Regeneration of Bovine Pancreatic Ribonuclease A. 3. Dependence on the Nature of the Redox Reagent†

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ABSTRACT: Monothiol reagents such as oxidized and reduced glutathione (GSSG and GSH, respectively) form stable mixed disulfides with protein thiols while dithiol reagents such as oxidized and reduced dithiothreitol (DTT^{ox} and DTT^{red}, respectively) do not. This large difference in the stabilities of the mixed disulfides is reflected in much greater rates of formation and reduction of protein disulfide bonds with GSSG/GSH than with DTT^{ox}/DTT^{red}. With dithiothreitol, the concentrations of intermediate species in the steady state depend on the redox potential, i.e., on the $[DTT^{ox}]/[DTT^{red}]$ ratio, and not on the absolute concentrations of these reagents. With glutathione, the redox potential and hence the concentrations of intermediate species in the steady state depend on the $[GSSG]/[GSH]^2$ ratio; hence, with glutathione, in contrast to dithiothreitol, the absolute values of the concentrations do affect the steady-state concentrations. Consequently, the regeneration pathways of bovine pancreatic ribonuclease A depend on the nature of the redox reagent as well as the redox potential at which they are used. The use of GSSG/GSH favors multiple regeneration pathways, while the use of DTT^{ox}/DTT^{red} favors regeneration through fewer pathways. These concepts are also illustrated by an analysis of literature data for the regeneration pathways of bovine pancreatic trypsin inhibitor.

In studies of the regeneration of native ribonuclease A (RNase A)[1] from its reduced form, mixtures of oxidized and reduced glutathione (GSSG and GSH, respectively) have been used almost exclusively as the redox reagent to convert protein thiols to internal disulfide bonds (Hantgan et al., 1974; Ahmed et al., 1975; Creighton, 1977b, 1979; Konishi & Scheraga, 1980a,b; Konishi et al., 1981, 1982a,b,c). An alternative redox system, a mixture of oxidized and reduced dithiothreitol (DTT^{ox} and DTT^{red}, respectively), was generally avoided because, until recently (Rothwarf & Scheraga, 1991), it was widely accepted that DTT^{ox} was incapable of regenerating native RNase A (Creighton, 1979; Wearne & Creighton, 1988). Recent measurements of the DTT^{ox}/DTT^{red} redox potential, however, indicated that literature estimates of the oxidizing power of DTT^{ox} compared to GSSG were too low by about 2 orders of magnitude and that DTT^{ox} is indeed strong enough to oxidize RNase A (Chau & Nelson, 1991; Rothwarf & Scheraga, 1992). Furthermore, as will be shown in this paper, the inability of DTT^{ox}/DTT^{red} to form stable mixed disulfides with the protein thiols results in an effective increase in the apparent redox potential of DTT^{ox}/DTT^{red} as judged by the formation of intramolecular protein disulfide bonds; i.e., more three- and four-disulfide species are formed when regeneration is carried out with DTT^{ox}/DTT^{red} than with GSSG/GSH at the *same* redox potential.

Earlier conclusions about regeneration of RNase A with GSSG and GSH had been inferred (Creighton, 1979, 1988; Wearne & Creighton, 1988) from the apparent inability of DTT^{ox} to regenerate RNase A, without taking into account possible differences in the mechanism of action with the two types of redox systems. Therefore, in this paper, we consider the differences in the interactions of the two types of reagents for the regeneration process. A comprehensive understanding of the regeneration of RNase A requires that the fundamental differences between the regeneration process carried out with glutathione and dithiothreitol, respectively, be elaborated.

While DTT^{ox}/DTT^{red} has not been used widely for the regeneration of most proteins, it has been used extensively in studies of BPTI, and a detailed folding mechanism for this protein has been proposed (Creighton, 1985), although some aspects of the model have been brought into question (Weissman & Kim, 1991, 1992). Therefore, in addition to the detailed analysis of the regeneration process of RNase A, comparison will be made between previously reported regeneration results on BPTI obtained with GSSG/GSH and DTT^{ox}/DTT^{red}, respectively.

