Regeneration of Bovine Pancreatic Ribonuclease A. 4. Temperature Dependence of the Regeneration Rate†

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ABSTRACT: The rate of regeneration of bovine pancreatic ribonuclease A with oxidized and reduced dithiothreitol (DTT^{ox} and DTT^{red}, respectively) decreases by a factor of 10 when the temperature is increased from 25 to 37 °C. The rate of regeneration of RNase A with oxidized and reduced glutathione increases slightly over that same range of temperature. This suggests that the regeneration processes with the two types of redox reagents proceed through different pathways. There is a significant change in the distribution of three-disulfide intermediates populated during regeneration with DTT^{ox}/DTT^{red} over the range of temperature 15–37 °C that suggests that the three-disulfide species populated at 15 °C are directly involved in the major regeneration pathway observed at 25 °C.

The pathways for regeneration of bovine pancreatic ribonuclease A (RNase A)[1] have been studied by using a variety of different redox systems and solution conditions (Hantgan et al., 1974; Ahmed et al., 1975; Creighton, 1977, 1979; Konishi & Scheraga, 1980a,b; Konishi et al., 1981, 1982a,b,c; Scheraga et al., 1984; Wearne & Creighton, 1988; Rothwarf & Scheraga, 1993a,b). In the three preceding papers (Rothwarf & Scheraga, 1993a,b,c), conclusions were drawn about multiple vs single regeneration pathways. However, these conclusions depend on the definition of what constitutes a separate pathway. We have defined a pathway by the types of species that are involved in the rate-determining step and the type of process involved, viz., oxidation, reduction, or intramolecular rearrangement (Rothwarf & Scheraga, 1993b). The inference, based on experimental considerations and interpretations thereof, has been made in the preceding papers that regeneration of RNase A occurs through more pathways with GSSG/GSH than with DTT^{ox}/DTT^{red}. These deductions, however, are based on considerations of the kinetic and thermodynamic quantities associated with the bimolecular reactions involved in the interaction of the redox reagent with the sulfhydryl and disulfide groups of the protein. Therefore, these differences in regeneration pathways with different redox reagents provide information about formation of disulfide bonds, but very little information about the conformational processes occurring during regeneration.

It is the structural transitions in the protein that occur in a pathway that are at the heart of the refolding process. However, without identifying the specific conformations of individual disulfide-bonded intermediates involved in rate-determining pathways, it is impossible to distinguish one regeneration pathway from another based on conformational differences. While the determination of specific disulfide pairs and structures is our ultimate goal, for the present a less detailed method is necessary to evaluate the conformational transitions that occur during the regeneration process. Furthermore, given the complexity of the process for regeneration of RNase A, we need to exploit conformational differences among intermediates, as well as the redox properties of the protein thiols, if we are to achieve that goal. Therefore, we have investigated the conformational properties of the intermediates populated during the regeneration of RNase A by examining the temperature dependence of the regeneration process with both DTT^{ox}/DTT^{red} and GSSG/GSH. If the regeneration pathways with both types of redox reagents proceed through a single type of conformational transition, then a similar temperature dependence should be observed with both reagents.

Similar studies were carried out by Ahmed et al. (1975) in which the regeneration of RNase A was studied as a function of temperature, and the differences between regeneration with GSSG/GSH and with air oxidation were compared. Their results showed that the rate of regeneration of RNase A with GSSG/GSH increased slightly as the temperature was raised from 14 to 37 °C while the rate of regeneration with air oxidation decreased by a factor of approximately 6 over the temperature range of 25–37 °C. No explanation was presented to account for these results, for which those with air oxidation closely mirror those presented here for the temperature dependence of the regeneration process with DTT^{ox}/DTT^{red}. Therefore, in addition to discussing the significance of the temperature dependence presented here, the significance of the results of the earlier work with air oxidation will also be considered.

MATERIALS AND METHODS

Materials. GSH and GSSG were obtained from Boehringer-Mannheim and were checked for purity by NMR and HPLC assays, as described previously (Rothwarf & Scheraga, 1992). All other reagents are described in a previous paper (Rothwarf & Scheraga, 1993a).

