Folding of a Disulfide-Bonded Protein Species with Free Thiol(s): Competition between Conformational Folding and Disulfide Reshuffling in an Intermediate of Bovine Pancreatic Ribonuclease A†

Kohei Saito, Ervin Welker, and Harold A. Scheraga*

Baker Laboratory of Chemistry and Chemical Biology, Cornell University, Ithaca, New York 14853-1301

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ABSTRACT: The conformational folding of the nativelike intermediate des-[40−95] on the major oxidative folding pathway of bovine pancreatic ribonuclease A (RNase A) has been examined at various pHs and temperatures in the absence of a redox reagent. Des-[40−95] has three of the four disulfide bonds of native RNase A and lacks the bond between Cys40 and Cys95. This three-disulfide species was unfolded at low pH to inhibit any disulfide reshuffling and was refolded at higher pH, allowing both conformational folding and disulfide-reshuffling reactions to take place. As a result of this competition, 15−85% of des-[40−95], depending on the experimental conditions, undergoes intramolecular disulfide-reshuffling reactions. That portion of the des-[40−95] population which has native isomers of essential proline residues appears to fold faster than the disulfide reaction can occur. However, when the folding is retarded, conceivably by the presence of non-native isomers of essential proline residues, des-[40−95] may reshuffle before completing the conformational folding process. These results enable us to distinguish among current models for the critical structure-forming step in oxidative folding and reveal a new model for coupling proline isomerization to disulfide-bond formation. These experiments also demonstrate that the reshuffling−folding competition assay is a useful tool for detecting structured populations in conformational folding intermediates.

The most important posttranslational event that may provide functionality to freshly synthesized polypeptide chains is the folding process in which the polypeptide chains proceed from mainly unfolded to mainly folded conformations. (*1*−*3*). Although disulfide bonds complicate the folding process considerably, many proteins use disulfide bonds to ensure extra thermodynamic stability for their folded conformations. However, not all the disulfide bonds are essential for maintaining such a folded conformation (*4*, *5*). Disulfide-bonded proteins frequently retain their folds even after the reduction of one of their disulfide bonds and often fold into a nativelike conformation before regenerating the last native disulfide bond (*4*−*6*). It has been shown that the effect of the structure of such an intermediate on the disulfide reactions is the critical factor that determines the basic characteristic of oxidative folding pathways (*7*, *8*). Despite this critical behavier, it is generally the disulfide-intact proteins for which conformational folding has been investigated, but the specific conditions under which a disulfide intermediate may fold conformationally have received little attention. In this study, we focus on this aspect, using des-[40−95], the major disulfide intermediate in the oxidative regeneration of bovine pancreatic ribonuclease A (RNase A), as a model.

RNase A contains four disulfide bonds, and both oxidative and disulfide-intact folding have been studied extensively (*7*−*13*). The conformational folding of the disulfide-intact protein has revealed several refolding phases with time constants ranging from less than 100 ms to more than 1 min (*14*−*16*). The diversity of the folding phases arises from the heterogeneity of the unfolded state, due mainly to the different isomeric states of essential X-Pro peptide groups. RNase A has four proline residues, two of which are in the cis conformation in the native state (*17*). However, in the unfolded polypeptide chain, the cis and trans isomers are in equilibrium; thus, non-native X-Pro isomers are also populated. The wrong isomeric states of three of the four proline residues were shown to retard the conformational refolding process (*16*). In particular, the cis isomers of Pro93 and Pro114 and the trans isomer of Pro117 appear to be obligatory for fast conformational folding. The fluorescent spectra of the flanking tyrosine residues 92 and 115 report directly on the isomeric states of the 93 and 114 X-Pro peptide groups, making the fluorescence spectra very complex. By contrast, the UV absorbance spectra are not sensitive to the cis−trans isomerization of the X-Pro peptide groups

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* Corresponding author. Phone: (607) 255-4034. Fax: (607) 254-4700. E-mail: has5@cornell.edu.

[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A.; des-[40−95], RNase A lacking the 40−95 disulfide bond; 3S ensemble, molecules with three disulfide bonds but no stable tertiary structure; CD, circular dichroism; HPLC, high performance liquid chromatography; DTT^{red}, DL-dithiothreitol; DTT^{ox}, *trans*-4,5-dihydroxy-1,2-dithiane; AEMTS, (2-aminoethyl)-methanethiosulfonate; GdnHCl, guanidine hydrochloride; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); TAPS, tris(hydroxymethyl)methylaminopropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PPI, peptidylprolyl isomerase.

