Regeneration of Bovine Pancreatic Ribonuclease A: Detailed Kinetic Analysis of Two Independent Folding Pathways†

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ABSTRACT: The regeneration of bovine pancreatic ribonuclease A (RNase A) from the reduced to the native form with mixtures of oxidized and reduced dithiothreitol at 25 °C, pH 8.0, proceeds through two separate pathways in which separate nativelike three-disulfide species are populated. The populations of these two three-disulfide species during the regeneration process have been monitored directly through the use of a reduction pulse. A detailed kinetic analysis of the regeneration process using improved experimental procedures and data analysis has been carried out to obtain rate constants for disulfide interconversion among the various disulfide-bonded intermediates. This analysis indicates that these two pathways can account for essentially 100% of the native protein regenerated and that the relative amount of native protein regenerated through these two pathways is insensitive to the redox conditions used. These results indicate that the rate-determining step in both pathways involves formation of the nativelike three-disulfide species, a step in which most of the conformational folding takes place. The experimentally determined rate constants indicate that these two pathways are sufficient to explain the differences in the temperature dependence of the regeneration rate with different redox reagents. In addition, the population of a fully oxidized species that contains three native disulfide bonded pairs and a dithiothreitol bridging cysteines 65 and 72 has been observed.

A general solution of the protein folding problem requires the identification of specific structural folding pathways and, in the case of disulfide-bond-coupled folding (i.e., a regeneration process), the identity and kinetic importance of the specific disulfide-bonded species that define that pathway. The preceding paper (*1*) has indicated that the regeneration of bovine pancreatic ribonuclease A (RNase A)[1] proceeds through two parallel pathways involving the formation of two nativelike three-disulfide intermediates, des-[65−72] and des-[40−95], which are missing the 65−72 and 40−95 disulfide bonds, respectively. These species are formed during the rate-determining step in the refolding process. The identification of specific intermediates, however, does not answer questions as to the role that they play in the folding pathway. In most experimental procedures that observe stable intermediates, including the type of disulfide trapping experiments carried out in the studies presented here and in the preceding paper, a thermodynamic distribution is measured. The mere presence of stable or metastable intermediates does not necessarily mean that these species are kinetically significant. Consequently, detailed kinetic studies are required to elucidate the role of isolated intermediates in the regeneration process. The importance of such kinetic studies has been demonstrated in the regeneration studies of BPTI, where it has been shown that non-native intermediates accumulate and lie on the direct regeneration pathway but are not involved in directing the subsequent folding (*2*). In this paper, we will examine in detail the kinetic significance of des-[65−72] and des-[40−95] and determine their relative contributions to the regeneration of native RNase A.

An earlier study (*3*) demonstrated that the rate of regeneration of RNase A with oxidized and reduced dithiothreitol (DTT^{ox} and DTT^{red}, respectively) decreased by a factor of 10 when the temperature was increased from 25 to 37 °C. In contrast, over that same temperature range the rate of regeneration of RNase A with oxidized and reduced glutathione (GSSG and GSH, respectively) increased by ∼10%. This initially suggested the existence of additional regeneration pathways kinetically accessible with GSSG/GSH but not with DTT^{ox}/DTT^{red}. From the kinetic data obtained at 25 °C and consideration of the temperature dependence of des-[65−72] and des-[40−95], the origin of the unusual temperature dependence can be explained. The results presented here, interpreted in conjunction with earlier studies of the reduction (*4*) and regeneration (*3*, *5*) of RNase A, suggest that the lower rate of regeneration at higher temperatures with DTT^{ox}/DTT^{red} can be explained as a consequence of the decrease in stability of des-[40−95] and des-[65−72] relative to the entire ensemble of 3S species at higher temperatures. This leads to a situation where the rate of rearrangement of des-[65−72] and des-[40−95] back to the

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; BPTI, bovine pancreatic trypsin inhibitor; des-[40−95], RNase A lacking the 40−95 disulfide bond; des-[65−72], RNase A lacking the 65−72 disulfide bond; AEMTS, 2-aminoethyl methanethiosulfonate [(NH_2)-$C_2H_5-S-SO_2-CH_3$]; DDS, disulfide detection system; DTT^{ox}, oxidized dithiothreitol; DTT^{red}, DL-dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography.

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3S species is comparable to the rate of oxidation to native protein. By contrast, under optimal regeneration conditions when using GSSG/GSH as the redox couple, the rates of disulfide-bond formation are much greater (1−3 orders of magnitude) (*5*) than the rates of disulfide-bond formation when DTT^{ox}/DTT^{red} is used as the redox couple. Therefore, when regeneration is carried out with GSSG/GSH the contribution of the rearrangement of des-[65−72] and des-[40−95] back to the 3S species is less significant, and the rate of formation of native protein is dominated by the rate of formation of des-[65−72] and des-[40−95]. Consequently, it is unnecessary to introduce any additional pathways to explain the differences in the temperature dependence between regeneration with DTT^{ox}/DTT^{red} and GSSG/GSH as had been proposed in an earlier study (*3*).

We will discuss how the kinetic studies presented here and the resultant information expands our knowledge of the protein folding process and enhances our goal of attaining the complete sequence of disulfide-bonded intermediates by which RNase A regenerates from the reduced unfolded state to the native fully oxidized form. In particular, we will discuss how these results establish the groundwork for the next stages in the general procedure to elucidate the regeneration pathways of RNase A.

MATERIALS AND METHODS

Materials. All materials are described by Rothwarf et al. (*1*) with the exception that the HPLC separations were carried out here on a Rainin Hydropore SCX 4.6 mm × 1.5 cm column. The gradient was the same as that reported previously (*1*), but the flow rate was reduced from 1 to 0.25 mL/min.

Detection of Nativelike Species. The regeneration process was carried out as described previously (*1*), although a wider range of redox conditions was used here (25−200 mM DTT^{ox}; 0−66 μM DTT^{red}). However, in addition to the usual blocking procedure, aliquots were subjected to a reduction pulse. This pulse consisted of the addition of an aliquot of a 200 mM solution of DTT^{red} (*before* adding the blocking agent) to yield a final concentration of 10 or 20 mM; after either 60 or 30 s, respectively, the mixture was blocked with AEMTS. Having determined the rate constants for reduction of native RNase A and of the nativelike species des-[40−95] and des-[65−72] to high accuracy (*4*), it follows that these species are not reduced to any appreciable extent by this reduction pulse. However, the other disulfide-bonded intermediates are reduced very rapidly under these redox conditions. The HPLC cation-exchange chromatogram for the resultant mixture should, therefore, contain only native RNase A, des-[65−72], des-[40−95], and totally reduced protein. But, based on previous values of the equilibrium constant for the R ⇔ 1S process (*6*), some small population of one-disulfide species should be observed under high redox conditions because the concentration of DTT^{ox} will be sufficiently high so that the equilibrium population of 1S, even at 10 or 20 mM DTT^{red}, will still be detectable. Because these 1S species are well resolved from des-[40−95] and des-[65−72] in the chromatographic separation, their presence in no way affects the analysis. Using this technique, it is therefore possible to obtain an accurate determination of the concentration of des-[65−72] and des-[40−95] as a function of time and redox conditions.