Regeneration of RNase A. Regeneration experiments with DTT^{ox}/DTT^{red} were carried out as described previously (Rothwarf & Scheraga, 1993a) at a starting concentration of 100 mM DTT^{ox} and 32 μM reduced RNase A. Regeneration experiments with GSSG/GSH were carried out with 2.2 mM GSH, 2.2 mM GSSG, and 32 μM RNase A. The pH of all solutions was adjusted to 8.0 at the temperature at which the regeneration was carried out. All regeneration studies were carried out in 100 mM Tris/2 mM EDTA.

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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.

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Table I: Rate Constant for Formation of Native RNase A at Various Temperatures, pH 8.0

redox couple	observed rate constant for regeneration ($\times 10^4$ min^{-1}) at temp (°C)						
	15	25	28	31	33	35	37
GSSG/GSH		80 ± 4[a]					94 ± 6
DTT^{ox}/DTT^{red}	7.2 ± 0.5	12.5 ± 0.6	9.8 ± 0.5	5.1 ± 1.0	4.4 ± 0.3	3.1 ± 0.6	1.3 ± 0.2

[a] The error is calculated at the 95% confidence limit.

Table II: Equilibrium Constants, K_{eq}^{obs}, at Various Temperatures, pH 8.0, for Regeneration with DTT^{ox}/DTT^{red}

reaction	15 °C	25 °C[a]	28 °C	31 °C	33 °C	35 °C	37 °C
R ⇄ 1S	30 ± 3[b]	31 ± 2	31 ± 2	29 ± 3	33 ± 4	33 ± 4	31 ± 2
1S ⇄ 2S	6.3 ± 0.6	5.7 ± 0.3	7.9 ± 0.7	7.0 ± 0.8	7.9 ± 0.6	7.8 ± 0.8	7.3 ± 0.6
2S ⇄ 3S	3.0 ± 0.4	1.7 ± 0.1	2.0 ± 0.2	1.8 ± 0.2	2.0 ± 0.2	2.0 ± 0.3	1.9 ± 0.2
3S ⇄ 4S		0.33 ± 0.01					

[a] Data taken from Rothwarf and Scheraga (1993a). [b] The error is calculated at the 95% confidence limit.

The rate of regeneration of RNase A with GSSG/GSH was assayed by measurement of the enzymatic activity of the entire regeneration mixture, without fractionation of the reaction mixture. At various time intervals (20–30 min), 40-μL aliquots were removed and added to 3.0 mL of "activity" solution at 22 °C, and the initial rate of hydrolysis of substrate (cytidine cyclic monophosphate) was monitored at 286 nm as described previously (Rothwarf & Scheraga, 1993a). The "activity" solution consisted of 100 mM acetic acid, 200 mM NaCl, and 500 μM cytidine cyclic monophosphate, pH 5.0. The first time interval in the regeneration process was taken after 25 min to assure that the steady-state distribution of intermediates had been achieved (Konishi et al., 1981; D. M. Rothwarf and H. A. Scheraga, unpublished results). This assured that the rate constant for regeneration of native protein was constant with time. The linearity of the activity response was determined with a calibration curve by using a series of concentrations of native RNase A over the range of concentrations of native protein attainable in the regeneration studies, i.e., 8–32 μM. The correlation coefficient to a straight line was greater than 0.999. Because the regeneration rate depends on the total concentration of protein, the amount of activity was measured after 24 h to determine that the initial determination of the concentration of protein was correct. The concentration of native protein corresponding to the measured activity was within 5% of the expected value (32 μM) in all cases.