DIFFERENCES IN REACTIVITY

As illustrated in Figure 1 of a preceding paper (Rothwarf & Scheraga, 1993a), formation of a disulfide bond with both reagents proceeds through the formation of mixed disulfides between the redox reagent and the protein. The stability of these mixed disulfides is reflected in the equilibrium constants K_{GSSG} and K_{DTT} (defined in the aforementioned Figure 1). As will be shown below, the value of the ratio K_{GSSG}/K_{DTT} is ~620 M at 25 °C, pH 8, the stability of the mixed disulfide with dithiothreitol being much lower because of the rapid recyclization to form DTT^{ox}. This large ratio strongly influences the pathways through which a protein is regenerated with either of these two types of reagents, primarily because of either of two effects arising from the formation of mixed disulfides.

The first effect arises from the fact that the rate of formation of protein disulfide bonds is 1–3 orders of magnitude slower with DTT^{ox}/DTT^{red} than it is with GSSG/GSH because the concentration of mixed disulfides is lower with DTT^{ox}/DTT^{red} than with GSSG/GSH. As can be seen in Figure 1 of

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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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Rothwarf and Scheraga (1993a), the mixed-disulfide form is a necessary intermediate in the formation of a protein disulfide bond. The second effect arises from the fact that the formation of excessive mixed disulfides (with GSSG) reduces the rate of formation of intramolecular protein disulfides by reducing the concentration of the protein thiols, as shown in the third step of Figure 1A of Rothwarf and Scheraga (1993a). The two effects appear to produce opposite results for the regeneration rate; i.e., the first increases the regeneration rate with GSSG/GSH vs DTT^{ox}/DTT^{red}, whereas the second decreases it with GSSG/GSH. However, as will be developed in this paper, both effects lead to a common result; viz., the existence of multiple pathways is favored for regeneration with GSSG/GSH, while regeneration with DTT^{ox}/DTT^{red} proceeds through a smaller number of pathways. To discuss these differences, we must first consider the differences between K_{GSSG} and K_{DTT}, cited above.

Value[2] *of* K_{GSSG}. The equation describing the equilibrium constant, K_{GSSG}, shown in the first step of Figure 1A of Rothwarf and Scheraga (1993a), can be rewritten in terms of the rate constants:

$$GSSG + P_{SH}^{SH} \underset{k_{EX_G}}{\overset{4k_{EX_P}}{\rightleftarrows}} P_{SH}^{S\text{-}S\text{-}G} + GSH \qquad (1)$$

where k_{EX} refers to the rate constant for intermolecular thiol–disulfide exchange and is presumed to be independent of the nature of the thiol in a thiol–disulfide exchange reaction when the thiol is completely ionized; however, since the thiols are generally not completely ionized, k_{EX} does depend on the nature of the thiol because the pK's of the various sulfhydryl species differ. The subscripts P and G refer to the type of thiolate, protein and glutathione, respectively, involved in the exchange reaction. The distinction is necessary because it is only the thiolate anion that is involved in the exchange reaction. It is, therefore, necessary to correct for differences in the ionization behavior of the different thiols. From eq 1, K_{GSSG} can be written in terms of the rate constants, i.e.

$$K_{GSSG} = 4k_{EX_P}/k_{EX_G} \qquad (2)$$

The factor of 4 in eq 1 and 2 is a statistical factor resulting from the existence of two equivalent sulfurs in GSSG and two equivalent thiols in the protein.