In addition to the reduction-pulse method, alternative methods were used to determine the concentrations of des-[65−72] and des-[40−95]. As discussed in the preceding paper (*1*), the elution position of des-[40−95] relative to the groupings of intermediates is very sensitive to the age of the column, eluting in the 2S region on a "new" column and in the 1S region on an "old" (heavily used) column. Therefore, by analyzing aliquots of the same regeneration mixture on new and old columns and determining the difference in the apparent amount of 1S and 2S species between the two chromatograms, the concentration of des-[40−95] was determined. In the case of des-[65−72], we found that the elution position of des-[65−72] relative to the other 3S species could be modulated slightly by changing the gradient and by adjusting the temperature within the range of temperatures in which quantitative recovery of all intermediates could be obtained (less than 30 °C). By maintaining the column temperature at 26 °C and adding a 20-min isocratic step under the initial chromatographic conditions prior to the gradient on a new column, it was possible to shift the distribution of 3S peaks such that des-[65−72] would elute earlier than the other species and could therefore be determined quantitatively. To minimize errors, these types of experiments were carried out under conditions in which the concentrations of des-[40−95] and des-[65−72] are known, from the reduction-pulse experiments, to be large. The concentrations of des-[40−95] and des-[65−72] measured by these techniques were always within 5% of the values determined from the reduction-pulse method.

Measurements of N′. N′ is a species that coelutes on the cation-exchange column with N. It is formed during the oxidation of des-[65−72] and is rapidly reduced back to des-[65−72] by the application of a reduction pulse; i.e., during the reduction pulse, N′ is reduced to des-[65−72], but N is not. Mixtures of N′ and N were prepared by direct oxidation of purified des-[65−72] at 25 °C, as described in the preceding paper (*1*). At various times, aliquots were removed and immediately subjected to the reduction-pulse procedure. Comparison of the peak areas of des-[65−72] and N (actually N + N′) obtained in the normal and in the reduction-pulse experiments was used to determine the appearance and disappearance of N′ as a function of time and redox conditions.

The rate of reduction of N′ to des-[65−72] was also measured directly using material that was purified by cation-exchange chromatography to remove des-[65−72]. For these studies, to enhance the concentration of N′ in the mixture, 175−200 mM DTT^{ox} was used, the protein concentration was kept at 20 μM or lower, and the oxidation was carried out for 50 min. The reduction was carried out with 1 mM DTT^{red}, and aliquots were removed for analysis at 3-min intervals.

Fitting of Experimental Data. The data were fit to various models using a simplex algorithm similar to that described previously (*7*). However, unlike the earlier procedure, all of the data were fit simultaneously rather than only those obtained under a given starting redox condition. This allowed more data to be included and a wider range of conditions to be used because even data obtained in single time measurements could be used in this improved procedure. This also allowed the reduction data for N′ and data obtained in experiments starting from isolated des-[65−72] and des-

[40−95] to be included. Each measurement at a given time was repeated at least twice, and in most cases, was repeated three times. To provide a better reflection of the quality of the data, error weighting was based on the measured standard deviations obtained from the repeats of the individual measurements. An additional factor was added to these standard deviations to account for the ±5% uncertainty of the relative extinction coefficients of the different species (*6*). Because of these improvements, the experimentally determined rate constants are more accurate than those of our previous studies, and the standard deviations of these rate constants better reflect the true standard deviations without potential systematic errors arising from averaging different data sets without explicitly accounting for errors in individual measurements. The error analysis was carried out by using a Monte Carlo procedure (*8*) in which data sets are generated by adding, to the experimentally determined data at each time, pseudo-random noise with a Gaussian distribution width equal to the standard deviation of the measurement. Two thousand five hundred such data sets were generated, and the results of the 2500 simplex fits were averaged to determine the standard deviation of the individual rate constants. Reduced χ^2 was close to 1 in all cases. The errors are given at the 95% confidence limit.

RESULTS

Reduction-Pulse Experiments. As shown in Figure 1, the application of a brief reduction pulse with DTT^{red} leads to the straightforward detection of both des-[65−72] and des-[40−95]. As expected, some of the 1S grouping is detectable at its equilibrium concentration with the reduced protein. The concentrations of des-[65−72] and des-[40−95] as well as that of the native protein and the various groupings of intermediates as a function of regeneration time and redox conditions are shown in Figure 2.

Kinetic Analysis. Initially, all of the kinetic data were fit to the model shown in Figure 2 of the preceding paper (*1*). The rate constants for the reduction of des-[65−72] and des-[40−95] and the reduction of the native protein are those obtained from direct reduction experiments (*4*). They are not obtainable from the regeneration data because of the low concentrations of DTT^{red} at which an appreciable regeneration rate can be obtained; i.e., the reduction of des-[65−72] and des-[40−95] make a negligible contribution to the concentration dependence of des-[65−72] and des-[40−95] or the rate of regeneration of native RNase A. The rate constants for rearrangement of des-[65−72] and des-[40−95] were also obtained from direct reduction experiments (*4*) and were used along with their standard deviations in the global fitting procedure.

The initial fitting of the experimental regeneration data resulted in the determination of a set of rate constants (data not shown) for the oxidation and reduction of the various groupings of intermediates as well as des-[65−72] and des-[40−95]. The rate constant for the oxidation of des-[65−72] obtained by this analysis was 0.087 ± 0.009 M^{-1} min^{-1} which is significantly different from the value of 0.139 ± 0.002 obtained by direct oxidation of purified des-[65−72] (*1*). The rate constant for the oxidation of des-[40−95] was 0.49 ± 0.03, which also does not agree with the value of 0.42 ± 0.01 obtained by direct oxidation of purified des-[40−95] (*1*). There are several possible explanations for these discrepancies. One possibility is that they are the result of a systematic error in the fitting of the data arising from the need to compute best-fit parameters for all species. However, computer simulations using the experimental rate constants demonstrated that the rate constants for interconversion among groupings of intermediates were not the cause of the discrepancy.