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and, thus, report only on the burial of the tyrosine residues in the folded conformation, thereby facilitating easier interpretation. The observed absorbance changes during disulfide-intact conformational folding are attributed mainly to three (Tyr25, Tyr92, Tyr97) out of the six tyrosine residues of RNase A (*16*, *18*).

In oxidative folding experiments, the native disulfide bonds are regenerated from the reduced protein with an accompanying formation of the tertiary structure of the native protein. In the initial stages of regeneration (under the frequently used conditions of 25−100 mM DTT^{ox}, pH 8.0 at 25 °C), the reduced protein molecules are oxidized successively to 1S, 2S, 3S, and 4S species, establishing a quasi-equilibrium among the 1S−4S ensembles (*19*, *20*). The nomenclature *n*S refers to an ensemble of species in which *n* disulfide bonds are already formed regardless of whether the bonds are native or nonnative. The species in the ensembles are unfolded and interconvert relatively rapidly by disulfide-reshuffling reactions. The 4S species is exceptional because, not having any free thiols, it does not reshuffle but can only be reduced to a 3S species.

In the final step, native RNase A is formed by oxidation from one of the four des species (*19*, *20*) (des refers to a species in which three native disulfide bonds are formed but one is missing). Two of these des species, des-[65−72] and des-[40−95], are structured (*4*, *5*) while the other two des species (des-[26−84] and des-[58−110]) are not stable enough to accumulate at 25 °C, pH 8.0 (*21*−*23*). The regeneration at 25 °C, pH 8.0, proceeds through the two structured des species (>85% through des-[40−95] and <15% through des-[65−72]) rather than through the unstable counterparts (*19*, *20*). The formation of these two structured species from the 3S ensemble by intramolecular reshuffling reactions, accompanied by two very minor oxidative steps from 2S to these same des species (9, *10*), is the rate-determining step in the oxidative folding of RNase A (*19*, *20*). Although it is the key step, the mechanism of this main intramolecular rearrangement step, i.e., how the protein gains its nativelike structured conformation, is not clearly understood (*24*, *25*).

In one model, the precursor intermediate of the des species folds first (conformationaly) and brings the reactive cysteines into close proximity. Then, the subsequent disulfide reaction, which forms the third native bond of the des species, stabilizes this already formed conformation (*24*, *25*). In one variant of this model, local regions of the protein adopt their native structure, which is then stabilized by forming nearby disulfide bonds. To simplify the discussion, we refer to this mechanism in this article as a *piecemeal model* of oxidative folding because, in this scenario, global folding results from the little-by-little accumulation of local "microfolding" events (*25*, *26*). In an alternative model, the third native disulfide bond forms first in the unfolded precursor species. Then, this bond formation induces conformational folding to produce the folded des intermediate. The resulting structure protects the formed native bonds from destruction by further disulfide reactions, thereby stabilizing and, thus, populating the des species (*25*). This model is called *quasi-stochastic* because the formation of disulfide bonds (either native or non-native) is governed primarily by the loop entropies and also by the (usually local) enthalpic interactions within each unfolded species.

Although our model experiments cannot provide direct information about how the structure-formation step occurs during regeneration, they reveal many important features of the regeneration process. Our aims here are (i) to establish whether conformational folding of des-[40−95] in the absence of the Cys40−Cys95 disulfide bond involves various folding phases such as those of the disulfide-intact protein, (ii) to confirm the feasibility of the quasi-stochastic model, and (iii) to characterize and determine the extent of the competition between conformational folding and disulfide-reshuffling reactions which may occur under different oxidative folding conditions. This information may also help to distinguish among current models of oxidative folding. Aim iii can be addressed with des-[40−95], which contains two free thiol groups to facilitate reshuffling, but not with wild-type RNase A, which contains no free thiol group.

Des-[40−95] is relatively well characterized (*5*, *27*−*30*), which helps in the interpretation of kinetic experiments. Its structure is nativelike, as revealed by NMR experiments using a Cys-to-Ala mutant analogue (*5*), with lower overall stability and local perturbations around the missing disulfide bond compared to the wild-type protein. However, the proline residues, especially Pro93, which is close to the region of Cys95, are locked in their native isomeric states (*5*).

In this study, the denatured des-[40−95] was kept at low pH to block any disulfide rearrangement completely, and the folding reaction was carried out at higher pH values (pH 5 to 9) allowing both conformational folding and disulfide-reshuffling reactions to take place at several different temperatures. The data presented here not only provide information about the mechanism of oxidative folding but also show the capability of the reshuffling−folding competition experiments to detect structure in conformational-folding intermediates.