The value of K_{GSSG} as expressed in eq 2 depends only on the pK's of the protein thiols and of GSH, since we have made the assumption that differences in k_{EX} arise exclusively from the extent of ionization of the thiol. The value of the ratio k_{EX_P}/k_{EX_G} can be expressed in terms of the pK's of the thiols of the protein and of GSH:

$$\frac{k_{EX_P}}{k_{EX_G}} = \frac{1 + 10^{pK_{GSH}-pH}}{1 + 10^{pK_{Prot}-pH}} \qquad (3)$$

The pK of GSH is 8.72 at 30 °C (Reuben & Bruice, 1976). The precise values of the pK's of the sulfhydryls of RNase A are not known. Few pK's of protein sulfhydryl groups have been determined; the problem is complicated because the internal environment and conformation of a protein affect the observed pK's (Laskowski & Scheraga, 1954, 1956), and the accessibility of the thiol affects the kinetic measurements. Estimates of sulfhydryl pK's have been made for deoxyribonuclease (pK = 8.8), lysozyme (pK = 11), adenylate kinase (pK = 7.5), and papain (pK = 8.4 at pH 9) at 30 °C (Shaked et al. 1980), and for BPTI (pK = 8.8) at 25 °C (Creighton, 1975a). Since GSH is a small cysteine-containing peptide and should therefore be similar to a protein, and since the pK of GSH falls within the limited range of protein thiol pK's determined, we make the approximation that $k_{EX_P} \approx k_{EX_G}$. This leads to the value of K_{GSSG} of 4 in the temperature range of 20–30 °C.

This value can be compared to the value of K_{GSSG} observed experimentally [see Figure 1 of Konishi et al. (1981)]. By measuring the preequilibrium constants between groups of intermediates that involved formation of a mixed disulfide, values of K_{GSSG} were determined. These values varied from 0.37 to 7.8 at 22 °C, pH 8.0. However, since these preequilibrium constants were based on groupings of intermediates, there are statistical factors that contribute to the observed values of K_{GSSG}. Normalizing the observed values for the statistical factors leads to values in the range[3] 0.46–7.6. These are the average values of K_{GSSG} for a cysteine of RNase A involved in a mixed disulfide with GSSG. While there are other factors that contribute to the range of observed values of K_{GSSG}, such as different protein conformations having different interaction energies with GSSG, the largest contribution to the observed range of K_{GSSG} is presumably due to differences in the pK's of the cysteine residues in the protein. In light of these factors, the above estimate of K_{GSSG}, ~4 from the pK's, is within the range of the values deduced from regeneration studies.

Value of K_{DTT}. The equation describing the equilibrium constant K_{DTT}, shown in Figure 1B of Rothwarf and Scheraga (1993a), can be rewritten in terms of the rate constants:

$$DTT\begin{matrix}S\\|\\S\end{matrix} + P\begin{matrix}SH\\|\\SH\end{matrix} \underset{k_{EX_D}C_{eff_{DTT}}}{\overset{4k_{EX_P}}{\rightleftharpoons}} P\begin{matrix}S\text{—}S\text{—}DTT\text{—}SH\\|\\SH\end{matrix} \qquad (4)$$

where k_{EX} is defined as for eq 1 and the subscript D refers to the exchange reaction involving a thiol of dithiothreitol. $C_{eff_{DTT}}$ is the effective concentration of the second thiol of dithiothreitol. C_{eff} is simply a number that describes the increase in an equilibrium constant for an intramolecular process relative to a closely related intermolecular process (Burns & Whitesides, 1990). In this particular case, it represents the concentration of the monothiol that would be necessary to provide the same rate as for the back-reaction in the bimolecular process. From eq 4, K_{DTT} may be written in terms of the rate constants as

$$K_{DTT} = 4k_{EX_P}/k_{EX_D}C_{eff_{DTT}} \qquad (5)$$

The factor of 4 in eq 4 and 5 is again a statistical factor resulting from the existence of two equivalent sulfurs in DTT^{ox}, and two thiols in the protein.

[2] The analysis presented here is similar to that presented by Creighton and Goldenberg (1984), and we have used a notation similar to theirs.