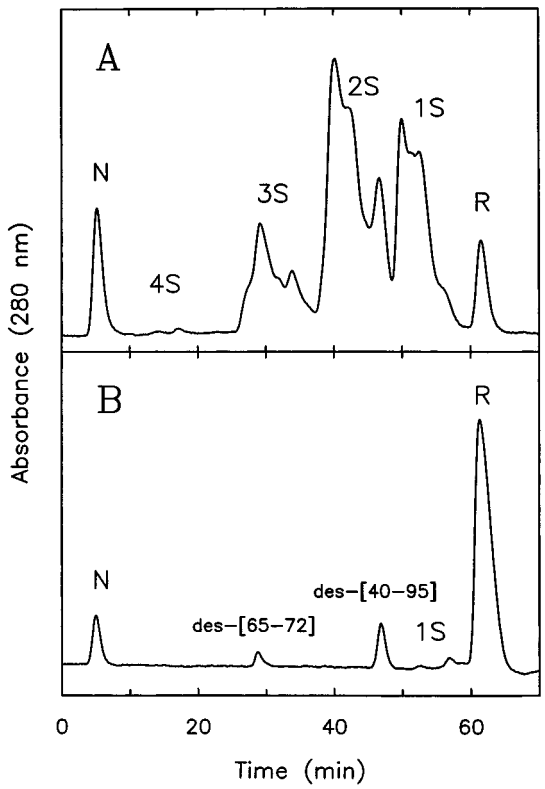

FIGURE 1: Chromatograms illustrating the reduction pulse experiment. (A) A typical regeneration mixture: 50 mM DTT^{ox} and 10 μM RNase A, pH 8.0, 25 °C, 90 min. (B) The same mixture that had been subjected to a 60 s pulse of DTT^{red} prior to blocking. The reduced protein is designated R, and the native protein is N. An Arabic numeral corresponding to the number of intramolecular disulfide bonds followed by S denotes the various groupings of disulfide-bonded intermediates. des-[40−95] and des-[65−72] correspond to species that contain three native disulfide bonds but lack the 40−95 and the 65−72 disulfide bonds, respectively.

A second possibility is that the differences are a result of an impurity in the DTT^{ox}. As discussed by Rothwarf and Scheraga (*5*), even small amounts (less than 0.1%) of linear disulfides can lead to large errors in the measurement of rate constants. However, this would imply that the rate constants measured for the early stages of the oxidation process, especially the R + DTT^{ox} → 1S step, contain significant contributions from the impurity. Since the impurity is expected to be a linear disulfide, the magnitude of its contribution is expected to be largely independent of the specific disulfide bond being oxidized (*5*). Consequently, it should result in a contribution of approximately 0.05 M^{-1} min^{-1} (i.e., the difference between 0.139 and 0.087) to the average rate constant, k_f^{avg}. Given that the measured value of k_f^{avg} in the preliminary regeneration analysis being discussed here (data not shown) and in our earlier study is approximately 0.05, this would suggest that essentially all of the reduced protein is oxidized as a result of the impurity, which is unreasonable.

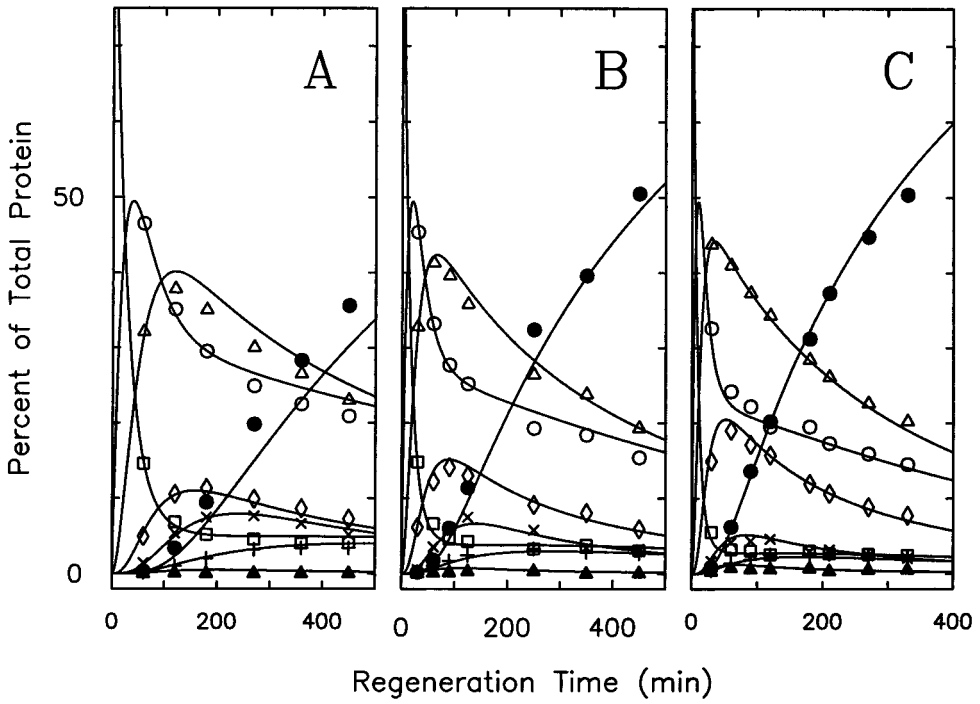

FIGURE 2: Representative curves showing the appearance of intermediates and native protein as a function of regeneration time at pH 8.0, 25 °C. (□) reduced protein; (○) one-disulfides; (△) two-disulfides; (◇) three-disulfides; (+) des-[65−72]; (×) des-[40−95]; (▲) four-disulfides; (●) native. Starting concentrations were (A) 25 mM DTT^{ox} and 6.0 μM RNase A; (B) 50 mM DTT^{ox} and 10 μM RNase A; (C) 100 mM DTT^{ox} and 16 μM RNase A. Solid lines are theoretical curves drawn using the model shown in Figure 3 and the rate constants shown in Table 1.