EXPERIMENTAL PROCEDURES

Materials. Native RNase A (type 1-A, Sigma) was purified by cation-exchange chromatography (SP Sepharose, Amersham Pharmacia Biotech) according to the procedure of Rothwarf et al. (*19*). DTT^{red} (ultrapure) was obtained from Sigma. The thiol-blocking reagent AEMTS was synthesized by the procedure described by Bruice and Kenyon (*31*).

Preparation of Intermediate. The folding intermediate des-[40−95] was produced from native RNase A by incubating the latter in a degassed solution (400 mM DTT^{red}, 100 mM Tris-HCl, pH 8.0, and 1 mM EDTA) at 15 °C for 16 h and purified by HPLC using a cation-exchange column (Hydropore SCX, Rainin) as described by Li et al. (*27*). Fully reduced RNase A was produced from native RNase A by incubating the latter in a buffer (4 M GdnHCl, 100 mM DTT^{red}, 100 mM Tris-HCl, pH 8.0, and 1 mM EDTA) for 4 h at room temperature. All final products were purified by HPLC using a reverse phase column (ODS-AQ column, YMC).

pH Transition. A series of protein solutions at different pHs was prepared by mixing 0.4 mM protein solutions in varying relative amounts of 100 mM glycine at pH 1.7 and 100 mM sodium acetate at pH 5.0. The absorbance of these protein solutions at 287 nm was measured at 25 °C with a Cary model 14 UV spectrophotometer.

Circular Dichroism Spectroscopy. Far-UV CD measurements were carried out on a modified Cary model 14

spectrophotometer (*32*) at 25 °C. The protein concentration was 33 μM, and a cuvette of 1 mm path length was used. Each spectrum was the average of three scans.

Stopped-Flow Kinetic Measurements. The experimental setup, using a Hi-Tech PQ/SF-53 stopped-flow apparatus, has been described by Houry et al. (*15*). The flow cell had a path length of 10 mm and a width of 2 mm. Data were collected every 0.5 ms for the first 1 min and every 50 ms thereafter.

For absorption measurements, a deuterium lamp (Hellma) was used as a light source, with a monochromator for the incident light set at 287 nm. Single-jump refolding and reshuffling experiments were carried out at 25 °C. For the single-jump refolding, 11 μL of protein solution (0.7 mM) in 50 mM glycine at pH 1.7 was mixed with 110 μL of refolding buffer, 100 mM sodium acetate, and 2 mM EDTA at pH 5.0. For the single-jump refolding and reshuffling, the same protein solution was mixed with a reshuffling buffer, 100 mM HEPES, and 2 mM EDTA at pH 8.0. The data obtained from single-jump refolding measurements were fit to a sum of exponentials plus a constant baseline with the program PLOT from New Unit (Ithaca, NY), using a Levenberg−Marquardt algorithm (*33*) for nonlinear least-squares fitting.

Refolding and Reshuffling Experiments. The protein solution [100 μL (0.8 mM) in 100 mM glycine at pH 1.7] was incubated at 25 °C for 1 h. After the incubation, the solution was diluted with 2 mL of the above refolding buffer or reshuffling buffer, respectively, which were preincubated in a water bath at the same temperature. Reshuffling buffers of pH 7.0 and pH 9.0 were prepared by using 100 mM HEPES and 100 mM TAPS buffers, respectively. For the GdnHCl study, the initial pH 1.7 buffer was prepared with 3 M GdnHCl. After mixing, the pH of the final solution was decreased by less than 0.2 units. At various times, 300 μL of the solution was quenched with 1 mg of solid AEMTS. After mixing with 20 μL of glacial acetic acid, the solution was desalted with 100 mM acetic acid on a Sephadex G25 (Amersham-Pharmacia Biotech) column. The sample was then loaded onto an analytical cation exchange HPLC column (Hydropore SCX 4.6 mm 10 cm, Rainin). The HPLC profile was obtained at pH 7.0 in 25 mM HEPES buffer with 1 mM EDTA and increasing concentrations of sodium chloride (*34*).

RESULTS

Stability of Des-[40−95]. Although des-[40−95] has a nativelike tertiary structure (*5*) in which the three native disulfide bonds are protected from reaction with its free thiol groups (Cys40 and Cys95), its structure is less stable than that of native wild-type RNase A [the midpoint of the GdnHCl transition is 0.8 M (at 25 °C, pH 8.0) and t_m is 34 °C (at pH 4.6) for the des analogue, the Cys40/95Ala mutant, compared to 3.0 M and 56 °C, respectively, for wild-type RNase A (*5*, *29*)]. The pH transition curve in Figure 1a suggests that des-[40−95] is unfolded around pH 2 and largely folded around pH 5 at 25 °C. The far-UV circular dichroism (CD) analyses at pH 1.7 and pH 5.0 at 25 °C further support this conclusion. The spectra at pH 1.7 and pH 5.0 closely resemble those of reduced RNase A and native RNase A, respectively (Figure 1b).