[3] The possibility might have existed that the range of values of K_{GSSG} observed by Konishi et al. (1981) results from experimental errors. We have, however, independently measured average values of K_{GSSG} between 0.8 and 2.8 (D. M. Rothwarf and H. A. Scheraga, unpublished results), thereby eliminating this possibility. These values, however, are less than 4, suggesting, on the basis of eq 2 and 4, that the pK's of the sulfhydryl groups of RNase A may be greater than 8.7. The values of K_{GSSG} were determined by regenerating the reduced protein (30 μM) with 2.2 mM GSH and 2.2 mM GSSG. At various times, the regeneration was quenched by addition of negatively-charged 2-sulfoethyl methanethiosulfonate ($^-SO_3C_2H_5SSO_2CH_3$) and fractionated at pH 7.0 by cation-exchange chromatography. Disulfide-containing groupings were identified and their relative concentrations determined by disulfide analysis as described in Rothwarf and Scheraga (1993a). The two- and three-disulfide species were further fractionated by cation-exchange chromatography at pH 2.0 to determine the relative concentrations of the species which contained mixed disulfides with glutathione.

In order to evaluate K_{DTT} from eq 5, we must determine $C_{eff_{DTT}}$. This quantity can be calculated from the experimentally determined value of the dithiothreitol–glutathione equilibrium constant, K_{G-T} (Rothwarf & Scheraga, 1993b). The equations describing the dithiothreitol–glutathione equilibrium in terms of the rate constants are

$$\mathrm{GSSG} + \mathrm{DTT}\begin{matrix}\mathrm{SH}\\ \mathrm{SH}\end{matrix} \underset{k_{EX_G}}{\overset{2k_{EX_D}}{\rightleftharpoons}} \mathrm{DTT}\begin{matrix}\mathrm{S{-}S{-}G}\\ \mathrm{SH}\end{matrix} + \mathrm{GSH} \qquad (6)$$

$$\mathrm{DTT}\begin{matrix}\mathrm{S{-}S{-}G}\\ \mathrm{SH}\end{matrix} \underset{2k_{EX_G}}{\overset{k_{EX_D}C_{eff_{DTT}}}{\rightleftharpoons}} \mathrm{DTT}\begin{matrix}\mathrm{S}\\ \mathrm{S}\end{matrix} + \mathrm{GSH} \qquad (7)$$

Therefore, the equilibrium constant for the glutathione–dithiothreitol equilibrium, with the aid of eq 6 and 7, is

$$K_{G-T} = \frac{[\mathrm{GSH}]^2[\mathrm{DTT^{ox}}]}{[\mathrm{GSSG}][\mathrm{DTT^{red}}]} = \left[\frac{k_{EX_D}}{k_{EX_G}}\right]^2 C_{eff_{DTT}} \qquad (8)$$

The value of the ratio k_{EX_D}/k_{EX_G} can be calculated from the known pK's of GSH and $\mathrm{DTT^{red}}$. The pK of GSH is 8.72 at 30 °C (Reuben & Bruice, 1976). The first and second pK's of $\mathrm{DTT^{red}}$ are 9.2 and 10.1, respectively, at 30 °C (Whitesides et al., 1977). In this analysis, we have treated $\mathrm{DTT^{red}}$ as a monothiol since its second pK is 10.1, and, at pH 8.0, the concentration of the dithiolate is negligible; this is the reason why a factor of 2, instead of 4, appears in eq 6. The value of the ratio k_{EX_D}/k_{EX_G} is therefore a simple function of the pK's:

$$\frac{k_{EX_D}}{k_{EX_G}} = \frac{1 + 10^{pK_{GSH}-pH}}{1 + 10^{pK_{DTT}-pH}} \qquad (9)$$

At pH 8.0, this value is 0.37. The value of the glutathione–dithiothreitol equilibrium constant, K_{G-T}, at pH 8.0 and 25 °C is 229 M (Rothwarf & Scheraga, 1992). Insertion of these values into eq 8 leads to a value of 1670 M for $C_{eff_{DTT}}$. Using this value, and the assumption of the previous section that the pK of a sulfhydryl group in the protein is the same as that of GSH (hence, that $k_{EX_G} \approx k_{EX_P}$ and therefore, according to eq 9, $k_{EX_P}/k_{EX_P} = 1/0.37$), a value of K_{DTT} of 6.5×10^{-3} $\mathrm{M^{-1}}$ is obtained from eq 5.