Furthermore, the values of k_f^{avg} are relatively constant in all of the data sets, both in this study and in our previous investigation (*7*). This would seem to be an unlikely result, if an impurity were contributing the bulk of the oxidizing potential, because the observed rate constants would be expected to fluctuate from one batch of DTT^{ox} to another and between samples of DTT^{ox} purified by methods other than the procedures used in these studies. No significant difference, however, has been found when regeneration was carried out with DTT^{ox} purified by recrystallization, HPLC, or unpurified straight from the bottle (D.M.R. and H.A.S., unpublished results). In addition, studies have been carried out on BPTI using DTT^{ox} without any evidence for the presence of a significant impurity (*9*). Lastly, we have independently measured the glutathione/dithiothreitol equilibrium constant using purified reagents (*10*) and also computed it from the equilibrium constants obtained for the R ⇔ 1S and 1S ⇔ 2S processes using GSSG/GSH and DTT^{ox}/DTT^{red} as the redox couple (D.M.R. and H.A.S., unpublished results) and obtained consistent results. These points strongly suggest that impurities make a negligible contribution to the rate of oxidation and that an alternative explanation is necessary.

One possible explanation is that des-[65−72] does not oxidize to the native state but rather to an intermediate that is susceptible to reduction (e.g., des-[65−72] ⇔ N′ → N) in which the rate of reduction of N′ to des-[65−72] is significant even at low concentrations of DTT^{red}. We tested this hypothesis, which arose from our *preliminary* analysis of the rate data, by oxidizing des-[65−72] with very high concentrations of DTT^{ox} (175−200 mM) and then carrying out the reduction-pulse procedure. The results indicated that a significant portion of the material that elutes in the position of native protein, N, is rapidly reduced to des-[65−72]. Consequently, since N is stable under these reduction conditions, this reducible material is *not* native, and we refer to it as N′.

We further investigated the nature of N′. The N + N′ mixture was purified by cation-exchange HPLC and allowed to equilibrate at pH 8. At various times, aliquots were removed and subjected to a reduction pulse to monitor the possible conversion of N′ → N. No change in the distribution of N/N′ was observed even after 10 days. This indicates that N′ does not convert to N directly. We, therefore, revised our model to a parallel rather than a sequential one, i.e., to include the steps des[65−72] ⇔ N′ and des-[65−72] → N. Addition of a mixture of DTT^{red} and DTT^{ox} (100 mM DTT^{ox}, 0.4 mM DTT^{red}) to the (N + N′) mixture results in complete conversion to N when allowed to equilibrate overnight (15 h), further supporting the need for a redox reagent to convert N′ to N.

Chemical characterization of N′ (*11*) revealed that it contains a dithiothreitol moiety that bridges Cys-65 and Cys-72 by means of disulfide bonds. This would suggest that the rate of formation of N′ from des-[65−72] should depend on $[DTT^{ox}]^2$. The global fitting of the data, however, suggests a linear dependence. More importantly, data obtained from direct oxidation of purified des-[65−72] (*1*) strongly indicate a linear dependence on $[DTT^{ox}]$. Best-fit parameters using a $[DTT^{ox}]^2$ dependence for the rate of formation of N′ result in predicted values that are over 5 standard deviations from the experimentally measured values. Finally, data obtained from direct oxidation of purified des-[65−72] in the presence of 4 mM DTT^{red}, conditions under which N′ does not form, provide the same value for the des-[65−72] → N rate constant (0.091 ± 0.006 min^{-1} M^{-1} at 95% confidence) as does the global analysis carried out assuming a linear dependence on $[DTT^{ox}]$ for des-[65−72]

Table 1: Rate Constants at 25 °C, pH 8.0

reaction	k_f^{obs} [a] ($\times 10^2$ min^{-1} M^{-1})	k_f^{avg} [b] ($\times 10^2$ min^{-1} M^{-1})	k_{intra}^{obs} [c] (min^{-1})	k_{intra}^{avg} [d] (min^{-1})	k_r^{obs} [e] (min^{-1} M^{-1})	k_r^{avg} [f] (min^{-1} M^{-1})
R ⇔ 1S	146 ± 2[g]	5.2	225	8.0	520 ± 20	520
1S ⇔ 2S	75 ± 2	5.0	116	7.7	1100 ± 40	560
2S ⇔ 3S	37 ± 2	6.2	58	9.6	1900 ± 200	630
3S ⇔ 4S	4.3 ± 0.4	4.3	6.7	6.7	2000 ± 300	490
3S → des-[65−72]	0.21 ± 0.02[h]					
des-[65−72] → 3S	0.030 ± 0.006[h,i]					
3S → des-[40−95]	1.4 ± 0.1[h]					
des-[40−95] → 3S	0.64 ± 0.03[h,i]					
des-[65−72] ⇔ N	9.4 ± 0.9	9.4	15	15	0.0065 ± 0.0004[i]	0.0065[i]
des-[65−72] ⇔ N′	4.7 ± 0.9	4.7	7.3	7.3	160 ± 30	160
des-[40−95] ⇔ N	43 ± 3	43	67	67	0.0086 ± 0.0004[i]	0.0086[i]

[a] k_f^{obs} is the observed rate constant for formation of a protein disulfide bond with DTT^{ox} for the reaction indicated. [b] k_f^{avg} is k_f^{obs} corrected for statistical factors, as described in the text. [c] k_{intra}^{obs} is the rate constant for formation of intramolecular protein disulfide bonds, determined from k_f^{obs} using eq 4 of ref 7. [d] k_{intra}^{avg} is k_{intra}^{obs} corrected for statistical factors as described in the text. [e] k_r^{obs} is the observed rate constant for reduction of a protein disulfide bond with DTT^{red} for the reaction indicated. [f] k_r^{avg} is k_r^{obs} corrected for statistical factors as described in the text. [g] The error is calculated at the 95% confidence limit. [h] Units are min^{-1} ($\times 10^2$). [i] Rate constants taken from ref 4.

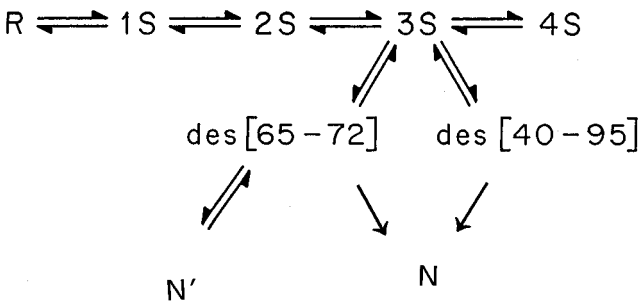

FIGURE 3: Final regeneration model; rate constants are given in Table 1.

→ N′. The mechanistic significance of this redox dependence is discussed by Li et al. (*11*). Consequently, a linear dependence on [DTT^{ox}] was used in the final kinetic analysis. It should be noted that des-[40−95] does not lead to a species such as N′, presumably because it lacks the specific interactions that stabilize the adduct with DTT.

Table 1 lists the experimentally determined rate constants for the final regeneration model shown in Figure 3.