Stopped-Flow Study. On the basis of the pH transition of des-[40−95], single-jump refolding stopped-flow experiments, followed by the change of the UV absorbance of the tyrosine residues, were carried out at 25 °C (Figure 2). The protein, which was previously denatured by lowering the pH to 1.7, was refolded by mixing with pH 5 (refolding) or pH 8 (reshuffling) buffer, and the kinetic data are shown in Figure 2a,b, respectively.

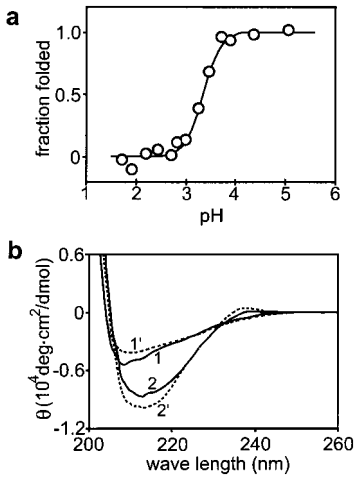

FIGURE 1: (a) pH transition of des-[40−95] at 25 °C. All data were calculated from the absorption at 287 nm. (b) Far-UV CD spectra of des-[40−95] at 25 °C and at pH 1.7 (curve 1) and 5.0 (curve 2), solid lines. As controls, the CD spectra of reduced (curve 1′) and native (curve 2′) RNase A at 25 °C and at pH 5.0 are presented as dashed lines.

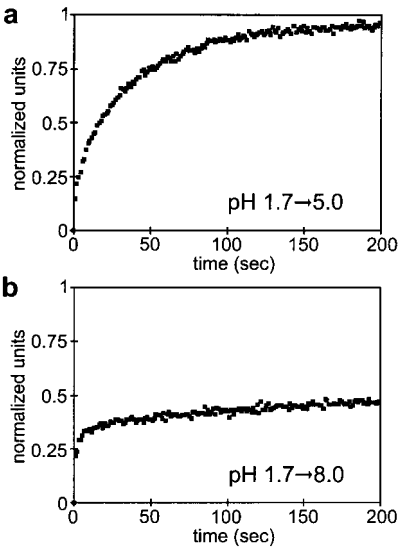

FIGURE 2: Representative data for the single-jump refolding and reshuffling experiments with des-[40−95] at 25 °C. The (a) refolding (jump from pH 1.7 to pH 5.0) and (b) competitive refolding and disulfide reshuffling (jump from pH 1.7 to pH 8.0) are shown on a normalized scale. From the refolding data at pH 5.0, time constants of refolding were calculated as 52 ms (fast), 2.8 s (medium), 32 s (slow), and 114 s (very slow) phases.

Acid-denatured des-[40−95], refolded by raising the pH to 5.0, shows multiple phases (Figure 2a). As shown previously in disulfide-intact studies (*15*, *16*), the multiple phases result from the presence of non-native isomers of essential proline residues in the denatured species. The time constants for refolding of the des-[40−95] species were observed to be 52 ms, 2.8 s, 32 s, and 114 s and resemble those of the disulfide-intact wild-type protein in that there are multiple refolding phases. For folding of wild-type protein at pH 5.0, 0.6 M GdnHCl, 15 °C, the respective

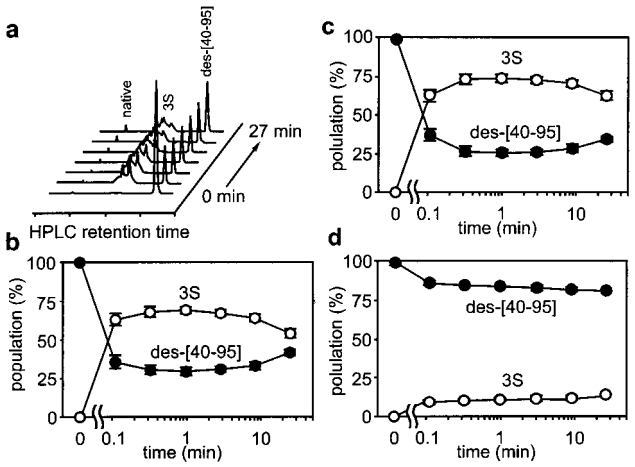

FIGURE 3: Relative changes in the population of species as a function of disulfide-reshuffling time from 0 to 27 min at 25 °C: (a) chromatographic changes in the refolding and reshuffling experiment (pH 8.0); relative changes in the population of species as a function of disulfide-reshuffling time after the pH jump (b) from 1.7 to 8.0, (c) from pH 1.7 and 3 M GdnHCl to pH 8.0 and 0.15 M GdnHCl, and (d) from pH 5.0 to 8.0. The relative populations of des-[40−95] (closed circles) and 3S (open circles) are presented. Those of the byproducts (native and other ensembles) were less than 5% during these experiments and are not presented here.