No direct measurements of K_{DTT} for the regeneration process of RNase A (for comparison with this value) have been made, since the mixed disulfide between the protein and dithiothreitol is highly unstable, making measurements difficult. However, since the value of k_{EX_D} cancels in the ratio of K_{GSSG}/K_{DTT}, the value of K_{DTT} relative to K_{GSSG} can be estimated, without making the above assumption that the pK's of the sulfhydryls of the protein and of GSH are the same. Combining eq 2 and 5, we obtain

$$K_{GSSG}/K_{DTT} = k_{EX_D}C_{eff_{DTT}}/k_{EX_G} \qquad (10)$$

Substitution of k_{EX_D}/k_{EX_G} from eq 9, and the value of $C_{eff_{DTT}}$ computed above, leads to a value of 620 M for this ratio.

Values of k_{intra} and k_{EX}. In order to evaluate the relative rates of formation of disulfide bonds in RNase A with GSSG/GSH and $\mathrm{DTT^{ox}}/\mathrm{DTT^{red}}$, respectively, it is also necessary to know the values of k_{intra} [see Figure 1 of Rothwarf and Scheraga (1993a)], the rate constant for formation of intramolecular protein disulfides, and k_{EX}, the rate constant for intermolecular thiol–disulfide exchange. The first of these quantities was estimated in the previous paper (Rothwarf & Scheraga, 1993b) to be in the range of 5.9–7.6 $\mathrm{min^{-1}}$.

The value of k_{EX_D} at 25 °C and pH 8.0 is 90 $\mathrm{M^{-1}\ min^{-1}}$, based on the rate of reduction of GSSG by $\mathrm{DTT^{red}}$, 180 $\mathrm{M^{-1}\ min^{-1}}$ (Rothwarf & Scheraga, 1992), and eq 6 (since the forward rate in eq 6 is much less than that in eq 7 by a factor of 1670/2 and hence rate-determining). Using the pK values for $\mathrm{DTT^{red}}$ and GSH, and eq 9, the value of k_{EX_G} can be estimated as $\sim$250 $\mathrm{M^{-1}\ min^{-1}}$ at pH 8.0 and 25 °C.

Rates of Formation of Disulfide Bonds. The overall rate of formation of protein disulfide bonds depends on which of the forward steps, k_{intra}, or formation of the mixed disulfide, is rate-determining. We will, therefore, consider both situations.

When k_{intra} is rate-determining, the initial rate of formation of protein disulfides with GSSG [the combined first two steps of Figure 1A of Rothwarf and Scheraga (1993a)] can be written as

$$\mathrm{rate} = K_{GSSG}k_{intra}\frac{[\mathrm{GSSG}]}{[\mathrm{GSH}]}[\mathrm{Prot_{SH}^{SH}}] \qquad (11)$$

Similarly, the initial rate of formation of protein disulfides with $\mathrm{DTT^{ox}}$ [as in Figure 1B of Rothwarf and Scheraga, (1993a)] is

$$\mathrm{rate} = K_{DTT}k_{intra}[\mathrm{DTT^{ox}}][\mathrm{Prot_{SH}^{SH}}] \qquad (12)$$

