Regeneration of Bovine Pancreatic Ribonuclease A. 2. Kinetics of Regeneration†

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ABSTRACT: Analysis of the experimental data of the previous paper [Rothwarf, D. M., & Scheraga, H. A. (1993) *Biochemistry* (first of four papers in this issue)], using the method of Konishi et al. [Konishi, Y., Ooi, T., & Scheraga, H. A. (1981) *Biochemistry 20*, 3945–3955; Konishi, Y., Ooi, T., & Scheraga, H. A. (1982) *Biochemistry 21*, 4734–4740], and a discussion of the validity of the steady-state kinetic treatment of the data analyzed here are presented. The analysis reveals that RNase A regenerates with oxidized and reduced dithiothreitol (DTT^{ox} and DTT^{red}, respectively) through a rearrangement pathway involving one or more three-disulfide species; i.e., multiple pathways could be involved. This pathway is different from that observed when RNase A is regenerated with oxidized and reduced glutathione (GSSG and GSH, respectively). These differences result primarily from the very different characteristics of the oxidation of thiols with DTT^{ox} and GSSG, respectively. In addition, the concept of multiple pathways, as applied to the regeneration of RNase A, is developed.

In the previous paper (Rothwarf & Scheraga, 1993a), we presented experimental data for the regeneration of bovine pancreatic ribonuclease A (RNase A)[1] with a mixture of oxidized and reduced dithiothreitol (DTT^{ox} and DTT^{red}, respectively), and showed that the disulfide-containing protein intermediates achieve a steady-state distribution. In this paper, we analyze the kinetic data, and deduce a model for the regeneration of RNase A with the DTT^{ox}/DTT^{red} redox reagent.

ANALYSIS OF RATE DATA

Regeneration Rate. The rate of regeneration of a protein is known to depend on the concentrations of both the thiol and disulfide of the redox pair. However, the rate of regeneration is not a simple function of the redox potential. This has been well established experimentally (Konishi et al., 1981, 1982a; Wetlaufer et al., 1987), and is explained below and in much greater detail in an accompanying paper (Rothwarf & Scheraga, 1993b), although this behavior is often misunderstood (Wearne & Creighton, 1988). One of the advantages of the equilibrium treatment of the steady-state distribution is that it clarifies the relationship between the concentrations of the redox couple and their effect on the rate of regeneration. The relevant kinetic equations have been presented previously for the regeneration of RNase A with oxidized and reduced glutathione (GSSG and GSH, respectively) (Konishi et al., 1982a; Scheraga et al., 1987), but they are much simpler for regeneration with DTT^{ox} and DTT^{red} than with GSSG and GSH, and it is possible to consider the process clearly without resorting to a series of complex equations. Therefore, before providing a complete kinetic analysis, we will first present a simple example which will be shown below to be relevant to the results obtained here.

Consider the regeneration of RNase A using DTT^{ox} and DTT^{red} as the redox pair. In this preliminary illustrative example, it is assumed that only a single major regeneration pathway exists. The rate of formation of native protein, dN/dt, will depend on the concentration of the intermediate that precedes the rate-determining step in the regeneration process. Since dithiothreitol does not form stable mixed disulfides with the protein, then[2] [according to eq (1)-4 to (1)-7] the relative concentration of this intermediate in the steady state depends only on the redox potential, i.e., on the ratio DTT^{ox}/DTT^{red}. However, this does not mean that the regeneration *rate* depends only on the redox potential. The type of process involved in the rate-determining step will also determine the redox dependence. As presented in the introduction of the previous paper (Rothwarf & Scheraga, 1993a), there are three types of rate-determining steps that are relevant to the regeneration process with DTT^{ox} and DTT^{red}. Figure 1 lists all possible rate-determining steps and the corresponding rate equations (an asterisk indicates an activated intermediate that proceeds very rapidly to N). If the rate-determining step involves reduction of a disulfide bond, e.g., $3S \rightarrow 2S^*$, then, at a given redox potential, the regeneration rate will depend linearly on

STEP	RATE EQUATION
$R \rightarrow R^*$	$k_1[R]$
$R + DTT^{ox} \rightarrow 1S^*$	$k_2[R][DTT^{ox}]$
$1S + DTT^{red} \rightarrow R^*$	$k_3[1S][DTT^{red}] = k_3K_A[R][DTT^{ox}]$
$1S \rightarrow 1S^*$	$k_4[1S]$
$1S + DTT^{ox} \rightarrow 2S^*$	$k_5[1S][DTT^{ox}]$
$2S + DTT^{red} \rightarrow 1S^*$	$k_6[2S][DTT^{red}] = k_6K_B[1S][DTT^{ox}]$
$2S \rightarrow 2S^*$	$k_7[2S]$
$2S + DTT^{ox} \rightarrow 3S^*$	$k_8[2S][DTT^{ox}]$
$3S + DTT^{red} \rightarrow 2S^*$	$k_9[3S][DTT^{red}] = k_9K_C[2S][DTT^{ox}]$
$3S \rightarrow 3S^*$	$k_{10}[3S]$
$3S + DTT^{ox} \rightarrow 4S^*$	$k_{11}[3S][DTT^{ox}]$
$4S + DTT^{red} \rightarrow 3S^*$	$k_{12}[4S][DTT^{red}] = k_{12}K_D[3S][DTT^{ox}]$
$4S \rightarrow 4S^*$	$k_{13}[4S]$

FIGURE 1: The 13 possible rate-limiting steps and the 9 groupings of these steps [making use of eq (1)-4 to (1)-7] as used in the kinetic analysis. The asterisk indicates a species that can proceed to N more rapidly than any of the other processes shown. The species 4S* is kinetically indistinguishable from native protein because it goes rapidly to N.

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; BPTI, bovine pancreatic trypsin inhibitor; DTT^{ox}, oxidized dithiothreitol; DTT^{red}, DL-dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione.

[2] Equations, figures, and tables in the previous paper (Rothwarf & Scheraga, 1993a) are designated as (1)-1, (1)-2, etc.

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the absolute concentration of DTT^{red}, i.e., $dN/dt = k[3S][DTT^{red}]$. Similarly, if the rate-determining step involves oxidation, e.g., $3S \rightarrow 4S^*$, then the regeneration rate would depend linearly on $[DTT^{ox}]$, i.e., $dN/dt = k[3S][DTT^{ox}]$. Finally, if the rate-determining step involves intramolecular reshuffling, e.g., $3S \rightarrow 3S^*$, then the regeneration rate would not depend on the absolute concentration of either DTT^{red} or DTT^{ox}, i.e., $dN/dT = k[3S]$.

It is important to note that, under steady-state conditions, it would be impossible to distinguish between rate-determining steps involving oxidation of a three-disulfide species from those involving the reduction of a four-disulfide species, because [according to eq (1)-7] the ratio of the concentrations of 3S and 4S is proportional to the redox ratio. This is clearly shown in Figure 1. For example, the use of the equilibrium constant between the 3S and 4S species, K_D from eq (1)-7, results in a rate equation for the $4S \rightarrow 3S^*$ process which is indistinguishable from the rate equation for the $3S \rightarrow 4S^*$ process. The same considerations apply to the other steps, e.g., $R \rightarrow 1S^*$ and $1S \rightarrow R^*$ etc. (see Figure 1).