Average Rate Constants. Observed rate constants derived from the fit of the experimental data are listed in columns 2 and 6 of Table 1. Average rate constants for disulfide-bond formation and disulfide reduction are listed in columns 3 and 7, respectively. Average rate constants reflect the average rate at which individual disulfide bonds are formed or reduced. They are calculated from the observed rate constants by normalizing the observed value by the appropriate statistical factor. These statistical factors arise because each of the groupings of the disulfide-bonded intermediates contain many species. There are 28 possible 1S species that can form from oxidation of the reduced protein. Therefore, the observed rate constant for the R → 1S step is actually the sum of 28 rate constants. Dividing the observed rate constant (k_f^{obs}), by 28 provides a value (k_f^{avg}), that is the average of the 28 individual rate constants. In a similar manner, average rate constants for the oxidation of the other groupings of intermediates can be determined. There are 15 2S species that can be formed from any 1S species, six 3S species that can be formed from any 2S species, and only one 4S species that can be formed from any 3S species. Likewise, for reduction, there are four 3S species that can be formed from any 4S species, three 2S species that can be formed from any 3S, two 1S species that can be formed from any 2S species, and only the reduced state can be formed from reduction of a 1S species.

DISCUSSION

The Model. The model shown in Figure 3 shows the pathways by which RNase A regenerates. The groupings of intermediates achieve a steady-state distribution as indicated in the top portion of the model. Native protein is formed through two separate pathways involving rearrangement of 3S species. The model does not include the reduction of des-[65−72], des-[40−95], and native protein by DTT^{red}. The value of the rate constant for those processes that are listed in Table 1 were obtained from studies of the reduction of native RNase A by DTT^{red} (*4*). Given the small value of these reduction rate constants and the low starting concentrations of DTT^{red} (≤66 μM) and low protein concentration (≤32 μM) used in the regeneration studies presented here, reduction of des-[65−72], des-[40−95], and the native protein make a negligible contribution to the regeneration process.

The model also does not include the possibility that there may be direct oxidation of 2S species to des-[65−72] and des-[40−95]. This is primarily because we have no way to measure this rate constant directly. However, as discussed in great detail in previous studies (*7*), intramolecular processes such as the rearrangements of 3S species to form des-[65−72] and des-[40−95] should be at least 2 orders of magnitude more rapid than an analogous intermolecular process involving the formation of des-[65−72] and des-[40−95] through the oxidation of 2S species by DTT^{ox}. This difference between intermolecular and intramolecular processes can also be seen clearly from a comparison of k_f and k_{intra}. Furthermore, a detailed kinetic analysis in the earlier study (*7*) failed to determine any significant contribution of 2S + DTT^{ox} → 3S* steps. Consequently, the contribution of direct oxidation processes to the formation of either des-[65−72] or des-[40−95] is probably negligible. It must be remembered that the difference in rate for oxidation and rearrangement processes arises from the use of DTT^{ox}/DTT^{red} as the redox couple (*5*). Therefore, regeneration pathways measured using other more potent redox couples such as GSSG/GSH may exhibit a significant contribution from the

direct oxidation of 2S species. Finally, regeneration studies with mutants, lacking either the 65−72 (12) or 40−95 (13) disulfide bond, showed that the rate-limiting step for the mutants is 2S → 3S* but that this pathway is indeed negligible for the regeneration of wild-type RNase A.

Comparison to Earlier Studies. The rate constants shown in Table 1 can be compared to the data obtained in an earlier work (*7*). Previously, we did not observe directly the species that formed in the rate-determining step, but rather inferred their existence from the kinetic analysis. Therefore, unlike the current model shown in Figure 3, the earlier work used a model that did not account explicitly for the presence of either des-[65−72] or des-[40−95], i.e., both des-[65−72] and des-[40−95] were grouped together as 3S*. Consequently, values of the rate constants reported here involving the 3S grouping, in particular those for the 2S ⇔ 3S process, are significantly different from those reported in the earlier work. All of the other rate constants involved in the oxidation and reduction of groupings of intermediates are the same, within error, as those reported previously (*7*). The value of k_f^{obs} for the 2S ⇔ 3S step is 0.37 min^{-1} M^{-1} as opposed to 0.23 min^{-1} M^{-1} in the previous study. In the case of k_r^{obs} for the 2S ⇔ 3S step, the current value is 1900 min^{-1} M^{-1} as opposed to 1100 min^{-1} M^{-1} in the previous study. These differences and the greater accuracy of the data presented here result in some modifications of the conclusions of those earlier studies. These will be developed in the following sections.

The average rate constants for reduction of the various intermediates in the various disulfide groupings are very similar (the same within error). This is consistent with the conclusion that the groupings of intermediates are disordered (*7*, *14*). Furthermore, as can be seen in Table 1, the rate constants for reduction of the groupings of intermediates are some 100 000-fold larger than the rate constants for reduction of the conformationally folded species, des-[65−72], des-[40−95], and the native protein (*4*), indicating the absence of significant structure within the groupings of intermediates.

k_{intra}. As discussed in detail in an earlier paper (*7*), k_{intra} is the rate constant for formation of an intramolecular disulfide bond and reflects the conformational properties of the protein. There are three entropic contributions to the observed value of k_{intra} that arise from the formation of intramolecular disulfide bonds that can be used to explain the observed values of k_{intra} of the conformationally disordered groupings of intermediates (*7*).

In the earlier study (*7*), there was no discernible difference between the values of k_{intra}^{avg} for any of the groupings of intermediates. In the present study, the value has a maximum for the 2S ⇔ 3S step and has a minimum for the 3S ⇔ 4S step. Such results suggest that the reason for the increase in the value of k_{intra}^{avg} for the 2S ⇔ 3S step arises mainly from the formation of overlapping loops involved in the formation of 2S, which reduce the entropy loss for the formation of the third disulfide bond. Formation of the fourth disulfide bond, however, is less favorable (except in the case of the native protein) since formation of three disulfide bonds has decreased the conformational freedom of the remaining thiols to a level that more than compensates for the decrease in entropy loss provided by the overlapping loops. Such a trend is quite reasonable given that the magnitude of the contribution arising from decreasing conformational freedom should increase with each successive disulfide bond formed, while the magnitude of the entropy loss due to overlapping loops should decrease. Therefore, one would normally expect the value of k_{intra}^{avg} to increase to a maximum value at some level of disulfide bond formation and then to decrease beyond that point. From the point of view of optimal folding, this point should directly precede the formation of the native protein. For example, in the regeneration of a four-disulfide protein, optimal oxidation to the three-disulfide form is preferred while subsequent oxidation to non-native four-disulfide species is less desirable. This is in agreement with the experimentally determined rate constants obtained from the regeneration studies of RNase A presented here (Table 1). The reason for the slightly more rapid formation of 1S than 2S species, when (from the above discussion) we would expect the opposite behavior, probably arises from the preferential formation of the very small loops (those involving formation of the 65−72 or 58−65 disulfide bond) in the 1S species. This explanation is consistent with experimental measurements of the distribution of 1S species (*15*).