The ratio of the rates of formation of disulfide bonds depends on the absolute concentrations of the redox couple and not simply on the redox potential itself (the latter would depend on $[\mathrm{GSSG}]/[\mathrm{GSH}]^2$ and $[\mathrm{DTT^{ox}}]/[\mathrm{DTT^{red}}]$ instead of the functionalities shown in eq 11 and 12). Therefore, no direct comparison of the rates in eq 11 and 12 can be made without consideration of the absolute concentrations of the reagents typically employed for regeneration of RNase A. Konishi et al. (1981) found that the maximum rate of regeneration of RNase A with glutathione is obtained with 0.77 mM GSSG and 2.3 mM GSH. The highest $\mathrm{DTT^{ox}}$ concentration used, and hence the greatest oxidation rate obtainable in the accompanying paper (Rothwarf & Scheraga, 1993a), was 200 mM (this concentration also corresponded, coincidently, to the conditions of the maximum rate of regeneration of native protein). The relative rates (obtained by dividing eq 11 by eq 12) are

$$\frac{\text{rate with GSSG}}{\text{rate with } \mathrm{DTT^{ox}}} = \frac{K_{GSSG}[\mathrm{GSSG}]}{K_{DTT}[\mathrm{GSH}][\mathrm{DTT^{ox}}]} \qquad (13)$$

Substituting the above numerical values for these quantities, we obtain a value of 1040. This large value arises because the concentration of mixed disulfides with GSSG is much greater than that with $\mathrm{DTT^{ox}}$, i.e., because $K_{GSSG} > K_{DTT}$.

We next consider the case in which the rate-determining step is the formation of the mixed disulfide [see Figure 1 of Rothwarf and Scheraga (1993a)]. In such a case, the initial rate of formation of disulfide bonds with GSSG is obtained from eq 1 as

$$\mathrm{rate} = 4k_{EX_P}[\mathrm{GSSG}][\mathrm{Prot_{SH}^{SH}}] \qquad (14)$$

In this case, the initial forward rate does not depend on the concentration of GSH. For formation of disulfide bonds with $\mathrm{DTT^{ox}}$, the rate of reclosing the ring to re-form $\mathrm{DTT^{ox}}$ from the mixed-disulfide form is significantly greater (1.5×10^5 $\mathrm{min^{-1}}$, computed from the back-reaction of eq 4, $k_{EX_D}C_{eff_{DTT}}$) than any of the observed k_{intra}'s [see column 5 of Table I of Rothwarf and Scheraga (1993b)]. Therefore, the free thiol and the mixed-disulfide form are in rapid equilibrium, and the rate expression given in eq 12 will be applicable under both conditions considered here; i.e., the formation of the mixed disulfide with $\mathrm{DTT^{ox}}$ cannot be rate-limiting. The ratio of

the rates for large k_{intra} (eq 14 and 12) becomes

$$\frac{\text{rate with GSSG}}{\text{rate with DTT}^{ox}} = \frac{4k_{EX_P}[GSSG]}{K_{DTT}k_{intra}[DTT^{ox}]} \quad (15)$$

It is important to note that no assumptions about the pK's of the protein thiols are necessary to evaluate the ratio in eq 15 since substitution of eq 5 into eq 15 yields

$$\frac{\text{rate with GSSG}}{\text{rate with DTT}^{ox}} = \frac{k_{EX_D}C_{eff_{DTT}}[GSSG]}{k_{intra}[DTT^{ox}]} \quad (16)$$

Having derived the two expressions for the relative rates of formation of disulfide bonds (eq 13 and 16), we must now consider the conditions under which either of these two cases applies. First, consider the rate-determining step in the regeneration process after the attainment of the steady-state condition discussed in a previous paper (Rothwarf & Scheraga, 1993a). Under these steady-state conditions, the concentration fractions of all species in the total population of intermediates, including those involving mixed disulfides with the redox couple, are not changing with time. The existence of this equilibrium implies that the step involving k_{intra} is rate-determining for pathways involving formation of disulfide bonds. Consequently, the rate equations for formation of disulfide bonds after the attainment of the steady state are those shown in eq 11 and 12. Hence, under conditions of maximum regeneration with both reagents (0.77 mM GSSG, 2.3 mM GSH, and 200 mM DTT^{ox}, respectively), regeneration through pathways involving formation of an intramolecular disulfide bond, e.g., 2S → 3S* of Rothwarf and Scheraga (1993b), would be approximately 1040 times faster with GSSG/GSH than with DTT^{ox}/DTT^{red}. This analysis has ignored the role of excessive formation of mixed-disulfide bonds, an issue that will be addressed in the next subsection.