Blocked intermediates obtained during the regeneration with DTT^{ox}/DTT^{red} were fractionated on a Rainin Hydropore SCX 4.8 mm × 10 cm column in addition to the Bakerbond CBX column used in earlier studies (Rothwarf & Scheraga, 1991, 1993a). The fractionation of disulfide-bonded groupings was similar for both types of columns. The fractionation of intermediate species within groups, however, is noticeably different on the two columns as can be seen by comparing Figure 1B of this paper with Figure 2A of Rothwarf and Scheraga (1993a). The concentration of native protein was determined from the HPLC chromatograms, as described by Rothwarf and Scheraga (1993a). In this paper, only regeneration samples, which were taken after the attainment of the steady-state condition, were used to determine the rate constant for the formation of native RNase A; however, data taken before the attainment of the steady-state condition were used to determine rate constants for formation and reduction of disulfide bonds among the intermediates.

For some of the regeneration experiments using DTT^{ox}/DTT^{red}, aliquots were withdrawn and assayed for activity as described above for the regeneration studies using GSSG/GSH. Within experimental error (~5%), there was no difference between the amount of native protein regenerated when measured by relative chromatographic area or by enzymatic activity.

All other procedures are as described previously (Rothwarf & Scheraga, 1993a).

RESULTS

Regeneration Rate. The rate constants for regeneration were determined by assuming a first-order rate of appearance of native protein, N, and integrating the rate equation:

$$\ln[1 - N] = -kt \quad (1)$$

where ln[1 – N] is the concentration fraction of *all* nonnative species. The values of the rate constant for formation of native protein with both GSSG/GSH and DTT^{ox}/DTT^{red} are shown in Table I. The rate constant decreases by a factor of 10 with increasing temperature for regeneration with DTT^{ox}/DTT^{red} over the temperature range 25–37 °C, while it increases slightly when regeneration is carried out with GSSG/GSH over that same range of temperature.

Equilibrium Constants. The observed steady-state equilibrium constant, K_{eq}^{obs}, was determined between groupings at each temperature for the regeneration experiments carried out with DTT^{ox}/DTT^{red}. They are listed in Table II. While the equilibrium constants for the R ⇄ 1S process do not change over the entire range of temperature studied, 15–37 °C, those for the 1S ⇄ 2S process are smaller at 15 and 25 °C than at the higher temperatures where the value is constant within the error of the measurements. The most significant observation, however, is that the value of the equilibrium constant for the 2S ⇄ 3S process is approximately 50% larger at 15 °C than at the other temperatures. The absence of a value of K_{eq}^{obs} for the 3S ⇄ 4S process at temperatures other than at 25 °C arises because no significant population of four-disulfide species appears under the redox conditions used at the other temperatures (100 mM DTT^{ox}, 32 μM reduced RNase A).

Distribution of Intermediates within Groupings. Chromatograms of the intermediates populated during the regeneration with DTT^{ox}/DTT^{red} at 15, 25, and 37 °C are shown in Figure 1. Significant differences in the distribution within groupings at various temperatures can be seen in both the two- and three-disulfide species. The most dramatic differences are seen in the distribution of the three-disulfide species as a function of temperature. Figure 2 shows a blowup of the three-disulfide region from Figure 1 to highlight these differences. At 15 °C, there are only two major three-disulfide species, while at the higher temperatures there are too many species to distinguish.

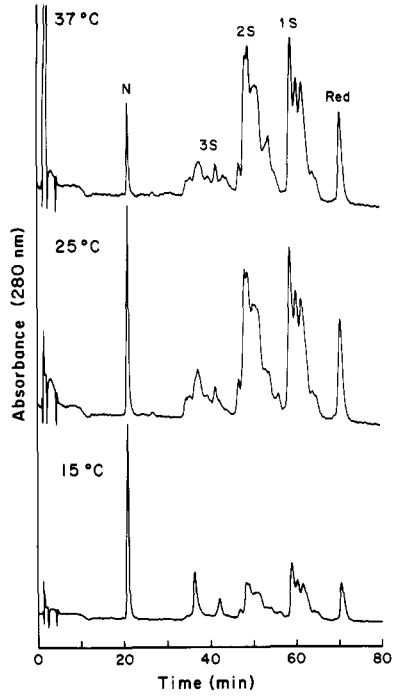

FIGURE 1: Distribution of regeneration intermediates at different temperatures. Starting regeneration conditions were 100 mM DTT^{ox}, 32 μM reduced RNase A, pH 8.0. 37 °C, 180 min; 25 °C, 90 min; 15 °C, 270 min.

