–23). The alternative explanation (namely, that these des species are in a rapid reshuffling equilibrium with the 3S ensemble) can be ruled out because of the absence of an effect of 260 μM DTT^{red} (which should reduce any unstructured 3S species). Moreover, the fast oxidation of these des species with 100 mM DTT^{ox} clearly indicates that their thiols are available to the redox reagent without exposing the disulfide bonds, which is consistent with reductive unfolding data (12); thus, these species are *disulfide-secure*. On the other hand, des[26–84] and des[58–110] back-reshuffle in the absence of a redox reagent, indicating that their disulfide bonds are available for intramolecular attack by their own thiols. However, this reshuffling is very slow, and neither the addition of 260 μM DTT^{red} nor the addition of 260 μM DTT^{red} and 100 mM DTT^{ox} affects the disappearance of these des species. This insensitivity to the solution redox conditions indicates that these species are long-lived metastable species and the thiols are exposed in concert with the disulfide bonds, presumably by infrequent global unfolding, which is likewise consistent with reductive unfolding data (12). Thus, these species are *disulfide-insecure* at 15°C and pH 8.0.

Under typical oxidative folding conditions, disulfide-secure species preferentially oxidize to the native protein, whereas disulfide-insecure species preferentially reshuffle to the unstructured ensemble. Obviously, the ratio between reshuffling and oxidation depends on the redox conditions as well; at very low concentrations of an oxidizing agent, even disulfide-secure species will preferentially reshuffle, albeit at slow rates. We stress that the distinction disulfide-secure/disulfide-insecure is a *conformational* distinction that is independent of the redox conditions, depending only on the relative free energies for structural fluctuations that expose the thiol groups and disulfide bonds. In disulfide species where the protein disulfide bonds and mixed disulfide bonds are equally accessible, the reshuffling reaction typically competes effectively with the oxidation reaction because of the conformational similarity between a reshuffling reaction and the second step of an oxidation reaction (2, 11, 24).

Structural and kinetic data suggest that des[40–95] has exposed thiol groups at 15–25°C and pH 8.0, where it is a structured intermediate (2, 5). By contrast, des[65–72] may bury at least one of its thiols (probably Cys72) under folding conditions, judging from NMR data (19) and kinetic studies that suggest a buried mixed disulfide in des[65–72] (2, 25). This conclusion is consistent with the temperature dependence of the oxidation rate

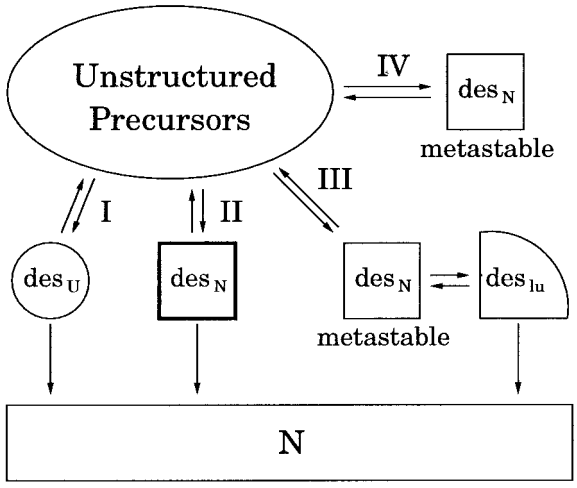

Fig. 3. The four generic pathways of oxidative folding (11). Structured native-like species such as des_N and N are indicated by squares, whereas unstructured intermediates such as des_U are represented by circles and ovals. The locally unfolded species des_{lu} is indicated by a quarter-circle. For simplicity, we assume that des species are the only species capable of folding to a native-like structure.

observed in these two des species. On the one hand, the ratio (0.21) of the oxidation rates of des[40–95] at 15°C (Fig. 1) and at 25°C (2) resembles the ratio (0.20) for unstructured 2S species (2, 18), consistent with structural data indicating that the thiols of des[40–95] are in flexible loop regions that are as solvent-exposed as the thiols of 2S species. On the other hand, the ratio (0.64) of the oxidation rates of des[65–72] at 15°C (Fig. 1) and at 25°C (2) is much larger, indicating that des[65–72] is much less sensitive to temperature. Taken together, the data suggest that des[65–72] may require local unfolding near its thiols to become oxidized to the native protein, whereas its disulfide bonds remain protected during the structural fluctuations required to expose its thiols; i.e., des[65–72] is a disulfide-secure species.