Rate Constants. The rate constants listed in Table 1, which are defined by the model shown in Figure 3, are of two types. The first type is an individual rate constant that corresponds to a process involving a chemically distinct species and a unique chemical reaction. The rate constants involving the oxidation of des-[65−72] and des-[40−95] fall into this category. The second type is an apparent rate constant (*16*), which corresponds to a weight-averaged sum of many individual rate constants. The rate constants that describe the interconversion among groups of intermediates are of this type. Even the rate constants involving pure species may be apparent ones, as for the rate constants for the interconversion of des-[65−72] and des-[40−95] back to 3S. They are apparent rate constants because there are two free thiols in both des-[65−72] and des-[40−95] that can interact with any of three disulfide bonds; hence, they are the sum of 12 individual rate constants.

Rate-Determining Step.The rate-determining step(s) in the folding reaction define the processes that are critical for attainment of the native structure and specify the folding pathway(s). Generally, the rate-determining step is considered to be the slowest step in a multistep reaction (*17*). In the regeneration pathways of RNase A, the assignment of the rate-determining step is complicated by two factors. The first is that the oxidation and reduction processes are bimolecular and involve the redox reagent, while the rates of the rearrangement steps are intramolecular and therefore independent of the redox reagent. The second complication is that some rate constants are individual and others are apparent ones as explained in the previous section.

When considering the effect of the redox conditions on the determination of the rate-determining step, it is important to recognize that, under extreme redox conditions, the determination is simple but meaningless. For example, if we consider the situation in which the concentrations of the redox components are infinite, oxidation and reduction processes will be much faster than any rearrangement processes and a rearrangement step will be rate-determining. Similarly, under infinitely dilute concentrations of the redox couple, oxidation and reduction will be much slower than

rearrangement and hence rate-determining. Rather than deal with such physically unrealistic conditions, let us initially assume experimentally attainable redox conditions under which a significant rate of regeneration is obtained, such as those used to generate the experimental data shown in Figure 2C, 100 mM DTT^{ox} and 16 μM RNase A. Furthermore, for the sake of this initial discussion, we will be concerned only with the processes that occur after the establishment of the steady-state distribution among the groupings of intermediates, and we will focus on the major regeneration pathway involving des-[40−95].

Under these conditions, the pseudo-first-order rate constant for oxidation of des-[40−95] to native ($k[DTT^{ox}]$) is ∼3-fold larger than the rate constant for formation of des-[40−95] from 3S (0.043 vs 0.014). Consequently, it would appear that formation of des-[40−95] would be the rate-determining step. However, the rate constant for oxidation of des-[40−95] to native is an individual rate constant while the rate constant for formation of des-[40−95] is an apparent one. There are 420 possible different disulfide-bonded intermediates in the 3S grouping, only 12 of which could directly precede des-[40−95]; hence, the individual rate constants that contribute to formation of des-[40−95] are likely to be 1 order of magnitude or more larger than the observed value. Therefore, at the level of the individual rate constants, the slowest step would be the oxidation of des-[40−95]. Furthermore, viewed in this light, the slowness of the 3S → des-[40−95] process arises primarily from the large number of 3S species that result in a low population of the productive 3S species that can directly precede des-[40−95].

While this is one way to view the rate-determining process, there are others. The definition of the rate-determining step in protein folding is generally considered to be the step with the highest free-energy barrier along the reaction coordinate (*18*) and to correspond to the transition state of highest energy relative to the lowest energy ground state that precedes it (*19*). To apply this definition, we must specify a common ground state, i.e., des-[40−95]. If we consider the relative heights of the transition states (the magnitude of the rate constants) that directly precede (3S → des-[40−95]) and follow (des-[40−95] → N) des-[40−95], we can consider the consequences of this definition. The relative height of the barrier for 3S → des-[40−95] can be determined from the rate constant for des-[40−95] → 3S, since microscopic reversibility applies. Since the rate constant for the des-[40−95] → 3S reaction is actually a sum of 12 rate constants, it represents a minimum height for the transition state. However, since the pseudo-first-order rate constant for the des-[40−95] → N step is greater than the rate constant for des-[40−95] → 3S for all $[DTT^{ox}] > 15$ mM, the height of the transition state barrier that precedes des-[40−95] is greater than the transition state barrier that follows it under all the redox conditions used here. Thus, the rate-determining step would be the formation of des-[40−95].

From the above discussion, it is obvious that the conventional definitions of rate-determining step have very little meaning when dealing with a system such as the one under consideration here. A clearer and more sensible method of assigning the rate-determining step in a manner that provides some insight into the conformational folding process is to consider only the value of the intramolecular rate constant, k_{intra}. Using this criterion, the rate-determining step is clearly the formation of des-[40−95] and des-[65−72] and not their subsequent oxidation. As shown in Table 1, the values of k_{intra}^{avg} for the oxidation of either of the nativelike three-disulfide species to the native protein are larger than the values for interconversion among the various intermediates, and in the case of oxidation of des-[40−95], it is an order of magnitude larger. With regard to the formation of des-[65−72] and des-[40−95] from 3S, the rate constants are much smaller than the values of k_{intra}^{avg} among groupings and are approximately 5000 times smaller than the corresponding values of k_{intra}^{avg} for the oxidation of des-[65−72] and des-[40−95], respectively. Given that 12 out of 420 of the 3S species can directly precede any other 3S species, it appears that the most likely explanation for the low value of k_{intra} for these steps arises as much from a small value of the individual rate constant as from the low concentration of the intermediate.

It must be mentioned that much of the ambiguity in the definition of the rate-determining step arises from the use of a poor oxidizing agent such as DTT^{ox}. In the case of regeneration with linear redox reagents such as GSSG/GSH, where the oxidation step would typically be 2 orders of magnitude or more faster than with DTT^{ox} (assuming typical redox concentrations of these reagents), the rearrangement step would clearly be rate-determining under any criterion.