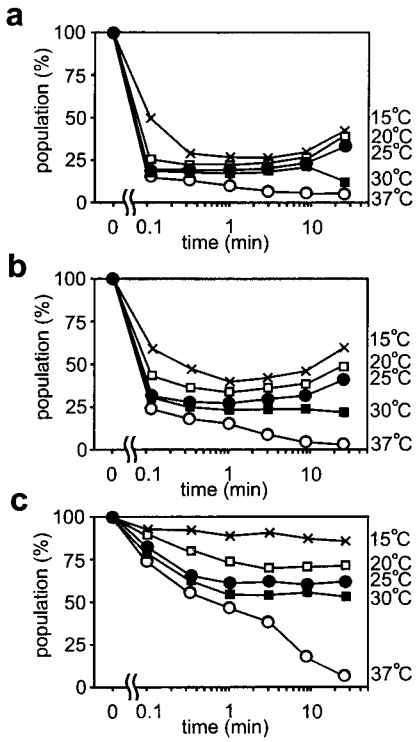

FIGURE 4: Temperature and pH dependence of the population of des-[40−95] in the refolding and reshuffling experiments: 15 °C (crosses); 20 °C (open squares); 25 °C (closed circles); 30 °C (closed squares); 37 °C (open circles); (a) pH 9.0; (b) pH 8.0; (c) pH 7.0. Only relative populations of des-[40−95] are presented.

values are 37 ms, 3.0 s, 30 s, and 129 s (*16*). The relative amplitudes are 111, 101, 612, and 182%, respectively, compared to 21, 9, 56, and 14% for the wild-type protein (*16*).

However, the refolding scenario at pH 8.0 was significantly different from that at pH 5.0 (Figure 2b). Most of the changes in UV absorbance occurred during the first seconds, in contrast to the pH 5.0 experiment in which about 70% of the changes in UV absorbance took place within a few seconds to minutes. The large difference in the conformational folding between these two final-pH conditions suggests that disulfide reactions might play an important role in the process.

Refolding and Reshuffling Experiments. To check this observation, identical pH jump experiments were carried out by manual mixing. However, the disulfide reaction was quenched by AEMTS at different times, and the aliquots were analyzed by cation-exchange chromatography. Des-[40−95] is well-separated from the other unstructured 3S species on the chromatogram (Figure 3a). About 70% of des-[40−95] was converted to other 3S species during the first 20 s (Figure 3b). The des-[40−95] population did not change significantly in the next few minutes. However, it starts to increase slowly with an accompanying decrease in the 3S population, after 9 min.

The 30% of des-[40−95] that survives the first 1 min of the experiment appears to be folded judging from its resistance against disulfide-reshuffling (Figure 3b). To ascertain whether this 30% folded des-[40−95] population is a result of incomplete pH denaturation or not, a similar experiment was carried out using GdnHCl (Figure 3c). The protein was unfolded in 3 M GdnHCl at pH 1.7 and was refolded at pH 8.0 in 0.15 M GdnHCl. Des-[40−95] is completely unfolded in 3 M GdnHCl under these conditions, judging from the GdnHCl transition of the mutant analogue (*5*, *29*). The result of the pH jump experiments was basically the same regardless of whether GdnHCl was included or not in the pH 1.7 buffer.

As a control, the same pH jump was carried out except that both solutions (pH 1.7 and 5.0) contained 3 M GdnHCl (data not shown). Under these conditions, most of the des-[40−95] reshuffled to 3S within 20 s. In another control, a jump from pH 5.0, where the species is largely folded, to pH 8.0 was carried out (Figure 3d). Less than 15% of des-[40−95] was reshuffled during the first 20 s. Afterward the des-[40−95] continuously decreased slightly. This 15% decrease of des-[40−95] due to reshuffling may arise because of a fluctuation of the structure by a pH jump and/or because some percentage of the des-[40−95] was possibly in the unfolded state at pH 5.0, 25 °C.