Since the concentration (relative to the total concentration of intermediates) of the three-disulfide species, [3S], as well as the relative concentrations of all of the other disulfide-bonded intermediates in a preequilibrium or steady state, depends only on the redox potential when using DTT^{ox} and DTT^{red}, their concentrations would not be affected by changes in the absolute concentrations of the redox couple, but only by changes in the ratio DTT^{ox}/DTT^{red}. Thus, by variation of the absolute concentrations of DTT^{ox} and DTT^{red} without variation of the ratios, the concentrations of the various disulfide groupings would remain constant as would the rate of formation of native protein through rearrangement pathways ($dN/dt = k[3S]$). However, formation of native protein through an oxidation or reduction pathway would change linearly with the absolute concentrations of the redox couple, e.g., $dN/dt = k[3S][DTT^{ox}]$. Therefore, even without precise knowledge of the concentrations of the relevant intermediates, or the preequilibrium constants, conclusions can be drawn about the nature of the rate-determining step and the existence of multiple pathways by determining the dependence of the rate of regeneration on DTT^{ox}/DTT^{red}, as long as (i) a steady state is established and (ii) the equilibrium constants do not vary over the range of redox concentrations used. A similar analysis could be used to explain the regeneration process using GSSG and GSH as the redox couple. However, because glutathione forms stable mixed disulfides with the protein, it is substantially more complex, although the mathematics have been treated extensively (Konishi et al., 1982a; Scheraga et al., 1987).

Having presented the above simple illustrative analysis, in the next subsection we consider the establishment of the rate-determining step in the regeneration of RNase A with DTT^{ox} and DTT^{red}. The much lower oxidizing potential of the DTT^{ox}/DTT^{red} redox pair as compared to the GSSG/GSH redox pair [see Table (1)-I] limits the range of redox conditions that can be used. Because RNase A cannot be regenerated at appreciable rates at DTT^{red} concentrations above 500 μM (no matter what the concentration of DTT^{ox} is), it was difficult to work at protein concentrations low enough so that the concentration of the DTT^{red} did not change appreciably during the regeneration process (Rothwarf & Scheraga, 1993a). Therefore, the experimentally attainable range of redox conditions is much smaller for regeneration with DTT^{ox} and DTT^{red} than for regeneration with GSSG and GSH.

Determination of the Rate-Determining Step. Let us first consider the proper kinetic form for the regeneration mechanism. Some of the 13 possible types of rate-determining steps shown in Figure 1 are obviously unlikely to contribute to formation of a significant fraction of native protein. Now, by imposing the steady-state condition[3] which allows use of the equilibrium constants (Rothwarf & Scheraga, 1993a) to reduce the number of variables, these 13 groups are reduced further to just 9, as shown in Figure 1 where rate equations with a dependence on the concentration of DTT^{ox} and involving the same disulfide intermediate are combined in braces. In this way, steps with a dependence on the concentration of the redox reagent can be expressed only in terms of DTT^{ox}. The relevant equation is

$$\frac{dN}{dt} = \sum_{i=1}^{4} k_i[I_i][DTT^{ox}] + \sum_{i=1}^{5} k_i[I_i] \qquad (1)$$

where dN/dt is the rate of regeneration of native protein, k_i is the apparent rate constant of the ith pathway, and $[I_i]$ is the concentration of the ith group of intermediates. The first and second summation terms on the right-hand side of eq 1 pertain to the steps in Figure 1 with and without brace-groupings, respectively; e.g., one of the terms in the first sum is $(k_2 + k_3K_A)[R][DTT^{ox}]$. Applying steady-state conditions, i.e., substituting $f_i(1 - N)$ for $[I_i]$, we obtain

$$\frac{dN}{dt} = \sum_{i=1}^{4} k_i f_i (1 - N)[DTT^{ox}] + \sum_{i=1}^{5} k_i f_i (1 - N) \qquad (2)$$

where f_i is the fractional concentration of the ith group among the intermediates in the steady-state distribution (computed from the equilibrium constants). Since f_i is constant (in the steady state), integration, keeping $[DTT^{ox}]$ constant, yields

$$\frac{-\ln(1 - N)}{t} = \sum_{i=1}^{4} k_i f_i [DTT^{ox}] + \sum_{i=1}^{5} k_i f_i \qquad (3)$$

In deriving these equations, we have made two assumptions. The first of these is that back-reactions from asterisked intermediates to intermediates involved in the steady state are negligible; i.e., the rate of formation of native RNase A from asterisked intermediates is more rapid than any process in which asterisked intermediates go back to intermediates. This first assumption is not strictly valid and will be addressed at the end of the subsection Some Properties of the Steady State.

The second assumption that is made, in accordance with the observation that the concentrations of intermediates change very slowly in the steady state, is that these concentrations remain constant. This assumption, while not quite accurate because of the slow production of DTT^{red} in the steady state, will have very little effect on the analysis presented here for the following reason. As was seen in Figure (1)-4, the relative concentrations of intermediates change slowly with time once the steady state is reached, and the equations will be valid for time intervals in which only a *small* amount of native protein is generated. Only data from time intervals corresponding to small increases in the concentration of native protein were used in the *initial* determination of the rate-determining step(s).

An additional and equally important point is that the entire kinetic analysis is based on the premise that each grouping

[3] In a sense, it has already been used (Rothwarf & Scheraga, 1993a), since groups of intermediates were treated as single kinetic species with rapid interconversion within each group. This condition holds when an equilibrium, preequilibrium, or steady state is shown to exist.

of intermediates can be considered as a single kinetic species. In order for this to be valid, the distribution of intermediates within a group must be invariant with changing time and redox conditions; this means that the interconversion among intermediates within groups must be rapid with respect to the rates of oxidation, reduction, and regeneration (Scheraga et al., 1987; Creighton, 1988). This condition will be shown to exist for the intermediates populated in the steady state. However, we defer until the next subsection and the Discussion the presentation of the evidence that the interconversion within groups is rapid enough, since such considerations benefit from a complete analysis of the data and are, therefore, most clearly presented after all of the analysis has been provided.

Various conclusions can be drawn from the data for regeneration, some of which are shown in Figures (1)-4 to (1)-6. Several of the potential pathways are unlikely to be significantly populated, in particular any pathway involving the totally reduced protein. The experimental data support this conclusion. No measurable concentration of native RNase A was formed under redox conditions (100 mM DTT^{ox}, 2 mM DTT^{red}, 32 μM RNase A, 425 h) where essentially only the reduced protein and one-disulfide species are populated at steady state. Therefore, we dismiss the potential pathways listed in Figure 1 that involve k_1–k_4. This then leaves six kinetically-distinguishable, potential types of pathways, involving k_5–k_{13}. The regeneration data were obtained under 10 distinctly different redox conditions some of which are shown in Figures (1)-4 to (1)-6. These data correspond to conditions in which the concentration of at least one of the redox reagents, DTT^{ox} or DTT^{red}, varied by a factor of 2 or more. Since the method of analysis requires that the data used correspond to intervals in which DTT^{red} did not change significantly, only a small portion of the total data was usable at this stage of the analysis.

Under some redox conditions, the concentration of a particular species was too small to be determined directly from the experimental data; in particular, the concentration of the 4S species was not populated under most regeneration conditions, e.g., 100 mM DTT^{ox}, 32 μM reduced RNase A. However, since we used 10 different redox conditions, each species was populated in at least 4 such experiments, thereby enabling all of the equilibrium constants to be determined. Therefore, in the determination of the k_i's, we took advantage of the equilibrium constants to calculate the concentrations of disulfide intermediates that were insufficiently populated in some cases to measure directly and accurately.