The second case, in which formation of mixed-disulfide bonds is rate-determining, can be important only in the time domain prior to the attainment of the steady state. Furthermore, by comparing k_{intra}^{avg}, given in column 5 of Table I of Rothwarf and Scheraga (1993b), which ranges from 5.9 to 7.6 min^{-1}, with $k_{EX_G}[GSH]$ [using the value $k_{EX_G} \sim 250\ M^{-1}\ min^{-1}$ and the concentration of GSH from the conditions for maximum regeneration (2.3 mM)], it is seen that $k_{intra} > k_{EX_G}[GSH]$: i.e., in the interconversion among intermediates, formation of mixed-disulfide bonds is rate-determining (i.e., is the slow step) when glutathione is used. Therefore, while, as discussed above, this second case is not relevant to the determination of rate-determining steps in the overall regeneration process, it is relevant to the rates of formation of disulfide bonds among the intermediates populated in the steady state, e.g., 2S → 3S of Rothwarf and Scheraga (1993b). Using the average values of k_{intra} for the individual steps [column 5 in Table I of Rothwarf and Scheraga (1993b)], the estimated values of k_{EX_D} and $C_{eff_{DTT}}$, and 0.77 mM GSSG and 200 mM DTT^{ox} as the starting concentrations, the relative rates of formation of disulfide bonds for the one-, two-, three-, and four-disulfide species from eq 16 are 76, 87, 98, and 85, respectively. These differences in the rates of formation of protein disulfide bonds among the steady-state intermediates with different redox reagents explain the experimental observation (Rothwarf & Scheraga, 1993a) that a lag in the appearance of native protein is much longer when regenerating RNase A with DTT^{ox}/DTT^{red} than with GSSG/GSH.

Excessive Formation of Mixed-Disulfide Bonds with GSSG. While the most significant outcome of the difference in the relative stabilities of mixed disulfides between the reagents is the effect on the rate of formation of disulfide bonds, the more commonly cited outcome of this enhanced stability of mixed disulfides with glutathione is that it leads to species that have multiple mixed disulfides, as illustrated in step 3 of Figure 1A of Rothwarf and Scheraga (1993a). It is well established that mixing reduced protein with very high concentrations of GSSG (0.1 M) results in completely glutathione-blocked reduced protein, and essentially blocks further oxidation (Odorzynski & Light, 1979; Konishi et al., 1981). Even under less extreme oxidizing conditions, excessive formation of mixed disulfides can result in preferential blockage of regeneration pathways. This leads to a pathway that was insignificant under one set of redox conditions becoming dominant under another set of redox conditions, and the observation of multiple regeneration pathways.

Since excessive formation of mixed disulfides reduces the concentration of protein thiols, it also complicates the analysis of relative rates of formation of disulfide bonds. The comparison of the rates of formation of disulfide bonds made in the previous section assumed that the concentration of free protein thiols was similar with DTT^{ox}/DTT^{red} and GSSG/GSH. However, this obviously would not be the case under all redox conditions (e.g., when [GSSG] ≫ [GSH]). Therefore, the comparisons of rates for k_{intra} being rate-determining were made by considering the conditions of maximum regeneration with GSSG/GSH, conditions under which excessive formation of mixed-disulfide bonds is not as pronounced as when uneder less favorable regeneration conditions. Specific examples of the influence that excessive mixed disulfides have on the distribution of species and regeneration pathways will be presented in the following sections.