To characterize the phenomena seen in these pH-jump experiments, the assays were carried out at several temperatures and pHs (Figure 4). The variations of pH and temperature affect the rates of both the disulfide-reshuffling reaction and conformational folding. Higher pH or temperature resulted in significantly higher population of 3S species, primarily by accelerating the disulfide-reshuffling reaction more than the conformational folding. The differences in the rate of disulfide-reshuffling under different conditions are evident in Figure 4; i.e., the population of des-[40−95] does not decrease any further after 6 s at 30 °C, pH 9.0, while it decreases until 3 min at 20 °C, pH 7.0. At 30 and 37 °C, the population of des-[40−95] keeps decreasing during the full course of the experiment, due to the instability of des-[40−95] at higher temperatures. At lower temperatures, where des-[40−95] is thermodynamically more stable than the 3S species, the des-[40−95] population increases after the first 1 min. In summary, Figure 4 suggests that the competition

of refolding and reshuffling of disulfide bonds can take place over a wide range of temperatures and pHs.

DISCUSSION

Unfolded Des-[40−95]. The starting conditions of these experiments, the unfolded state of des-[40−95], is critical for discussing the results. Reduced RNase A has no remaining stable secondary structure (*13*, *35*); however, it is not a statistical coil (*36*). Some conformational ordering is present in the reduced protein in the absence of denaturant (*13*, *36*) in accord with the differences in the far-UV CD spectra of the reduced protein in the presence and absence of GdnHCl (*13*, *35*). Similarly, most of the unfolded intermediates are likely to have similar conformational ordering, compared to the fully reduced protein. pH denaturation is usually not as complete as GdnHCl denaturation; therefore, the pH-denatured state of des-[40−95], and not the GdnHCl-denatured one, more closely resembles that of the unfolded intermediates populated in the oxidative regeneration of RNase A. Thus, pH denaturation was used in these experiments. Comparison of refolding of acid-denatured and GdnHCl-denatured des-[40−95] supports the conclusion that, in this particular case, the protein is unfolded at pH 1.7 without GdnHCl (Figure 3b and c). However, Figure 3c also revealed that any conformational order that may be present in the pH-denatured species but not in the GdnHCl-denatured one does not seem to cause any significant change in the conformational folding of the des species. Presumably the GdnHCl-denatured species equilibrate in less than a few milliseconds among the same conformations as in pH denaturation in the absence of GdnHCl, upon dilution to 0.15 M at pH 8.0. It is possible that the precursor species of des-[40−95] populated during oxidative folding at pH 8 (under our regeneration conditions) may have more conformational order than the pH-denatured species in this model system, thereby altering the conformational folding rates considerably during regeneration. Thus, our results for this model system may not be directly applicable to oxidative folding during the regeneration experiment.

Conformational Folding of Des-[40−95]. Conformational folding of the des species at pH 5.0 resembles that of the disulfide-intact protein (Figure 2a and ref *16*); i.e., the respective folding phases are present in both proteins. The thiols are mostly in the protonated form at pH 5.0; thus, the disulfide-reshuffling reactions are extremely slow and do not affect the conformational folding process significantly. Apparently, the absence of the disulfide bond between Cys40 and Cys95 has little effect on the conformational folding rates. A significant fraction of the des protein, as in the disulfide-intact wild-type protein, folds in the millisecond time range. This finding is interesting, considering the close proximity of Pro93 and the Cys40-Cys95 disulfide bond; however, only the non-native trans isomer of the X-Pro93 peptide group seems to slow the conformational folding process (*16*).

Competition of Conformational Folding and Disulfide-Reshuffling Reactions of Des-[40−95]. The refolding of the pH-unfolded species at higher pH (between 7 and 9), where disulfide-reshuffling reactions can also take place with an appreciable rate, shows different characteristics. When the protein is subjected to folding conditions by increasing the pH, the two processes (conformational folding and disulfide reshuffling) start to compete. The thiolate may attack the disulfide bonds resulting in species that have non-native disulfide bonds. At pHs between 7 and 9 at 25 °C, only those species that have a particular set of three native disulfide bonds can fold; the non-native disulfide-bonded species remain unfolded and undergo further disulfide-reshuffling reactions. This process is indicated by the appearance of the 3S population on the cation-exchange chromatogram in Figure 3.