Steady-state data obtained over time intervals in which the change in the concentration of native protein was small were first fit to eq 3 to determine if any single pathway (among those in Figure 1) could account for the formation of the majority of the regenerated native protein over the range of redox conditions studied. The data were fit to each of the possible single-pathway models by determining the value of k_i for each potential pathway (each term in eq 3) under each of the 10 distinctly different redox conditions used in these experiments. A consistent value of k_i was found only for the rate-determining step corresponding to $3S \rightarrow 3S^*$. The value of k_i for this step (k_{10} in Figure 1) from the initial limited set of data was determined to be $(1.0 \pm 0.1) \times 10^{-2}$ min^{-1} (95% confidence limit). This single pathway ($3S \rightarrow 3S^*$) can account for between 90 and 110% of the regenerated protein under each of the redox conditions used as input data. The data were also fit by a simplex minimization (Caceci & Cacheris, 1984) to eq 3 with all k_i's as variables. The minimization was carried out with different starting estimates

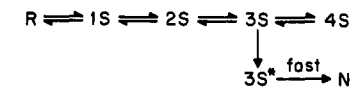

FIGURE 2: Final regeneration model; rate constants are given in Table I. The unimolecular rate constant for the rate-determining step, $3S \rightarrow 3S^*$, is $(9.8 \pm 0.5) \times 10^{-3}$ min^{-1}.

of the target variables, and these minimizations converged to different local minima. However, the best fit to the data, in all cases, was through the single pathway, $3S \rightarrow 3S^*$, and the value of the k_i for that step (k_{10}) was found to be within 5% of the single-pathway value (1.0×10^{-2} min^{-1}) for all minimizations.

Determination of Rate Constants. Having determined the existence of a single major pathway from a subset of the steady-state data, *all* the data (over a wide range of redox conditions) including those obtained prior to the attainment of the steady-state condition were then used in the final kinetic fit, which is described in this subsection. The final model is shown in Figure 2, and its associated rate constants are shown in Table I. Experimental data for the concentrations of each species as a function of time were fit to the model shown in Figure 2 by application of a simplex minimization (Caceci & Cacheris, 1984) of a fourth-order Runge–Kutta numerical solution (Wiberg, 1986) of the system of simultaneous rate equations based on the model of Figure 2. This model included the time dependence of the concentration of DTT^{red}.

The solutions for the forward rate constants, k_f, were determined by first taking the reverse rate constants (k_r) as k_f/K, where K is the equilibrium constant given in eq (1)-4 to (1)-7. The use of the equilibrium constants in this manner was necessary because significant amounts of each of the disulfide groupings did not appear under some of the experimental redox conditions. Specifically, experiments under conditions corresponding to low oxidizing potential, e.g., 40 mM DTT^{ox}, 32 μM RNase A [Figure (1)-4A], did not lead to significant concentrations of the 3S and nonnative 4S species, and experiments under conditions corresponding to high oxidizing potential, e.g., 200 mM DTT^{ox}, 3.2 μM RNase [Figure (1)-4C], did not lead to significant concentrations of the R and 1S species.

Forward rate constants determined by using the equilibrium constants to obtain k_r should not differ from those obtained by using k_r as a free variable unless the rate of oxidation or reduction of groups is slow compared to the rate of formation of native protein, i.e., if equilibration among intermediates were not rapid compared to the rate of formation of native. In the model of Figure 2, since only the 3S species is directly involved in the formation of native protein, it is the equilibrium constants involving the 3S species that would be most perturbed by the formation of native protein. Rate constants, k_f and k_r, were initially determined by using the equilibrium constants, and revealed (as expected) that the rates of formation and reduction of the one- and two-disulfide species were significantly larger than the rate of formation of native protein (through the $3S \rightarrow 3S^*$ step) under conditions where the one- and two-disulfide species were well populated and, therefore, should not be affected by use of the equilibrium constant to relate k_f and k_r. The rates of formation of the 3S and 4S species under conditions where they were well populated, however, were similar in magnitude to the rate of formation of the native protein, which would mean that the ratio of the forward and reverse rate constants, k_f/k_r, would not be equal to the observed equilibrium constant. Therefore, the data were refit to determine the rate constants, with the rate constants involving the three- and four-disulfide species varied

Table I: Rate Constants[a] at 25 °C, pH 8.0

reaction	k_f^{obs} [b] ($\times 10^2$) (min^{-1} M^{-1})	k_f^{avg} [c] ($\times 10^2$) (min^{-1} M^{-1})	k_{intra}^{obs} [d] (min^{-1})	k_{intra}^{avg} [e] (min^{-1})	k_r^{obs} [f] ($\times 10^{-2}$) (min^{-1} M^{-1})	k_r^{avg} [g] ($\times 10^{-2}$) (min^{-1} M^{-1})
R ⇄ 1S	140 ± 10[h]	5.0	210	7.6	4.5 ± 0.4	4.5
1S ⇄ 2S	65 ± 9	4.3	99	6.6	11 ± 2	5.7
2S ⇄ 3S	23 ± 3	3.8	35	5.9	11 ± 2	3.6
3S ⇄ 4S	4.4[i] ± 1.5	4.4	6.8	6.8	16 ± 3	4.0
				6.7 (avg)		

[a] These rate constants were determined from data for the *whole* time course under each of the 10 different experimental redox conditions. [b] k_f^{obs} is the observed rate constant for formation of a protein disulfide bond using DTT^{ox} for the reaction indicated. [c] k_f^{avg} is k_f^{obs} corrected for statistical factors, as described in the text. [d] k_{intra}^{obs} is the rate constant for formation of intramolecular protein disulfide bonds, determined from k_f^{obs} using eq 4. [e] k_{intra}^{avg} is k_{intra}^{obs} corrected for statistical factors as described in the text. [f] k_r^{obs} is the observed rate constant for reduction of a protein disulfide bond using DTT^{red} for the reaction indicated. [g] k_r^{avg} is k_r^{obs} corrected for statistical factors as described in the text. [h] The error is calculated at the 95% confidence limit. [i] The rate-determining step is 3S → 3S*, for which the unimolecular rate constant is $(9.8 \pm 0.5) \times 10^{-3}$ min^{-1}.

without making use of the equilibrium constants to determine k_r, but with the rate constants involving the reduced and one- and two-disulfide species still constrained by use of the equilibrium constants. It is these data that are shown in Table I. It is important to note that the resulting value of k_f/k_r is only ~20% different from the value of the equilibrium constant for the processes involving the 3S species. Convergence was confirmed by varying the starting estimates of the rate constants. Each set of experimental data corresponding to a single set of redox conditions was analyzed separately. The rate constants and their standard deviations (95% confidence limit) listed in Table I are based on the average of *all* data sets under different redox conditions and do not reflect the standard deviation within any individual data set. All curves shown in Figures (1)-4 to (1)-6 are theoretical ones, based on the rate constants in Table I and the model shown in Figure 2.

Examination of the regeneration rate data shown in Figure (1)-6 reveals that there is a lag before the appearance of significant amounts of native protein. This observation is consistent with the fact that the major regeneration pathway(s) involves species that form late in the establishment of the steady-state distribution. In addition, it is instructive to note that the kinetic analysis described in this subsection, based on a proposed single pathway, is in excellent agreement with the data prior to the attainment of the steady-state distribution, as shown in Figures (1)-4 and (1)-6. The significance of this will be made clear at the end of the next subsection.