Temperature Dependence. An earlier study (*3*) indicated that the rate of regeneration of native RNase A with DTT^{ox}/DTT^{red} decreases by a factor of 10 as the temperature is increased from 25 to 37 °C. In contrast to results obtained with DTT^{ox}/DTT^{red}, the rate of regeneration of native RNase A increases slightly over the temperature range 25−37 °C when GSSG/GSH is used as the redox couple. This suggested the existence of an additional pathway(s) when regenerating with GSSG/GSH. The possible origins of such an additional pathway(s) arising from the nature of the redox reagent have been discussed previously in detail (*3*, *5*). The rate constants obtained at 25 °C and shown in Table 1 suggest an alternative explanation. The rate of oxidation of des-[65−72] and des-[40−95] to native RNase A at 100 mM DTT^{ox} [the concentration used in the earlier temperature dependence study (*3*)] is only 30- and 7-fold, respectively, greater than the rate at which those species interconvert back to 3S. As the temperature increases, the rate of interconversion back to 3S increases significantly, particularly for des-[40−95] (*4*). In oxidation with GSSG/GSH, the effective rate of oxidation is 1−3 orders of magnitude more rapid than it is with DTT^{ox}/DTT^{red} at the concentrations of those redox couples used in the earlier temperature dependence studies (*5*). Therefore, the rate of des-[65−72] → N or des-[40−95] → N using GSSG/GSH will be 2 orders of magnitude or more greater than the rate of any of the rearrangement steps involving des-[65−72] or des-[40−95]. Consequently, the rates of formation of des-[65−72] and des-[40−95] largely determine the rate of formation of native protein, and the rate of rearrangement back to 3S is less significant. Hence, the observed temperature dependencies can be explained solely on the basis of the effect of the nature of the redox reagent on the des-[65−72] and des-[40−95] pathways without requiring any assumptions about the existence of additional regeneration pathways. This clarifies a major question arising from the difference in the temper-

ature dependencies of the rate of regeneration of RNase A when using different types of redox reagents. It is interesting to note that this explanation suggests that the des-[65−72] pathway is likely to be the dominant pathway at higher temperatures particularly when using DTT^{ox}/DTT^{red} as the redox couple.

One point that should be mentioned is that both des-[40−95] and des-[65−72] are expected to have T_m values well above 40 °C at neutral pH (*20*). However, when considering the regeneration process, the relevant issue is not the stability of des-[65−72] or des-[40−95] with respect to their denatured state but rather with respect to the entire ensemble of 3S species. Since there are 420 possible 3S species, there is a large entropic factor favoring 3S. In addition, there may be some favorable enthalpic interactions in the 3S grouping. Consequently, the experimentally measured T_m values for des-[65−72] and des-[40−95] represent an upper limit for the T_m values for des-[65−72] ⇔ 3S and des-[40−95] ⇔ 3S.

Multiple Pathways. The data clearly indicate the existence of two distinct major regeneration pathways each containing a different nativelike three-disulfide species. Because the rate-determining step in both pathways involves an intramolecular process that has no redox dependence, the relative amount of native protein regenerated through the two pathways is largely unaffected by the concentration of the redox reagents under redox conditions in which RNase A can be regenerated. However, some redox dependence exists since des-[65−72] and des-[40−95] interconvert with 3S and are reduced by DTT^{red}. This dependency becomes large only when considering redox conditions that do not lead to significant levels of regenerated native protein, as under very strongly reducing conditions and will be considered below. Numerical simulation of the regeneration through the two pathways using the rate constants shown in Table 1 indicates that 80−86% of native RNase A is regenerated through the pathway containing des-[40−95] while the remainder is produced through the pathway containing des-[65−72].

Detailed studies of the reduction of RNase A (*4*) reveal that the transitions involved in the breakage of the 65−72 and 40−95 disulfide bonds in the native protein are conformationally distinct. Hence, based on the principle of microscopic reversibility, the formation of the final disulfide bond in the two regeneration pathways also occurs through conformationally distinct transitions. However, as we have established above, the main structural folding event and the rate-determining one (in terms of redox independent intramolecular processes) is the formation of the nativelike three-disulfide species and not their subsequent oxidation to native. Whether the two regeneration pathways involving des-[65−72] and des-[40−95], respectively, differ in this step at 25 °C is not known. It is interesting to note, however, that reduction studies have shown that the conformational transitions from des-[65−72] and des-[40−95] to 2S at 15 °C involve separate structural transition states (*4*).

Given that the evidence presented here and in earlier studies (*7*, *14*) indicates that the majority of the intermediates are conformationally disordered and in relatively rapid equilibrium, it is likely that the pathways do not diverge until the 3S species. However, it is possible that the formation of the correct disulfide pairings of des-[65−72] and des-[40−95] arises as a result of the same conformational transition and then diverges as a result of a conformational folding event that is after the formation of the third native disulfide bond. This could, for example, involve formation of either of the two native disulfide bonds that des-[65−72] and des-[40−95] have in common, viz., 58−110 and 26−84. However, in such a case in which the conformational transitions are the same and involve formation of the same disulfide bond in the absence of conformational effects, the rates of formation would be expected to be closer than the experimentally determined factor of ∼7 (see Table 1). This is because the differences in loop entropy among the various three-disulfide species will be fairly small.

The analysis presented here is consistent with the conclusion that the des-[65−72] and the des-[40−95] pathways account for essentially 100% of the native RNase A regenerated. However, the experimental uncertainty in the kinetic analysis (∼6%) leaves open the possibility that other minor regeneration pathways are populated (*12*, *13*). In particular, it is quite likely that pathways of the type $2S + DTT^{ox} \rightarrow$ des-[65−72] and $2S + DTT^{ox} \rightarrow$ des-[40−95] are populated (*12*, *13*). Moreover, such pathways could contain transition state structures similar to those involved in the 3S → des-[65−72] and 3S → des-[40−95] pathways. The insignificant contribution from the pathways involving oxidation of 2S to the formation of native protein (*12*, *13*) arises from the fact that they involve bimolecular reactions with the redox couple. If a redox reagent that formed stable mixed disulfide bonds were used, such pathways would be more highly populated. Consequently, the use of DTT^{ox}/DTT^{red} as the redox couple reduces the number of significant pathways.