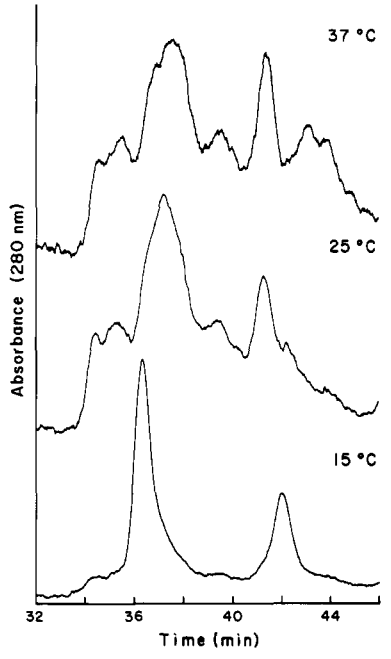

FIGURE 2: Blowup of the three-disulfide region of Figure 1.

Rates of Disulfide Bond Formation and Reduction among the Intermediates. From data obtained prior to the attainment of the steady state at 15 and 37 °C with DTT^{ox}/DTT^{red}, rate constants for formation and reduction of disulfide bonds among the intermediates were determined. Solutions for the observed forward rate constant, k_f^{obs}, were determined by using the expression for the observed rate constant for reduction, k_r^{obs}, in terms of the equilibrium constants, K, given in Table II, i.e., $k_r^{obs} = k_f^{obs}/K$. The rate constants were determined by using the simplex minimization and numerical integration method described in an accompanying paper (Rothwarf & Scheraga, 1993b). k_{intra}, the rate constant for formation of intramolecular disulfide bonds, was calculated by using eq 4 of Rothwarf and Scheraga (1993b). Average values of the rate constants k_f^{obs}, k_r^{obs}, and k_{intra} were determined as described previously (Rothwarf & Scheraga, 1993b).

Comparison of the average rate constants at 37 °C, as shown in Table III, indicates that they are independent of the number of intramolecular protein disulfides and are approximately 3-fold greater than those observed at 25 °C [Table I in Rothwarf and Scheraga (1993b)].

The average rate constants obtained at 15 °C, as shown in Table III, are similar for processes involving reduced and one- and two-disulfide species and are approximately half the values observed at 25 °C (Rothwarf & Scheraga, 1993b). The value of k_{intra}^{avg} for formation of the three-disulfide species is ~65% of the value of the other intermediates. The value of k_r^{avg} for reduction of the three-disulfide species is ~40% of that value for the other intermediates.

DISCUSSION

Regeneration Pathways. From the analysis in a previous paper (Rothwarf & Scheraga, 1993b), the regeneration process with DTT^{ox}/DTT^{red} occurs through a single major pathway (3S → 3S*) over the range of conditions employed. Therefore, the decrease in regeneration rate with increasing temperature, as shown in Table I, suggests that the species involved in this pathway are being melted out. There are two possibilities for the identity of the intermediate that is actually being melted out. The first is that the species 3S that precedes the rate-determining step (3S → 3S*) is being unfolded, or rather that the steady-state distribution is changing, and the concentration of the species immediately preceding the rate-determining step is decreasing. Since the rate of regeneration of native protein with DTT^{ox}/DTT^{red} decreases by a factor of 10 over the temperature range 25–37 °C while, as discussed above, there is approximately a 3-fold increase in the rate constant of oxidation and reduction among the intermediates, e.g., 1S → 2S, over the same temperature range, the concentration of the 3S species involved in the rate-determining step probably decreases by a factor of ~30. There is no reason to suppose that specific interactions between dithiothreitol or glutathione and the protein would lead to preferential stabilization of the species that precedes the rate-determining step; therefore, the same variation in the distribution of structural intermediates with temperature should occur with GSSG/GSH. If the regeneration of RNase A with GSSG/GSH proceeded exclusively through the same pathway(s) as with DTT^{ox}/DTT^{red}, then a similar proportional decrease in the regeneration rate (dN/dt) would be expected.