Some Properties of the Steady State. We have shown above that, during the regeneration of RNase A with DTT^{ox} and DTT^{red}, the intermediates appear to achieve a steady-state distribution (Rothwarf & Scheraga, 1993a). It is important to note that this steady-state condition is somewhat different from the conventional one in which the concentrations of species remain constant under steady-state conditions. $d[I]/dT$, the change in the concentration of an intermediate with time, is not zero, because N is forming continually; however, the change in the fraction of any intermediate species within the total population of intermediates with time, df/dt, is zero. In a strict sense, since the concentration of DTT^{red} is changing with time, even this statement is not completely accurate. These questions of terminology are not relevant to the validity of our analysis since the initial kinetic fit was applied only to data obtained during time intervals in which the change in the concentrations of all intermediates was small. Subsequent analysis was carried out by using rate equations in which any changes in concentration with time were accounted for correctly. As Figure (1)-4 and Table (1)-I illustrate, the curves generated from calculated equilibrium and rate constants accurately reflect the concentrations of the various groupings of disulfide-bonded intermediates at various times, and the kinetic analysis shown in Figure 1 and eq 3 is valid if the distribution of intermediates within groups is invariant with time and redox conditions.

Some limited information about the distribution within groupings in the steady state can be obtained from the experimental data. Within our ability to subdivide the groupings chromatographically, as shown in Figure (1)-2, the distribution of intermediates within each grouping does not change as a function of time or redox conditions. While this suggests that the rate of equilibration within groups is significantly faster than the rate of regeneration, we are not able to resolve the components to any greater extent than that shown in Figure (1)-2; the chromatograms are therefore insensitive to changes in subpopulations that correspond to less than ~20% of the grouping.

Therefore, the possibility that reshuffling of some species is slow with respect to the regeneration rate must be considered. Since the groupings achieve an approximate steady-state distribution, if any intermediate that represented a large percentage of any grouping were very slow to form, we would observe a large change in the distribution of groups with redox potential as well as a change in the distribution of species within groups with time and redox conditions. Since neither of these phenomena is observed, this situation does not arise. This, however, does not exclude intermediates whose concentrations are small but exchange slowly and hence are not in equilibrium with the rest of the species within the group. There is no way to evaluate this from our data, and undoubtedly it does occur. The major consideration is whether such a species is involved in the rate-determining step in folding. If it is not and, since, from our hypothesis above, it represents only a small portion of the total group, its effect is negligible. If it is involved in the rate-determining step in folding and is slow to form, then it can make a major contribution to the regeneration rate only if it proceeds rapidly to form native protein. If this occurs, then formation of that species is the rate-determining step (corresponding to one or more of the steps listed in Figure 1), and its appearance has therefore been correctly treated by the kinetic analysis. Furthermore, as will be shown under Discussion, the rate of internal rearrangement within groups is expected to be at least 1 order of magnitude greater than the rate of regeneration of native protein (dN/dt).

The discussion thus far has ignored the wrong four-disulfide species because they cannot reshuffle intramolecularly. Obviously they cannot interconvert rapidly since interconversion within that grouping requires a three-disulfide intermediate and, therefore, requires two steps, one involving reduction and the other oxidation, both of which are much slower than intramolecular rearrangement. While undoubtedly there are some four-disulfide species that could accumulate slowly, this

does not affect our kinetic analysis under the regeneration conditions that we have used for the following reasons. It is important to emphasize that the formation of both wrong four-disulfide species and the native protein depends on the concentration of 3S and that the rate of formation of the native protein is comparable to or greater than the rate of formation of wrong four-disulfide species [9.8×10^{-3} min^{-1} vs $(4.4 \times 10^{-2})[DTT^{ox}]$ min^{-1}] under all the concentrations of DTT^{ox} used (up to 200 mM). Therefore, under the redox conditions that favor the formation of wrong four-disulfide species (conditions where the concentration of 3S is large), the rate of regeneration of the native protein is so rapid [see Figure (1)-6] that no significant population of these slow-forming four-disulfide species occurs during the time of our analysis. Slow regeneration conditions, that could favor the accumulation of slow-forming wrong four-disulfide species, correspond to conditions of low redox potential in these experiments. However, under conditions of low redox potential, the concentration of *all* wrong four-disulfide species is negligible [see Figure (1)-4A,B].

The rate of appearance of native protein is well fit by our model even in the time regime prior to the attainment of the steady state [Figures (1)-4 and (1)-6], which is another indication that the rate of internal reshuffling is rapid with respect to the regeneration rate. Furthermore, the ability to fit the data to a single type of pathway over a greater than 50-fold range of the concentration of DTT^{red} suggests that reduction of species after the rate-determining step is negligible compared with the subsequent formation of native protein; e.g., $3S^* \rightarrow N$ is much faster than $3S^* \rightarrow 2S$.

DISCUSSION

Relative Rates of Formation of Disulfide Bonds. At the heart of any attempt to explain differences between the results that we have analyzed here using DTT^{ox}/DTT^{red} and earlier studies using GSSG/GSH is the difference in the mechanism of action of the two types of redox reagents. The main difference between the reagents arises from the stability of the mixed disulfides between the reagents and the protein. As a consequence of the much greater stability of mixed disulfides involving glutathione, the overall rates of formation and reduction of protein disulfide bonds are 1–3 orders of magnitude greater with GSSG/GSH than with DTT^{ox}/DTT^{red} under the redox conditions typically employed in regeneration studies. A detailed discussion of these differences in rate is presented in an accompanying paper (Rothwarf & Scheraga, 1993b). However, for the purposes of this Discussion section, it is important to keep in mind this difference in the rate of oxidation of protein thiols and of reduction of protein disulfide bonds.

Values of k_{intra}. While much of the discussion in this paper and the accompanying ones (Rothwarf & Scheraga, 1993a,b,c) deals with the interaction between the different types of redox reagent and the protein, there is a quantity that is independent of the redox reagent and reflects only the conformational properties of the protein. This quantity is k_{intra} [Figure (1)-1], the rate constant for formation of an intramolecular protein disulfide bond. Since k_{intra} reflects an intramolecular process that involves only the protein, it is unaffected by the type of redox reagent used [we assume that this intramolecular process is independent of the leaving group, i.e., that it is the same for GSSG/GSH and DTT^{ox}/DTT^{red}; however, as will be explained in a later section (Reduction of Disulfide Bonds among Intermediates in the Steady State), this is only an approximation]. It is possible to determine the values of k_{intra} from the observed forward rate constants for formation of 1S, 2S, 3S, and 4S species with DTT^{ox}. The observed forward rate constant for any of these steps [the combined reactions of Figure (1)-1B] is

$$k_f^{obs} = K_{DTT} k_{intra} \quad (4)$$

Using the value of K_{DTT} (6.5×10^{-3} M^{-1}) derived in an accompanying paper (Rothwarf & Scheraga, 1993b), values of k_{intra} can be determined from the values of k_f^{obs} shown in column 2 of Table I. The values of k_{intra} are shown in column 4 of Table I. These values, however, are not representative of any single process since each step actually involves a mixture of many species each of which is capable of forming a number of disulfide pairs. The observed k_{intra} is the weight-averaged sum of all the possible species involved in that type of disulfide.