Disulfide Rearrangement or Conformational Isomerization. In the above discussion, we have consistently referred to the 3S → des-[65−72] and 3S → des-[40−95] steps when discussing the regeneration pathways; however, we have not discussed the nature of those steps. In particular, is it a disulfide bond reshuffling step or is it a conformational folding process that does not involve disulfide chemistry. A conformational folding process could involve proline isomerization. Unfortunately, it is very difficult to distinguish these possibilities because the observed rate constants are apparent ones (since the concentration of the individual 3S species are not known). RNase A contains four proline residues, two of which are cis in the native state. These prolines give rise to very complicated refolding kinetics of the guanidine-denatured disulfide-intact form (*21*−*23*). The time constant for proline isomerization at 25 °C should be on the order of 10−100 s, which is about 2 orders of magnitude faster than the slow steps that we observe. However, given that only a small percentage of 3S would contain the correct disulfide pairings (1/420 if completely random), the proline hypothesis would seem reasonable. Given the fact that the concentrations of des-[65−72] and des-[40−95] detected are unaffected by the reduction pulse, either the isomeric state of the prolines does not affect the rate of reduction or the vast majority of those species (>∼95%) correspond to species containing the correct *cis*-proline conformations. However, whether the rate-determining step involves the isomerization is not clear. The regeneration conditions used here, 25 °C and pH 8.0 in the absence of any chemical denaturants, correspond to very strong conformational folding conditions. The vast majority (∼85%) of the disulfide-intact protein

would be expected to refold very rapidly under these conditions, viz., conformational folding is not prevented by the presence of non-native proline isomers (*22, 23*). des-[65−72] and des-[40−95] are somewhat less stable than native and could, therefore, be conformationally destabilized by the existence of non-native prolines. In such a case, we might reasonably suppose that lowering the temperature would increase the stability of des-[65−72] and des-[40−95] containing wrong proline isomers. However, no significant acceleration in the rate of regeneration is observed at 15 °C (*3*), which we would expect if this proline hypothesis is correct. Even if proline isomerization is not directly involved in the rate-determining step, an additional and somewhat subtle contribution could be made by proline isomerization to the regeneration pathways. The existence of non-native proline isomers could affect the formation of the nearby disulfide bonds, thus shifting the distribution of disulfide bonds within the groupings of intermediates and most importantly within the 3S grouping. For example, the population of the 40−95 disulfide bond could be decreased by the presence of non-native isomers at Pro-41 and Pro-93, thus increasing the likelihood of that disulfide occurring as the final step in the pathway.

N′ Pathway. The observation of N′ as a species containing a bridging dithiothreitol molecule (*11*) is quite unusual and represents the first example of such an experimentally observed species. A detailed analysis and discussion of N′ is found in ref *11* and will not be repeated here. However, the identification of N′ from the kinetic analysis of the regeneration data highlights the sensitivity of this experimental approach for the determination of regeneration pathways and specific disulfide-bonded intermediates. In particular, the maximum concentration of N′ that could be populated under regeneration conditions (starting from the fully reduced protein) optimized for formation of N′ would be less than 7% (obtained by computer simulation with the current model and the rate constants in Table 1). The kinetic analysis clearly indicated the presence of N′ even though its existence was not hypothesized during the original analysis. The attainment of high-quality kinetic data is the reason that this could be accomplished, and such data are critical for the further elucidation of the complete regeneration pathways of RNase A as discussed below.

Comparison to Reduction Pathways. Studies of the reduction pathways of RNase A (*4*) have revealed that RNase A is reduced through two parallel pathways involving des-[65−72] and des-[40−95]. Since the only difference between reduction and regeneration processes is the concentration of the redox reagent, the rate constants obtained from one study should be applicable to the other and microscopic reversibility should apply. (This assumes no significant interactions between the protein and the redox reagent other than those involving redox chemistry. This is still a reasonable assumption but, given the existence of N′, it is one that should be considered with care.) As a consequence of the application of microscopic reversibility, several conclusions can be drawn about the regeneration process, as noted above, including the one in which the final step in the two pathways (formation of the native protein) proceeds through conformationally distinct transition states.

It is important to remember that the regeneration and reductive pathways are dependent on bimolecular reactions involving the redox reagent. Consequently, while microscopic reversibility applies to the individual oxidation and reduction steps, it does not require that the population through the two pathways be similar in the two types of experiments and indeed they are not. During reduction, des-[65−72] is the major populated intermediate as opposed to des-[40−95] during regeneration. More importantly, under strongly reducing conditions, 54−60% (computed from the rate constants in Table 1 and in ref *4*) of the protein is reduced through the des-[40−95] pathway as opposed to the 80−86% of the protein regenerated through that pathway under favorable oxidizing conditions. These differences are not due to experimental error, and the fact that the values are in fact as close as they are is not a necessary condition. There are likely conditions of pH and temperature where regeneration would proceed primarily through one pathway and reduction through the other. This is a consequence of the very different concentrations of redox reagents required for the two types of processes. Moreover, the mechanisms describing the types of experiment presented here, i.e., regeneration or reduction, do not require that the same intermediates will be populated in both types of experiment. While by definition under identical solution conditions (including the concentration of the redox couple) the population of the two pathways will be the same under regeneration and reduction conditions, this is meaningless since the rate in at least one direction will be too small to be measured, i.e., either reduced protein will not be able to regenerate to native in any reasonable time and/or native protein will not be reduced. These differences are a necessary result of the practical limitations of such experiments, and it is important to avoid drawing unwarranted conclusions about one process based on qualitative observations from the other. Application of the results from one type of experiment to the other should be restricted to quantitative kinetic measurements.

General Method. The purpose of these studies is to elucidate the nature of the conformational transitions that direct the regeneration of RNase A. These studies can be divided into four major stages. The first is the determination of the type of rate-determining step. The second is the identification of the intermediate(s) that directly follow the rate-determining step in all major pathways. The third is the determination of the species that directly precede the rate-determining steps. The fourth is the detailed determination of the individual interactions that direct that step including the characterization of the transition state. An additional aspect is the identification of local structural preferences and their role in directing folding (*15*). The first two stages have largely been completed (*1*, *3*, *5*−*7*). To identify the intermediates that directly precede the rate-determining steps, it is necessary to determine the rate constants involved in the major pathways accurately. In addition, it is necessary to ascertain that all significant regeneration pathways have been identified. These objectives have been achieved in this paper. This information can then be used to design trapping experiments to identify the specific species (in this case, three-disulfide species) that directly precede the rate-determining step in each pathway. In particular, there are 12 possible 3S species that can directly precede any other 3S species in the regeneration pathway. Therefore, identification of des-[65−72] and des-[40−95] as the species that directly follow the rate-determining step reduces the number

of species that can directly precede the rate-determining step from 420 to 20 (there are four 3S species that can directly precede both des-[65−72] and des-[40−95]). Equally important is our determination of the rate constants for formation of des-[65−72] and des-[40−95] from 3S since this provides a means of screening subfractionated 3S species and thereby quantitatively determining the relative contribution of these species to the formation of des-[65−72] and des-[40−95].

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