Four Generic Pathways of Oxidative Folding. Based on our observations of RNase A, four generic pathways of oxidative folding can be proposed (Fig. 3), depending on whether any disulfide intermediates are structured, whether the structured disulfide species are metastable (i.e., bury both their disulfide bonds and thiol groups) and whether the metastable disulfide species are disulfide-secure or -insecure (11). In the simplest pathway (denoted as pathway I in Fig. 3), no structured intermediates form during the regeneration, such as occurs in RNase A under destabilizing conditions, such as high temperatures (2, 18). This pathway is denoted as a des_U pathway because the des species are unstructured (11). The remaining pathways (denoted as pathways II–IV in Fig. 3) have structured intermediates that differ in the relative accessibility of their thiol groups and disulfide bonds. The most efficient pathway (pathway II) is the des_N pathway, which involves a disulfide-secure des species where the thiols are easily accessible to the redox reagent. At the other extreme (the "dead-end" pathway IV), the structured intermediates are disulfide-insecure species that preferentially reshuffle to unstructured species. Finally, pathway III involves a metastable but disulfide-secure intermediate, i.e., one in which the thiols are buried (hence metastable, as in the dead-end pathway) but can be exposed by local unfolding without exposing the disulfide bonds. An example of pathway III may occur in a structured intermediate identified recently in the regeneration of epidermal growth factor (8); however, the data show that both local and global unfolding occur in this intermediate, possibly

indicating a small difference in the free energies for local and global unfolding (8).

Conclusions

The present results suggest a structural explanation for the diverse regeneration behavior of multiple-disulfide proteins (2–4, 7, 8, 26, 27) and, in combination with our earlier work (1, 11), lead to the following three-stage model of oxidative folding. In the first, *prefolding* stage, the protein oxidizes successively, generating mixtures of unstructured species with different redox states (2–4, 7, 8, 10, 27, 28). In these unstructured species, the reactive groups (thiolate and disulfide bonds) are accessible and can be freely reshuffled, reduced, and oxidized, leading to a quasi-equilibrium distribution of disulfide species (2, 4, 14, 28). The quasi-equilibrium distribution of unstructured disulfide species within each *n*S ensemble is determined primarily by their loop entropies but also by the enthalpic interactions within each species (29, 30), whereas the analogous pre-equilibrium distribution of the *n*S ensembles is also determined by the redox conditions. The second stage corresponds to the formation of stable tertiary structure in intermediates that locks in the native disulfide bonds, preventing the back-reaction and, thus, inducing such species to accumulate (1–5, 11). The final, *postfolding* stage of oxidative folding corresponds to the disulfide-bond reactions in the folded intermediates that cause those species either to oxidize to the native protein (pathways II and III in Fig. 3) or to reshuffle back to the unstructured species (pathway IV in Fig. 3; refs. 7–9). Naturally, there is no postfolding stage in the des_U pathway (pathway I in Fig. 3), because the native tertiary structure appears in the fully native protein where no further oxidative folding is possible.

Our results highlight the critical role of accessibility (both of the disulfide bonds and of the thiol groups) in oxidative folding. The two other general factors influencing oxidative folding are the proximity and reactivity of the reactive thiols and disulfide bonds. These factors determine the quasi-equilibrium of disulfide species in the prefolding stage, and are evidently not sufficiently specific to strongly favor the native disulfide bonds over the nonnative disulfide bonds, or to obtain high yields of the native protein (28). The characteristic of folded species (such as the native protein and the conformationally stable des species) that distinguishes them from the other members of their *n*S ensemble derives from their ability to form stable tertiary structure that excludes the solvent (and, hence, the redox reagent) from the vicinity of the reactive disulfide bonds and thiol groups, drastically altering the reaction rates. Thus, the critical step in efficient oxidative folding of single-domain proteins with multiple disulfide bonds seems to be the formation of stable tertiary structure that sequesters the native disulfide bonds (preventing their subsequent rearrangement) but leaves the thiol groups of intermediates relatively exposed (or exposable) for subsequent oxidation.

The role of structured disulfide-secure intermediates in efficient oxidative folding does not seem to have been widely appreciated. Typical oxidative folding studies either employ methods that are incapable of detecting intermediates with stable tertiary structure (10, 28, 31) or apply structurally sensitive methods to undiscriminated mixtures of disulfide species containing both structured and unstructured species (7, 10, 32). Such approaches make it difficult to discern the critical structural events in oxidative folding.