The simplest situation to discuss is the formation of the one-disulfide species since the starting (reduced) material is a single chemically distinct species. There are 28 1-disulfide species that can be formed by oxidation of the reduced protein. The observed k_{intra} is the sum of all 28 rate constants. Dividing the observed value of k_{intra} by 28, therefore, leads to an average value of k_{intra} for the formation of the one-disulfide species.

Similarly, there are 15 2-disulfides that can form from any 1-disulfide species, 6 3-disulfides that can form from any 2-disulfide species, and only 1 4-disulfide that can form from any 3-disulfide species. The average values are shown in column 5 in Table I. The close agreement between each of the four average values suggests that the rates of formation of intramolecular disulfide bonds are similar for most of the disulfides, and hence would appear to be consistent with the observation of Konishi and Scheraga (1980) that the intermediates populated during regeneration with oxidized and reduced glutathiones are largely disordered.

However, there are a variety of factors arising from entropic considerations that contribute to k_{intra}. Since the formation of each successive intramolecular protein disulfide bond decreases the conformational freedom in the protein, thereby restricting the mobility of protein thiols, the value of k_{intra} would be expected to decrease with the formation of each additional intramolecular protein disulfide bond. The value of k_{intra} would also decrease because entropically favored small disulfide loops form preferentially early in the regeneration process. Creighton (1979) has determined that the disulfides capable of forming the smallest loops, those involving cysteines-58, -65, and -72, are involved in the majority of one-disulfide species. Therefore, it is expected solely from entropic considerations about the formation of single loops that the average value of k_{intra} for the formation of the two-disulfide species would be less than for the formation of the one-disulfide species.

There is, however, another factor influencing k_{intra} that arises from overlapping loops (Poland & Scheraga, 1965; Lin et al., 1984). Each intramolecular protein disulfide bond in an overlapping loop decreases the entropy loss for subsequent disulfide bond formation. Solely on the basis of this consideration, the value of k_{intra} for any particular disulfide bond would be expected to *increase* as the protein becomes more oxidized. This effect would, therefore, be in a direction opposite to that predicted from the other factors. Undoubtedly, the observed trend is a combination of all three factors.

Rate of Intramolecular Thiol–Disulfide Exchange among the Protein Intermediates. A measure of the rate of intramolecular thiol–disulfide exchange among the protein intermediates [eq (1)-3] is crucial for a complete understanding of the regeneration process. Unfortunately, it is difficult to

measure this rate directly since it requires the identification of specific disulfide pairs. This has been done in the case of certain intramolecular rearrangements in the regeneration of bovine pancreatic trypsin inhibitor (BPTI) (Creighton & Goldenberg, 1984; Weissman & Kim, 1991), but these have involved the rearrangement of one nativelike species to another nativelike species rather than the interconversion among disordered intermediates. On the other hand, as explained in the introduction of the previous paper (Rothwarf & Scheraga, 1993a), the process of interest in protein folding is the conformational transition from the reduced form to the native. Therefore, rearrangements of disulfides among nativelike intermediates are not relevant to the regeneration process of RNase A where the intermediates are largely disordered (Konishi & Scheraga, 1980). However, an estimate of the rate of intramolecular thiol–disulfide exchange among intermediates can be made from the values of k_{intra} determined above.

There are three types of intramolecular protein disulfide rearrangements. The first type involves a mixed disulfide and a protein thiol exchanging to form a protein disulfide as shown in the second step of Figure (1)-1A,B. In this case, a protein thiolate attacks a protein mixed-disulfide bond and displaces the small molecular weight component (either DTT^{red} or GSH). The second is the reshuffling of one mixed disulfide to another, in which case a protein thiolate attacks a protein mixed disulfide, displacing a protein thiolate. To a first approximation, this second process should be equivalent to the first process with a rate constant, k_{intra}, for the formation of a disulfide bond. Both processes involve a close approach of a protein thiol to a mixed disulfide. While the geometries required for the two processes may be slightly different, it is reasonable to suppose that the sulfur of the small redox reagent thiol involved in the mixed disulfide would be no more restricted than the cysteine involved in the mixed disulfide. Therefore, the principal difference in rate constant would result from differences in the electrostatic properties of the species involved in the mixed disulfide.

The third type of process involves a rearrangement of intramolecular protein disulfide bonds, in which a protein thiolate attacks a protein disulfide, displacing a protein thiolate. It is similar to the first two processes, but differs in that a protein disulfide could be much more hindered than a mixed disulfide. However, the conclusion of this work and that of Konishi and Scheraga (1980), that the intermediates are disordered, and the observation that the values of k_{intra}^{avg}, k_f^{avg}, and k_r^{avg} are independent of the level of intramolecular disulfide bond formation suggest that there is no significant population of hindered disulfide bonds which would affect the rate of intramolecular protein disulfide reshuffling for the majority of species. An additional minor consideration is that, since there are two possible products of an intramolecular disulfide bond reshuffling step, there is a statistical factor of 2 which must be accounted for.

Since there seems to be little variation among the values of k_{intra}^{avg}, we conclude from the arguments given above that the average rate constant for intramolecular disulfide rearrangement in RNase A intermediates would be expected to be ~ 6.7 min^{-1} as shown in column 5 of Table I. This value of 6.7 min^{-1} can be compared to the average values of the rate constants for oxidation and reduction [k_f^{avg} from column 3 of Table I and k_r^{avg} (derived in the following subsection) from column 7 of Table I] multiplied by the concentration of the appropriate redox reagent, [DTT^{ox}] and [DTT^{red}], respectively. For example, for the 1S → 2S step, the rate is $(4.3 \times 10^{-2})(0.2) = 8.6 \times 10^{-3}$ min^{-1} at 200 mM DTT^{ox}. This comparison indicates that the rate of intramolecular rearrangement is 2–4 orders of magnitude greater than the rates of oxidation and reduction of the disulfide intermediate species formed under the conditions used in this study for the regeneration of RNase A. It is important to keep in mind that this discussion has focused on average properties; actual rates of intramolecular disulfide rearrangements for specific species could vary widely from such average values.

Reduction of Disulfide Bonds among Intermediates in the Steady State. We can compare the rate constants for reduction of disulfides among the protein intermediates in the steady state to that of the reduction of GSSG by DTT^{red}. In order to make such a comparison, the assumption is made that the average pK of a protein thiol is equivalent to the pK of the sulfhydryl group of GSH. This assumption is necessary because it has been shown experimentally (Creighton, 1975; Wilson et al., 1977; Szajewski & Whitesides, 1980) that the rate constant for reduction of disulfides depends on the electrostatic properties of the disulfide being reduced, as reflected in the pK of the thiol corresponding to this disulfide. A lower pK implies that a species will contribute a better leaving group, since it corresponds to a species that is better able to stabilize the negative charge which develops in the transition state. Therefore, the lower the pK of the thiols corresponding to the disulfide being reduced, the greater the rate of disulfide interchange. Other major factors that contribute to the observed rate and equilibrium constants are strain, accessibility, and other environmental factors in the disulfide being reduced (Creighton, 1975; Milburn et al., 1987; Milburn & Scheraga, 1988; Altmann & Scheraga, 1990; Falcomer et al., 1992).