The chromatograms in Figures 1 and 2 suggest that it is this first possibility that is occurring. The distribution within groupings changes significantly as the temperature is increased from 15 to 37 °C, particularly within the three-disulfide species. Furthermore, with respect to the rate constants observed at 25 °C, the large rate constant of regeneration of native protein at 15 °C (7.2×10^{-4} min^{-1}), coupled with the 3-fold lower rate constant of formation (k_f^{obs}) [7.7×10^{-2} min^{-1} M^{-1} from Table III as compared to 23×10^{-2} min^{-1} M^{-1} from Table I of Rothwarf and Scheraga (1993b)] of the three-disulfide species at 15 °C, suggests that one or more of the three-disulfide species, stabilized at the lower temperature, are involved in the rate-determining step.

The second possibility is that the rate of regeneration is decreased by the melting of an asterisked intermediate species (3S*), i.e., a species that follows the rate-determining step. In the previous papers (Rothwarf & Scheraga, 1993a,b), we largely ignored any transitions back from asterisked inter-

Table III: Rate Constants at pH 8.0 for Regeneration with DTT^{ox}/DTT^{red}

reaction	k_f^{obs} [a] ($\times 10^2$ min^{-1} M^{-1})	k_f^{avg} [b] ($\times 10^2$ min^{-1} M^{-1})	37 °C k_{intra}^{obs} [c] (min^{-1})	k_{intra}^{avg} [d] (min^{-1})	k_r^{obs} [e] ($\times 10^{-2}$ min^{-1} M^{-1})	k_r^{avg} [f] ($\times 10^{-2}$ min^{-1} M^{-1})
R ⇄ 1S	400[g]	14	610	22	13	13
1S ⇄ 2S	180	12	280	19	25	13
2S ⇄ 3S	82	14	130	21	43	14
reaction	k_f^{obs} [a] ($\times 10^2$ min^{-1} M^{-1})	k_f^{avg} [b] ($\times 10^2$ min^{-1} M^{-1})	15 °C k_{intra}^{obs} [c] (min^{-1})	k_{intra}^{avg} [d] (min^{-1})	k_r^{obs} [e] ($\times 10^{-2}$ min^{-1} M^{-1})	k_r^{avg} [f] ($\times 10^{-2}$ min^{-1} M^{-1})
R ⇄ 1S	60	2.1	92	3.3	2.0	2.0
1S ⇄ 2S	29	1.9	44	2.9	4.6	2.3
2S ⇄ 3S	7.7	1.3	12	2.0	2.6	0.85

[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 Rothwarf and Scheraga (1993b). [c] k_{intra}^{obs} is the rate constant for formation of intramolecular protein disulfide bonds, determined from k_f^{obs} using eq 4 of Rothwarf and Scheraga (1993b). [d] k_{intra}^{avg} is k_{intra}^{obs} corrected for statistical factors as described in Rothwarf and Scheraga (1993b). [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 Rothwarf and Scheraga (1993b). [g] On the basis of comparison with similar determinations made in Rothwarf and Scheraga (1993b), we estimate the error in these rate constants to be less than 15% at the 95% confidence limit.