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1. Wedemeyer, W. J., Welker, E., Narayan, M. & Scheraga, H. A. (2000) *Biochemistry* **39,** 4207–4216.
2. Rothwarf, D. M., Li, Y.-J. & Scheraga, H. A. (1998) *Biochemistry* **37,** 3767–3776.
3. Pace, C. N. & Creighton, T. E. (1986) *J. Mol. Biol.* **188,** 477–486.
4. Thannhauser, T. W., Rothwarf, D. M. & Scheraga, H. A. (1997) *Biochemistry* **36,** 2154–2165.
5. Rothwarf, D. M., Li, Y.-J. & Scheraga, H. A. (1998) *Biochemistry* **37,** 3760–3766.
6. Weissman, J. S. & Kim, P. S. (1995) *Nat. Struct. Biol.* **2,** 1123–1130.
7. van den Berg, B., Chung, E. W., Robinson, C. V., Mateo, P. L. & Dobson, C. M. (1999) *EMBO J.* **18,** 4794–4803.
8. Chang, J. Y., Li, L. & Lai, P.-H. (2001) *J. Biol. Chem.*, in press.
9. Rothwarf, D. M. & Scheraga, H. A. (1993) *Biochemistry* **32,** 2680–2689.
10. Roux, P., Ruoppolo, M., Chaffotte, A.-F. & Goldberg, M. E. (1999) *Protein Sci.* **8,** 2751–2760.
11. Narayan, M., Welker, E., Wedemeyer, W. J. & Scheraga, H. A. (2000) *Acc. Chem. Res.* **33,** 805–812.
12. Li, Y.-J., Rothwarf, D. M. & Scheraga, H. A. (1995) *Nat. Struct. Biol.* **2,** 489–494.
13. Laity, J. H., Montelione, G. T. & Scheraga, H. A. (1999) *Biochemistry* **38,** 16432–16442.
14. Rothwarf, D. M. & Scheraga, H. A. (1993) *Biochemistry* **32,** 2671–2679.
15. Creighton, T. E. (1977) *J. Mol. Biol.* **113,** 295–312.
16. Bruice, T. W. & Kenyon, G. L. (1982) *J. Protein Chem.* **1,** 47–58.
17. Welker, E., Narayan, M., Volles, M. J. & Scheraga, H. A. (1999) *FEBS Lett.* **460,** 477–479.
18. Rothwarf, D. M. & Scheraga, H. A. (1993) *Biochemistry* **32,** 2698–2703.
19. Talluri, S., Rothwarf, D. M. & Scheraga, H. A. (1994) *Biochemistry* **33,** 10437–10449.
20. Pearson, M. A., Karplus, P. A., Dodge, R. W., Laity, J. H. & Scheraga, H. A. (1998) *Protein Sci.* **7,** 1255–1258.
21. Laity, J. H., Lester, C. C., Shimotakahara, S., Zimmerman, D. E., Montelione, G. T. & Scheraga, H. A. (1997) *Biochemistry* **36,** 12683–12699.
22. Shimotakahara, S., Rios, C. B., Laity, J. H., Zimmerman, D. E., Scheraga, H. A. & Montelione, G. T. (1997) *Biochemistry* **36,** 6915–6929.
23. Iwaoka, M., Wedemeyer, W. J. & Scheraga, H. A. (1999) *Biochemistry* **38,** 2805–2815.
24. Weissman, J. S. & Kim, P. S. (1993) *Nature (London)* **365,** 185–188.
25. Li, Y.-J., Rothwarf, D. M. & Scheraga, H. A. (1998) *J. Am. Chem. Soc.* **120,** 2668–2669.
26. Weissman, J. S. & Kim, P. S. (1991) *Science* **253,** 1386–1393.
27. Creighton, T. E. (1997) *Biol. Chem. Hoppe-Seyler* **378,** 731–744.
28. Ruoppolo, M., Vinci, F., Klink, T. A., Raines, R. T. & Marino, G. (2000) *Biochemistry* **39,** 12033–12042.
29. Xu, X., Rothwarf, D. M. & Scheraga, H. A. (1996) *Biochemistry* **35,** 6406–6417.
30. Volles, M. J., Xu, X. & Scheraga, H. A. (1999) *Biochemistry* **38,** 7284–7293.
31. Ruoppolo, M., Freedman, R. B., Pucci, P. & Marino, G. (1996) *Biochemistry* **35,** 13636–13646.
32. Roux, P., Delepierre, M., Goldberg, M. E. & Chaffotte, A.-F. (1997) *J. Biol. Chem.* **272,** 24843–24849.