The assumption is also made that the reference disulfide, GSSG, is unstrained and is completely accessible and, as assumed before, that the pK of GSH is similar to that of the cysteines in RNase A. Comparison of the rate of reduction of GSSG, 186 ± 16 min^{-1} M^{-1} (Rothwarf & Scheraga, 1992), with the rates of reduction of the one-disulfide intermediates, 450 ± 40 min^{-1} M^{-1} (column 6 of Table I), indicates that this approximation is not quite exact. One likely explanation for this difference is that the pK of the protein thiols is higher than that of GSH (based on the above discussion of the dependence of reduction rates on the electrostatic properties of the disulfide being reduced). Such a conclusion about the pK is consistent with the values of K_{GSSG} that we have observed [see footnote 3 of Rothwarf and Scheraga (1993b)]. Additional factors that could affect the rate of reduction of disulfide bonds are accessibility and strain of the disulfide being reduced.

Given the poor agreement between the rate constants for reduction of GSSG and 1S, we conclude that 1S is a better reference for comparison to the rates of reduction of the 2S, 3S, and 4S species, since the one-disulfide species should be the most accessible and the least strained. The rate constants, k_r^{obs} (column 6 of Table I), for reduction of the other disulfide species are significantly larger than those of the one-disulfides. This, however, most likely results from the statistical factors which must be considered since the observed rate constant is the weight-averaged sum of the rate constants for all the disulfides in that grouping. To determine an average value for the reduction of a single disulfide, the observed rate must be divided by the number of intramolecular protein disulfide bonds. As shown in column 7 of Table I, the average values are similar for all disulfide species and, when coupled with the average value of k_{intra} (column 5 in Table I), suggests that the intrinsic formation and reduction of protein disulfides among

the intermediates is similar for all of the intermediates. This is compatible with the conclusion that the intermediates are disordered (Konishi & Scheraga, 1980).

The Rate-Determining Step. The rate-determining step is the slowest step in any particular pathway. However, when one considers the steady-state distribution of species observed here, the rate-determining step is the *fastest* step by which intermediates can regenerate to native. If an intermediate can proceed to native through *several* different slow steps, the fastest of the slow steps will be the one chosen. This is in essence the rate-determining step. The rate constant of the rate-determining step is shown in footnote *i* of Table I. This rate constant was derived under the assumption that the entire group of three-disulfide intermediates is the species that precedes the rate-determining step, when in actuality it is likely to be only a small percentage of the three-disulfide species (i.e., a minor component) that is involved. Hence, the rate constant of the rate-determining step involving this minor component could be much larger than the observed rate constant.

An additional point is that, unless the rate-determining step is the formation of the final disulfide bond, there will be steps that follow the rate-determining step. In the system investigated here, since the rate-determining step (3S → 3S*, as shown in Figure 2) involves a rearrangement of one or more three-disulfide species, there is at least one step, the formation of the final disulfide bond (3S* → N), that follows the rate-determining step. Hence, this oxidation step must be faster than the rate-determining step. This, coupled with the fact that the rate constant of the rate-determining step involving the hypothetical minor component (in the 3S → 3S* step) mentioned above could be much larger than the observed rate constant (9.8×10^{-3} min^{-1}), suggests that the rate constant for oxidation of the final disulfide bond to form the native protein in the 3S* → N step is likely to be significantly larger than the observed rate constant for formation of the three- or nonnative four-disulfide species (23×10^{-2} and 4.4×10^{-2} min^{-1} M^{-1}, respectively) and is probably faster than any of the oxidation steps that we have observed. This further suggests that, given the slow rate of oxidation of thiols by DTT^{ox}, the fastest route out of the steady state is one in which the 3S → 3S* step has led to a particular three-disulfide species (3S*) which has a very high rate of oxidation to native.

Given these considerations and the inference that the rate-determining step is a rearrangement that occurs within the three-disulfide species, there are three likely possibilities for the nature of the 3S → 3S* step that fit the experimental data, and we cannot distinguish among these three on the basis of the data presented here. The first of these is one in which the rate-determining step involves a conformational change within the 3S species. This could involve the exposure of a buried cysteine residue. Before the conformational change, this three-disulfide species presumably would have three native disulfide bonds. Alternatively, the conformational change could involve the same type of localized isomerization that gives rise to the slow-folding forms of the disulfide-intact enzyme, i.e., a cis–trans proline isomerization or perhaps an inversion of the chirality of one of the disulfide bonds (Mui et al., 1985).

The second type of rate-determining step could involve reshuffling from a three-disulfide species with native disulfide pairings to a three-disulfide species with nonnative disulfide pairings, followed by a rapid conversion to the native protein. A pathway of this type is similar to the one observed for the regeneration of BPTI (Creighton, 1985; Weissman & Kim, 1991). Such a pathway could arise because of the slow rate of oxidation of protein disulfide bonds with DTT^{ox}; i.e., the rate of formation of the final disulfide bond (3S → 4S*) could be slow compared to the rate of intramolecular disulfide rearrangement, e.g., 3S → 3S*. Therefore, it is possible that only one of the four possible three-disulfide species with native pairings can oxidize rapidly with respect to the rate of regeneration through 3S → 3S*; i.e., there may be only one 3S* species with three native disulfide bonds such that 3S* → N occurs rapidly with DTT^{ox}/DTT^{red}. Therefore, for 3S to rearrange to 3S*, the most rapid path to native could be for one three-disulfide species with native disulfide pairings to rearrange (through a nonnative three-disulfide species) to a different three-disulfide species with native disulfide pairings (3S*) that could rapidly oxidize to native. It is important to note that a pathway of this type might not be populated during regeneration with GSSG/GSH. While there still would be one three-disulfide species with native disulfide pairings that oxidized more rapidly to native than the others, the rate of the direct oxidation pathway, 3S → 4S*, would in all likelihood be faster than the rate of the disulfide rearrangement pathway, 3S → 3S*. This is a result of the greater rate of disulfide bond formation that occurs with GSSG/GSH as opposed to DTT^{ox}/DTT^{red}.

The third type of rate-determining step involves reshuffling from a wrong three-disulfide species to a native one. There is a possible kinetic advantage to a rate-determining step of this type since there are 12 ways for any 3-disulfide species to be formed from any other 3-disulfide species. Therefore, as many as 12 different 3-disulfide species could interconvert to form the native 3-disulfide species that oxidizes rapidly. This type of rate-determining step is, therefore, likely to involve multiple regeneration pathways.

In addition, since any combination of the three types of rate-determining steps could explain the observed regeneration kinetics, the single pathway that we observe could actually involve multiple pathways. However, since all of these potential pathways have the same dependence on the DTT^{ox}/DTT^{red} ratio, the relative population of these multiple pathways does not depend on the redox conditions, in contrast to those with GSSG/GSH that will be discussed in the next section.

Comparison of Current Analysis to Regeneration with GSSG and GSH. The fact that the values of K_{G-T} [Table (1)-I], measured by using the equilibrium constants presented here and those of Konishi et al. (1981), vary by as much as an order of magnitude from the experimentally determined value does not reflect on the validity of the preequilibrium treatment or the overall conclusions of Konishi et al. (1981, 1982a,b,c). Using GSSG and GSH for the redox reagent, there are 24 independent preequilibrium constants corresponding to 25 groupings, instead of the 4 preequilibrium constants and 5 groupings obtained when using DTT^{ox} and DTT^{red} as the redox reagent. In the regeneration studies with GSSG and GSH, fractionation of the intermediates was based on the formation of mixed disulfides with glutathione. Since this process identified the intermediates from only 9 chromatographic peaks, the concentrations of some of the 25 groupings were determined indirectly by measuring the concentrations of some of the groups under a variety of redox conditions. This involved some assumptions about the relative stability of mixed disulfides between glutathione and the protein. The fact that a preequilibrium condition existed, however, was shown directly by measuring the concentrations of species in the nine chromatographically distinct peaks as a function of time and redox conditions.