However, when the protein molecule folds, it protects its disulfide bonds from the attacking thiolates, slowing the disulfide-reshuffling rate considerably. This process results in the survival of the des-[40−95] population on the cation-exchange chromatogram. The absorbance change detected during the first 1 min in the stopped-flow experiment at pH 8.0 (Figure 2a) can be attributed to the appearance of this folded population. This conclusion is further supported by the pH-jump control experiments. In the pH-jump from one folded (pH 5.0) to another folded (pH 8.0) state (Figure 3d), the tertiary structure protects the des species from fast reshuffling. When conformational folding does not occur [in the jump from one guanidium-denatured (pH 1.7) to another guanidium-denatured state (pH 8.0)] (data not shown), most of the des-[40−95] reshuffles to 3S within 20 s. However, in the jump from the denatured (pH 1.7) to the folded (pH 8.0) state (Figure 3b,c), a significant fraction of the species kept its disulfide bonds intact, suggesting that it is the conformational folding of the des-[40−95] that protects its disulfides from fast reshuffling.

It should be noted, however, that the population of des-[40−95] in the chromatogram of Figure 3a is not necessarily equivalent to the folded des population. Molecules that are neither folded nor yet reshuffled at the moment of blocking at the beginning of the experiment still elute with the population of des-[40−95] species which are folded at the moment of blocking (unpublished observation). However, the competition is complete during the first 3 min depending on the conditions, as indicated by the absence of any further decrease in the des-[40−95] population at and below 25 °C (Figure 4).

After this first fast equilibration process under all conditions, in which the competition results in folded des-[40−95] species and other rapidly interconverting unfolded species with three disulfide bonds (i.e., the 3S species), a second slower equilibration process begins in which the 3S and des-[40−95] populations approach the thermodynamically stable equilibrium. At 25 °C and below, the more stable des-[40−95] species starts to increase (Figure 4). By contrast, at 30 and 37 °C where des-[40−95] is less stable, the 3S population increases with the accompanying decrease in the des-[40−95] population. However this decrease is apparently much slower than in the first fast-equilibration process (Figure 4). Because this second equilibration process is well-characterized (*8*, *13*, *19*, *37*), we did not collect later data to elucidate this process in the present work.

Before these experiments were carried out, it was not possible to anticipate the results and conclusion from this study. The (native) cis isomeric state of the Tyr92−Pro93 peptide group is essential for fast (millisecond) conformational folding (*15*, *16*). If the 40−95 disulfide bond were also essential for such fast conformational folding, no folded

species would be observed for des-[40−95] under conditions (high pH and temperature) in which the disulfide-reshuffling rate is fast. By contrast, if a possibly kinetically trapped intermediate with a (non-native) trans isomer of Pro93 is destabilized by the absence of the 40−95 disulfide bond, des-[40−95] would fold much faster than the disulfide-intact wild-type protein with a non-native Tyr92-Pro93 peptide group; thus, nearly all of the des-[40−95] population would fold without much reshuffling under all conditions.

This analysis has also demonstrated that these pH-jump experiments are a sensitive tool for detecting structure in conformational-folding intermediates. Considering that disulfide-bonded proteins frequently retain their folded conformations, even after the reduction of one of their disulfide bonds, the experimental approach described here is not restricted to RNase A but is applicable more generally to other disulfide-bonded proteins. A modified version of this model pH-jump experiment may make use of a reducing agent, instead of the intramolecular thiols of the protein, to detect the structural protection of the disulfide bonds in conformational folding intermediates of the *disulfide-intact* protein. It is worth noting that proteins having both disulfide bond(s) and free cysteine(s) in their native state have the same constraints to their conformational folding as the disulfide intermediates were shown to have in this study.

Implications for Oxidative Folding. Although the experiments presented here do not distinguish among possible mechanisms (*25*) of the structure-formation step (from 3S to des-[40−95]) of RNase A during oxidative folding, they do reveal some interesting features of the process. One of the most important conclusions from these pH-jump experiments is that a competition between conformational folding and disulfide reshuffling can occur and, as a result, the lifetime of the unfolded 3S intermediate species which is going to fold is very limited. The protein molecule has to fold faster than the thiolate can attack the disulfide bonds. At 25 °C, pH 8.0, where the regeneration experiments are usually carried out, the lifetime of the not-yet-folded des species is about a few seconds in our experiments as estimated from Figure 3b. Considering that there are several ways for two thiols to interact with the three disulfide bonds of des-[40−95], this value agrees reasonably well with the estimated average lifetime (6−9 s) for a single intramolecular thiol−disulfide-reshuffling reaction in unfolded RNase A species (*20*).