mediates to the steady-state distribution. Given the facts that the native state is the thermodynamically most stable one and that the regeneration model [Figure 2 of Rothwarf and Scheraga (1993b)] is valid over a greater than 50-fold range of the concentration of DTT^{red}, the assumption was made that reduction of asterisked intermediates did not occur to any significant extent. However, since we have no such detailed information on the regeneration rate over a wide range of redox conditions at other temperatures, this assumption may no longer be valid at 37 °C. Again, this consideration would also apply to regeneration with GSSG/GSH, but the absolute concentrations of the redox couple could influence the rates significantly. As discussed in the previous paper (Rothwarf & Scheraga, 1993c), the rate of reduction is proportional to the concentration of the thiol component of the redox couple. At the concentrations of GSH and DTT^{red} used in this study (2.2 and 0.060 mM, respectively), the rate of reduction of disulfide bonds is over 40-fold (2.2/0.060) greater[2] with GSH than with DTT^{red}. Therefore, if the species following the rate-determining step becomes more susceptible to unfolding with increasing temperature, then the rate of regeneration should decrease more rapidly with increasing temperature when using GSSG/GSH than with DTT^{ox}/DTT^{red} under the conditions used. This result, however, is not observed.

Regardless of which of these possibilities accounts for the observed decrease in the rate of regeneration with DTT^{ox}/DTT^{red}, a common conclusion is reached. The rate of regeneration through the pathway 3S → 3S*, observed during regeneration with DTT^{ox}/DTT^{red}, either should display the same temperature dependence during regeneration with GSSG/GSH or should decrease more rapidly with increasing temperature with GSSG/GSH than with DTT^{ox}/DTT^{red}. Given the experimental result that, over the range of temperature 25–37 °C, the rate of regeneration of native protein decreases by an order of magnitude for regeneration with DTT^{ox}/DTT^{red} while it increases slightly for regeneration with GSSG/GSH, the only conclusion is that regeneration with GSSG/GSH occurs through additional pathways. Furthermore, these pathways involve protein conformations that are distinct from those involved in the 3S → 3S* pathway observed with DTT^{ox}/DTT^{red}.

There are several possibilities for the type of additional pathways by which RNase A regenerates with GSSG/GSH. Since these pathways are not populated during regeneration with DTT^{ox}/DTT^{red}, they probably involve oxidation or reduction of protein disulfide bonds, e.g., 3S → N. However, as discussed above and in the preceding papers (Rothwarf & Scheraga, 1993b,c), the concentration of GSH is ~40-fold greater than the concentration of DTT^{red} used in these experiments. In a preceding paper (Rothwarf & Scheraga, 1993a), in which concentrations of DTT^{red} as much as 10-fold higher than those employed here were used, no pathways involving reduction, e.g., 2S → 1S*, were observed during the regeneration with DTT^{ox}/DTT^{red}. Under those redox conditions, only reduced and one- and two-disulfide species were well populated. This suggests that reductive pathways are probably not significant unless they involve three- or four-disulfide species.[3]

As discussed in a previous paper (Rothwarf & Scheraga, 1993b), Konishi et al. (1982a) found that a pathway involving the 4S species was involved in a major regeneration pathway, i.e., 4S → 3S*. This conclusion was based on the kinetics of formation of native protein when the regeneration was restarted from the isolated four-disulfide intermediate and is therefore independent of the validity of any kinetic models. Because of the much lower rate of formation of disulfide bonds with DTT^{ox}/DTT^{red} as opposed to GSSG/GSH, the 4S species is not populated significantly under any of the redox conditions employed here. Furthermore, the much greater redox potential of GSSG/GSH used in these experiments would favor the formation of four-disulfide species [as shown in Table II of Rothwarf and Scheraga (1993c) at 2.2 mM GSSG and 2.2 mM GSH, the concentration of the 4S species experimentally determined by Konishi et al. (1981) is ~25%]. This further supports the earlier conclusion (Konishi et al., 1982a) that the pathway corresponding to 4S → 3S* could be a major regeneration pathway with GSSG/GSH.

[2] Since it is only the thiolate anion that is involved in reduction and, from eq 9 of Rothwarf and Scheraga (1993c), at pH 8.0 the ionization of the first thiol of DTT^{red} is only 0.37 that of GSH, the rate of reduction with GSH should be ~110 (i.e., 40/0.37) times greater than the rate of reduction with DTT^{red} under the conditions used here.