A key point that can be deduced from the earlier work is that, regardless of the accuracy of the preequilibrium constants of Konishi et al. (1981), it would still be possible to determine whether a single rate-determining step existed, by fitting the 24 independent preequilibrium constants and the rate constants of the possible rate-determining steps to the regeneration rate data. This analysis was indeed carried out by Konishi et al. (1982a), and it showed that no single pathway could account for the regeneration kinetics. Hence, the overall conclusion that the regeneration intermediates achieve a preequilibrium state and regenerate to native protein through multiple pathways is well established.

As regards the six specific regeneration pathways proposed by Konishi et al. (1982a,b), we cannot use our current data to refine their preequilibrium constants since the data presented here using DTT^{ox}/DTT^{red} provide no direct information on the stability of mixed disulfides between glutathione and RNase A. However, on the basis of a comparison of the equilibrium constants determined here and in the earlier studies of Konishi et al. (1981), and using the experimentally determined value of K_{G-T} (Rothwarf & Scheraga, 1993a), some conclusions can be drawn. A possible overestimate of the concentration of the one-disulfide species by Konishi et al. (1981, 1982a) may have led to attributing too great an importance to pathways involving the one-disulfide species, i.e., $1S \rightarrow 1S^*$.

Konishi et al. (1982a) carried out further checks on some of their proposed pathways by restarting the regeneration from isolated intermediates. Such a procedure, including monitoring the initial regain of activity or formation of intermediates, represents one of the best methods to determine regeneration pathways, and recently has been used extensively in the determination of the regeneration pathways of BPTI (Weissman & Kim, 1991, 1992). Using this method, Konishi et al. (1982a) found that a wrong four-disulfide species was involved in a major regeneration pathway. While this would seem to be in conflict with our current results, it is easily reconciled by the much greater rates of formation and reduction of disulfide bonds that occur when the regeneration is carried out with GSSG/GSH as opposed to DTT^{ox}/DTT^{red} (Rothwarf & Scheraga, 1993b).

The much greater rate of reduction of disulfide bonds with GSSG/GSH than with DTT^{ox}/DTT^{red} indicates that a reductive pathway involving a four-disulfide species, i.e., $4S \rightarrow 3S^*$, will be much faster with GSH than with DTT^{red}. In addition, as discussed above, when considering the effects of slow-to-form disulfide species, the four-disulfide species cannot interconvert directly. Therefore, the distribution of four-disulfide species depends on the rate of formation of disulfide bonds, i.e., the rate of $3S \rightarrow 4S$. Given the much slower rate of formation of disulfide bonds with DTT^{ox}/DTT^{red}, the distribution of four-disulfide species could be very different with GSSG/GSH. Hence, the four-disulfide species involved in the rate-determining step with GSSG/GSH may not be significantly populated during regeneration with DTT^{ox}/DTT^{red}.

Concept of Multiple Regeneration Pathways. We define a regeneration pathway by the intermediates and type of process involved in the rate-determining step. By comparison of differences between regeneration pathways with different types of redox reagents, e.g., DTT^{ox}/DTT^{red} and GSSG/GSH, a clearer understanding of the concept of multiple regeneration pathways, and the role that the bimolecular character of many of the steps plays in determining the pathway, can be obtained.

A potentially important process, which is difficult to evaluate, is the perturbation of the pathway by formation of mixed disulfides; i.e., the mixed disulfide actually may direct the pathway by affecting the structure and stability of the intermediate. However, as will be developed in an accompanying paper (Rothwarf & Scheraga, 1993b), the most important effect of the difference in the stability of mixed disulfides is the greater rate of formation and reduction of disulfide bonds with GSSG/GSH. It is this difference that will be used in this section to illustrate how a regeneration pathway that is insignificant with DTT^{ox} and DTT^{red} could be the major pathway with GSSG and GSH.

An example of multiple pathways involves the possibility that the same conformational state and even the same disulfide pairings can be attained by different pathways. In our use of the DTT^{ox}/DTT^{red} system, we have determined that the rate-determining step is $3S \rightarrow 3S^*$. However, because of the 1–3 orders of magnitude greater rate of disulfide bond formation with GSSG/GSH, it is quite likely that the same $3S^*$ species involved in the regeneration with DTT^{ox}/DTT^{red} could be populated during regeneration with GSSG/GSH by an oxidative pathway, e.g., $2S \rightarrow 3S^*$, where $3S^*$ is the same as for regeneration with DTT^{ox}/DTT^{red}; i.e., the rapid oxidation with glutathione could favor a $2S \rightarrow 3S^*$ step, whereas the slower rate of oxidation with dithiothreitol would require that $3S^*$ be formed from 3S.

It is also possible that a particular conformational step is involved in a number of different pathways and that disulfide bonds not relevant to that conformational transition play no role. For example, consider three separate pathways, $2S \rightarrow 2S^*$, $3S \rightarrow 4S^*$, and $3S \rightarrow 3S^*$. Even though the asterisked intermediates have different numbers of disulfide bonds, their formation could depend on the same conformational transition and could also involve the formation of the same disulfide in any of the above three reactions. Alternatively, all three pathways could involve the isomerization of a particular proline. These examples illustrate the limitations of our current definition of a pathway.

Furthermore, conditions could exist under which identical folding pathways are taken with both types of reagents but proceed through different rate-determining steps. A hypothetical example would be one three-disulfide species, $3S_i$, rearranging to another three-disulfide species, $3S_j$, which then oxidizes to native, i.e., $3S_i \rightarrow 3S_j \rightarrow N$. In this example, if the rate-determining step with DTT^{ox}/DTT^{red} is the formation of the final disulfide bond, e.g., $3S_j \rightarrow N$, then, because oxidation with GSSG/GSH is 3 orders of magnitude faster, the rate-determining step during regeneration with GSSG/GSH could become $3S_i \rightarrow 3S_j$. It is important to note that, in this example, the regeneration rate for this hypothetical folding process would be significantly greater with GSSG/GSH than with DTT^{ox}/DTT^{red}.

It is, therefore, important to realize that the ability to assign the type of rate-determining step and the pathway is no substitute for knowing the structures of the intermediates involved in the pathway; it does, however, serve as an invaluable diagnostic tool for determining the conditions to isolate the critical species for subsequent studies of the structures of the important folding intermediates.

Finally, using our definition of a pathway, viz., that a change in the rate-determining step is indicative of a change in pathway, the data presented here indicate that multiple pathways must by definition exist for regeneration of RNase A with DTT^{ox} and DTT^{red}. Since at least one of the steps that follows the rate-determining step must depend on the con-

centration of DTT^{ox}, i.e., $3S^* \rightarrow N$, there will be conditions under which that step would be slower than the rate-determining step ($3S \rightarrow 3S^*$); since oxidation is a bimolecular process while intramolecular rearrangement is a unimolecular process, conditions can arise in which $3S^* \rightarrow N$ (which involves oxidation) is slower than $3S \rightarrow 3S^*$ (which involves intramolecular rearrangement). This type of situation may be irrelevant because the rate of regeneration under those conditions may be negligible; i.e., no regeneration occurs, or is experimentally unattainable; e.g., at infinite dilution where $[DTT^{ox}]$ is vanishingly small, the rate-determining step will shift to become the formation of the final disulfide bond, i.e., $3S^* \rightarrow N$, or some other oxidation step.