The very low population of the species that undergo conformational folding during the regeneration makes it difficult to obtain information about this step directly from the regeneration process. However, our results (Figure 3) suggest that it occurs within a few seconds, and the regeneration proceeds mainly through those species that are able to fold on this time scale. Oxidative folding models (*25, 26, 38−40*) that postulate that a slow conformational transition occurs in the unfolded intermediates, as well as the piecemeal model of oxidative folding that proposes successive formation of native disulfide bonds in one molecule, seem to be less compatible with the short lifetime of the unfolded intermediates that was shown in our study.

These competition experiments also showed that the quasi-stochastic model (*25*) of oxidative folding is a feasible one, because a significant fraction of des-[40−95] could fold under all conditions considered here. If, on the other hand, a folded population were absent, this would have indicated that this model is not realistic because des-[40−95] can readily be formed in the regeneration process under the same conditions. [As discussed in the previous section, the absence of a folded population of des-[40−95] could occur if the 40−95 disulfide bond played a critical role in fast conformational folding of the wild-type protein, and thus des-[40−95] (without the essential 40−95 disulfide bond) would not exhibit a fast (millisecond) folding phase. Thus, the whole population of des-[40−95] was reshuffled under several oxidative folding conditions (high pH and temperature) where reshuffling is fast.] Such a result, i.e., if folded populations of des-[40−95] were absent in the pH-jump experiment, would indicate an alternative mechanism, namely, that the freshly formed des species in the regeneration, in contrast to the pH-denatured des species, already has some structure at the moment of formation of the disulfide bond, which would promote faster conformational folding even in the absence of the 40−95 disulfide bond. Our results did not completely rule out this second alternative mechanism, but they showed that the quasi-stochastic mechanism is a feasible one.

Coupling of Proline Isomerization to the Regeneration Process. Our data demonstrate a new model (as in the quasi-stochastic mechanism) for coupling proline isomerization to the regeneration process. Although there is no direct indication that proline isomerization influences the oxidative folding of RNase A, proline isomerization is known to affect the rate of oxidative folding of proteins (*26, 38, 39*). Because the time scales of the regeneration (several hours) and proline isomerization (10−100 s) are quite different, it is not a priori obvious how proline isomerization can alter the oxidative folding rate. Two different mechanisms have been suggested to account for the influence of proline isomerization on disulfide regeneration (*26, 39*).

In one study, a piecemeal model of oxidative folding was proposed in which formation of the correct native X-Pro peptide group facilitates the formation of partial structure-(s) which promotes the subsequent formation of the native disulfide bonds (*26*). The enhancement of the regeneration rates by PPI leads to the suggestion in another study (*39*) that the rate-determining step of the process is a slow conformational transition. PPI speeds up proline isomerizations, thereby enhancing this conformational transition followed by formation of the correct disulfides (*39*).

By contrast, in the quasi-stochastic mechanism, the rate of formation of the three native bonds of des-[40−95] is postulated not to be altered by proline isomerization. However, proline isomerization affects the conformational folding rate in the freshly formed (but yet unfolded) des-[40−95] species and, thus, affects the *back-reaction*, the *destruction* of the native disulfide bonds in the competition step shown in this study. To understand this mechanism, we review the oxidative folding process.

In the first stage of oxidative folding (referred to as the pre-folding stage) the disulfide reactions are governed by loop-entropy and (usually local) enthalpic interactions in the unfolded intermediates (*8, 25*). Because these effects evidently are not sufficiently specific to favor the native disulfide bonds strongly over the non-native ones, many three disulfide species are formed, and the rate of formation of any particular set of disulfide bonds (native or non-native)

is slow. Once the three correct native disulfide bonds of des-[40−95] are formed at this slow rate, the same competition takes places in the des-[40−95] intermediate between conformational folding and the reshuffling reaction, as was seen in our pH-jump experiments. Increasing the proline-isomerization rate (e.g. by PPI) or the fraction of native proline isomer in the unfolded des-[40−95] species (e.g. by using a proline-mutant protein variant) may make the intermediates more competitive against reshuffling in this competition by enhancing their conformational folding rate; thus, the folded structure can protect the formed set of native disulfide bonds in a higher population, thereby increasing the rate of formation of the folded species, which is frequently the rate-determining step. Our experiments have shown that this model of the coupling of proline isomerization and disulfide formation is feasible; i.e., a significant fraction (70% at pH 8, 25 °C) of des-[40−95] is reshuffled, conceivably due to non-native isomers of essential proline residues.

Thus, to explain the accelerating effect of PPI in oxidative folding in the quasi-stochastic mechanism, by contrast to other mechanisms (*26*, *39*), there is no need to postulate (without any supporting data) that conformational folding occurs or even partial structure(s) is formed (*26*, *39*) in globally unfolded intermediates.

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