[3] As explained in Rothwarf and Scheraga (1993b,c), an alternative explanation is that pathways such as 2S → 1S* are not significantly populated during regeneration with DTT^{ox}/DTT^{red} because 1S* → N involves three oxidation steps which would be expected to be very slow with DTT^{ox}/DTT^{red}.

Equilibrium and Rate Constants for Regeneration with DTT^{ox}/DTT^{red}. All data for the equilibrium and rate constants among intermediates shown in Tables II and III are consistent with the conclusion that the intermediates are largely disordered (Rothwarf & Scheraga, 1993a,b; Konishi & Scheraga, 1980b) with the exception of the data involving the three-disulfide species populated at 15 °C, i.e., the 2S ⇄ 3S step. As presented under Results, both the equilibrium constant and the average forward and reverse rate constants involving that species deviate from the values for the other steps. However, the comparison of rate data is complicated by the use of the equilibrium constant to determine k_r^{obs}. As explained in an accompanying paper (Rothwarf & Scheraga, 1993b), k_f^{obs}/k_r^{obs} will not be equal to the steady-state equilibrium constant if one of the species contributing to the equilibrium constant is involved in a regeneration pathway (3S → 3S*) when DTT^{ox}/DTT^{red} is used. Therefore, if the regeneration proceeds through the same rearrangement pathway determined at 25 °C, then the values of k_f^{obs} and k_r^{obs} at 15 °C shown in Table III are incorrect. However, we have recalculated the rate constants shown in Table III without the use of the experimentally determined equilibrium constant to determine the value of k_r and have also recalculated the rate constants using the assumption that native RNase A is regenerated through a single pathway at 15 °C involving 3S → 3S*. With these changes, the values obtained (data not shown) for k_f^{obs} and k_r^{obs} at 15 °C for the 2S ⇄ 3S process were within 6% of the values shown in Table III. This indicates that, regardless of the regeneration model, the average rate constant of reduction (k_r^{avg}) of a three-disulfide species at 15 °C is ~2.5 times smaller than that observed for the other intermediates at 15 °C (0.85×10^2 min^{-1} M^{-1} compared to 2.3×10^2 and 2.0×10^2 min^{-1} M^{-1}), and indicates the existence of a significant amount of nonrandom structures at 15 °C that are not easily accessible to DTT^{red} within the three-disulfide species. Such a conclusion is in agreement with the chromatographic data presented in Figure 2, which indicate a smaller number of three-disulfide intermediates at 15 °C. The decrease in the value of k_f^{avg} for the formation of 3S with respect to the values of k_f^{avg} for formation of the 1S and 2S species also reflects some level of local conformational ordering in the 2S species.

Differences in Redox Potential. Two points that need addressing concern the difference in redox potential between the regeneration experiments carried out with GSSG/GSH and DTT^{ox}/DTT^{red}, respectively, and the role, if any, that these differences play. The first point is that the relative redox potential between DTT^{ox}/DTT^{red} and GSSG/GSH does not change with temperature. This has been shown experimentally through measurement of the dithiothreitol–glutathione equilibrium constant at 15, 25, and 37 °C. Within experimental error, there is no difference in the value of the equilibrium constant (Rothwarf & Scheraga, 1992). More importantly though, the distribution of intermediates, as reflected in the values of K_{eq}^{obs} given in Table II, does not vary significantly over the temperature range 25–37 °C. This suggests that the redox potential of RNase A relative to DTT^{ox}/DTT^{red} also does not change over that temperature range.

The second point is that the redox potentials which correspond to the redox conditions used in this paper are very different for the two types of reagents; the redox conditions with GSSG/GSH are at a much stronger oxidizing potential than those with DTT^{ox}/DTT^{red}. However, as shown in Table II of the preceding paper (Rothwarf & Scheraga, 1993c) and discussed above, the only major difference in the distribution of intermediates at 25 °C under the redox conditions used here involves the absence of the four-disulfide species when regenerating with DTT^{ox}/DTT^{red}.