Therefore, the fact that protein regeneration involves bimolecular processes (i.e., a reaction between the protein and a redox reagent) means that there will exist some set of redox conditions for which the pathway will change. However, while the pathway has changed, the actual sequence of disulfide-containing intermediates and the conformational transitions among those intermediates may not have changed. Consequently, the most significant origin of multiple regeneration pathways may be that the range of redox conditions under which significant regeneration of RNase A can be obtained is much greater with GSSG and GSH than with DTT^{ox} and DTT^{red}. Therefore, a single pathway can kinetically appear as multiple pathways.

Lastly, it is important to recognize that multiple regeneration pathways have also been observed in studies of the regeneration of BPTI and of modified BPTI lacking one or more thiols (Goldenberg, 1988; Marks et al., 1987; Weissman & Kim, 1992). A comparison of the regeneration pathways of BPTI with those of RNase A will be made in an accompanying paper (Rothwarf & Scheraga, 1993b).

Comparison with Earlier Studies of RNase A. One point that still needs addressing is how the results and analysis presented thus far can be reconciled with the experimental observations and conclusions of Creighton (1977, 1979, 1980, 1986, 1988) and Wearne and Creighton (1988). Our model, as shown in Figure 2, is essentially identical to the model proposed by Creighton (1988). The major difference in the form of the model involves Creighton's proposal that the pathways of formation and reduction of the native protein are the same. We have presented no data in this paper concerning the reversibility of the final regeneration step. $N \rightarrow 3S^*$. Creighton (1988) has stated that the reductive and regeneration pathways are the same even though they are conducted under very different redox conditions. There is no theoretical basis for such a conclusion since all the oxidation and reduction steps are bimolecular processes involving the redox couple. Since the theoretical and experimental basis of Creighton's model (1988) is called into question by the data presented here, as discussed below, we will not present any further comparison between his model and ours.

The Creighton model, which states that the rate-determining step is formation of the final disulfide bond, is based on three of his experimental observations: (1) a nativelike three-disulfide species accumulates prior to the establishment of steady-state conditions during regeneration with GSSG and GSH; (2) DTT^{ox} was incapable of oxidizing reduced RNase A beyond the two-disulfide form; and (3) no intermediates are populated on reduction of RNase A by DTT^{red}. In using these observations to determine a model for the regeneration of RNase A, the assumption was made that the regeneration process with GSSG and GSH and the regeneration process with DTT^{ox} and DTT^{red} were essentially identical. This assumption was based largely on the apparent similarity between the regeneration pathways observed during the regeneration of BPTI with both types of redox reagents. However, the tremendous thermodynamic conformational stability of the intermediates populated during the regeneration of BPTI serves to minimize the inherent differences between the two types of redox reagents. Nevertheless, as will be presented in the following paper (Rothwarf & Scheraga, 1993b), multiple regeneration pathways which depend on the type and concentration of the redox couple should be observed during the regeneration of BPTI. Similar comparisons while studying the regeneration of RNase A with both redox reagents (Wearne & Creighton, 1988) were flawed by use of an incorrect value for the dithiothreitol–glutathione equilibrium constant and the failure to observe either any three-disulfide species or native protein.

As discussed above and quite extensively in an accompanying paper (Rothwarf & Scheraga, 1993b), the very different nature of glutathione- and dithiothreitol-mediated regeneration processes is such that no general conclusions can be drawn about the one from the data of the other. Furthermore, as discussed in the introduction of the previous paper (Rothwarf & Scheraga, 1993a), the regeneration of RNase A with GSSG/GSH cannot be accounted for solely by pathways involving the nativelike three-disulfide species observed by Creighton.

In answer to the second of Creighton's experimental observations, the data presented here and in the accompanying papers (Rothwarf & Scheraga, 1993a,c) clearly show that DTT^{ox} is capable of regenerating RNase A and at a rate (from the steady state) comparable to that observed with GSSG/GSH.

Finally, the third experimental observation used by Creighton to arrive at his model for regeneration of RNase A has been shown (Rothwarf & Scheraga, 1991) to be due to inadequate blocking procedures, and it has been shown that a nativelike three-disulfide species is significantly populated (as much as ~8% of the total protein) during the reduction of RNase A by DTT^{red} (Rothwarf & Scheraga, 1991).

CONCLUSION

Through the application of a kinetic analysis applied to the data obtained after the attainment of steady-state conditions during the regeneration of RNase A with DTT^{ox}/DTT^{red}, the major regeneration pathway has been identified. Regeneration proceeds through a rate-determining step involving an intramolecular rearrangement of one or more three-disulfide species. We have shown that this model is able to fit *all* of our data, including data taken prior to the attainment of the steady-state conditions. Our model is able to account quantitatively for the rate of formation and the steady-state concentrations of all intermediates as well as the native protein over a wide range of redox conditions. While the pathway determined here is different from those observed by Konishi et al. (1982a) with GSSG/GSH, these differences arise from differences in the action of the redox reagents. In particular, regeneration with GSSG/GSH is more likely to proceed through multiple pathways than is regeneration with DTT^{ox}/DTT^{red}.

A relevant question is the following: "What is the general value of the type of analysis presented here?" It serves as a first step in the identification of the major regeneration pathways. However, because of all the theoretical and experimental complications, the method should be used to identify major regeneration pathways, those accounting for

more than 10% of the total regenerated protein. Therefore, while we observe only a single type of pathway, 3S → 3S*, it is possible that other pathways are populated and produce significant amounts of native protein but that these minor pathways are not significant in comparison with the major observed pathway.

In addition, at this point in the study of regeneration processes in proteins, we would recommend the use of DTT^{ox} and DTT^{red} or other cyclic disulfide reagents that do not form stable mixed disulfide bonds. As the results here have shown, the situation is already of sufficient complexity. Use of glutathiones or other linear disulfide reagents increases the number of species by a factor of 10, and favors multiple major regeneration pathways.

Furthermore, the use of DTT^{ox} and DTT^{red} results in a small enough number of groupings so that they can be well resolved, even in the case of complex regeneration data such as those obtained here with RNase A. Therefore, if any of the slow-forming intermediates led to significant changes in the distribution of intermediates as a function of time or redox conditions, it would be experimentally determinable.

Many of the ideas and concepts developed in this paper have focused on kinetic and thermodynamic considerations, but have provided no significant insight into the specific conformational changes and interactions that direct the regeneration process. However, the purpose of this work is to establish the kinetic and thermodynamic framework in which to investigate the conformational folding events which, when coupled with formation of disulfide bonds, lead to the attainment of the native protein. While we have determined the type of pathway(s) (3S → 3S*) through which RNase A regenerates with DTT^{ox}/DTT^{red}, no explanation of protein regeneration is complete until the specific species that precede the rate-determining step(s) in the folding are identified and structurally characterized and all the kinetic constants relevant to their formation and to the subsequent formation of native protein are determined.

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