While redox conditions with DTT^{ox}/DTT^{red} can be found such that a significant population of four-disulfide species occurs, such conditions may not provide any additional insight into the regeneration process because, as explained above and in the three preceding papers, pathways of the type 4S → 3S* have a direct dependence on the concentration of the thiol component of the redox couple. In order to populate the four-disulfide species significantly during regeneration with DTT^{ox}/DTT^{red}, the concentration of DTT^{red} must be kept very low (on the order of 10–20 μM). Therefore, regeneration through a 4S → 3S* pathway would be more than 2 orders of magnitude slower with DTT^{ox}/DTT^{red} than with GSSG/GSH.

An additional point is that, on the basis of the rate constant for the rate-determining step (3S → 3S*) determined in the previous paper, 9.8×10^{-3} min^{-1} at 25 °C, pH 8.0, the maximum rate of regeneration (corresponding to all of the protein being in the three-disulfide form) would be 9.8×10^{-3} min^{-1}. This rate is only ~25% greater than the rate observed here at 25 °C and 2.2 mM GSSG, 2.2 mM GSH. On the basis of the concentrations of three-disulfide species populated under these redox conditions [as shown in Table II of the preceding paper (Rothwarf & Scheraga, 1993c)], the amount of three-disulfide species (~30–50%) is insufficient to account for the observed regeneration rate with GSSG/GSH at 25 °C if the only populated pathway is 3S → 3S*. Therefore, the pathway observed at 25 °C during regeneration with DTT^{ox}/DTT^{red} (3S → 3S*) is incapable of explaining all of the observed regeneration rate with GSSG/GSH at that temperature, suggesting the existence of multiple regeneration pathways with GSSG/GSH at 25 °C, in agreement with the results of Konishi et al. (1982a).

Comparison with Earlier Studies Using GSSG/GSH and Air Oxidation. Another point that needs addressing is the comparison of these results to earlier measurements of the temperature dependence of the regeneration process. Ahmed et al. (1975) have observed that the rate of regeneration with GSSG/GSH increased with temperature over the range 14–37 °C but decreased slightly when the temperature was increased to 42 °C. This is consistent with the results obtained here. They carried out additional experiments regenerating RNase A by air oxidation, and found that the rate decreased by a factor of ~6 over the temperature range of 25–37 °C. A similar observation was made by Epstein et al. (1962). This is similar to the temperature dependence that we observe here with DTT^{ox}/DTT^{red} and suggests that the rate-determining processes with the two different regeneration methods (i.e., air and dithiothreitol oxidation) may be similar.

The question that needs to be answered is why is regeneration by air oxidation similar to regeneration with DTT^{ox}/DTT^{red}. A second and more important question is why are the pathways accessible during regeneration with GSSG/GSH not accessible during regeneration by air oxidation. While the precise mechanism for the oxidation of thiols by air is not well understood, there is no effective means to reduce a disulfide bond during regeneration by air oxidation. Consequently, rate-determining steps involving rearrangement and oxidation should be favored, and pathways involving reduction should be inaccessible. Therefore, the major regeneration pathway populated during regeneration with DTT^{ox}/DTT^{red} (3S → 3S*) should be populated during regeneration by air oxidation, since it is a rearrangement pathway, hence the similar temperature dependencies of the observed regeneration rate

observed with the two methods. In addition, the inability of the regeneration process by air oxidation to proceed through pathways involving reduction of a disulfide bond further supports the hypothesis presented above that one of the major pathways populated during regeneration of RNase A with GSSG/GSH at higher temperatures involves reduction of a three- or four-disulfide species.

CONCLUSION

The data for the temperature dependence of the rates of regeneration of native protein presented here clearly indicate that there is a fundamental difference in the regeneration pathways when using the two types of redox reagents, DTT^{ox}/DTT^{red} and GSSG/GSH, respectively. Furthermore, it suggests that these differences in pathways with the different redox reagents employed are not simply a result of our definition of "rate-determining step" as discussed in the preceding papers (Rothwarf & Scheraga, 1993a,b), but rather result from the population of different structural intermediates in these different pathways.

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