COMPARISON OF EXPERIMENTAL RESULTS FOR REGENERATION OF RNASE A WITH THE TWO REAGENTS

In order to make accurate comparisons between the relative rates through regeneration pathways with the two types of reagents, the various statistical factors and the quantitative effect of excessive formation of mixed disulfides must be considered. Furthermore, specific comparisons must be made for each type of rate-determining step. For the purposes of this discussion, we consider the simplest example, the rate-determining processes involving the three-disulfide species. There are three general types of rate-determining steps (formation of intramolecular disulfide bonds, reduction, and rearrangement, respectively), as discussed previously, each of which involves a number of distinct chemical processes. We consider each of these processes separately.

Before considering these processes, however, the following considerations must first be addressed. For the purpose of these comparisons, we assume that the total concentrations of three-disulfide species are equivalent for each of the redox conditions considered with each type of reagent. The concentration of groups of intermediates as a function of the redox potential is quite complex in the case of regeneration with GSSG/GSH, and will be considered separately in the next section.

Because these comparisons require a knowledge of the concentrations of DTT^{ox}/DTT^{red} and GSSG/GSH, we choose two redox conditions for each type of reagent. For GSSG/GSH, we use 0.77 mM GSSG, 2.3 mM GSH, and 2.2 mM GSSG, 2.2 mM GSH. The first set of conditions, which has also been used above, is chosen because it corresponds to the experimentally determined conditions of optimum regeneration

Table I: Distribution of Mixed-Disulfide Species in the 3S Species

[GSSG][a] (mM)	[GSH][a] (mM)	[DTT^{ox}][b] (mM)	[DTT^{red}][b] (mM)	% of 3S (3S,0M)	(3S,1M)	(3S,2M)
2.2	2.2			4.0	32	64
0.77	2.3			18	49	33
		100	0.060	99.9	0.13	8.5×10^{-5}
		200	0.010	99.7	0.26	3.4×10^{-4}

[a] The concentrations of intermediates with GSSG/GSH were calculated assuming $K_{GSSG} = 4$. Essentially identical values would be determined using the values of K_{GSSG} measured by Konishi et al. (1981). [b] The concentrations of intermediates with DTT^{ox}/DTT^{red} were calculated using the value of K_{DTT} of 6.5×10^{-3} M^{-1} determined in the text.

of native RNase A (Konishi et al., 1982a). The second condition corresponds to those used in the following paper (Rothwarf & Scheraga, 1993c) as well as conditions referred to explicitly in earlier work (Konishi et al., 1981). In addition, the 1/1 ratio of GSSG/GSH was used in other early studies (Hantgan et al., 1974; Ahmed et al., 1975).

We also consider two concentrations of DTT^{ox}/DTT^{red}. The first is the experimentally determined value corresponding to maximum regeneration as used in a previous paper (Rothwarf & Scheraga, 1993a): 200 mM DTT^{ox}, 3.2 μM RNase A. We arbitrarily choose 10 μM as the concentration of DTT^{red} as a convenient value which corresponds closely to the concentration under the steady-state conditions as shown in Figure 5 of Rothwarf and Scheraga (1993a). The second set of conditions is 100 mM DTT^{ox}, 32 μM RNase A. We choose 60 μM as the concentration of DTT^{red}, again based on the steady-state concentrations as shown in Figure 5 of Rothwarf and Scheraga (1993a). This particular redox ratio is chosen because it corresponds to a well-studied set of conditions in the preceding (Rothwarf & Scheraga, 1993a,b) and following (Rothwarf & Scheraga, 1993c) papers.

Using these concentrations of the redox couples, the concentrations of the three subgroups which comprise the three-disulfide species can be determined. These three subgroups (which participate in the three general types of rate-determining steps referred to above) are three-disulfide species with no mixed disulfides (3S,0M), three-disulfide species with one mixed disulfide (3S,1M), and three-disulfide species with two mixed disulfides (3S,2M). With the values of the concentrations of the redox couple chosen above, the values of K_{GSSG} and K_{DTT} determined above, and the appropriate statistical factors [the statistical factors arise from the two ways to form a (3S,1M) species from either thiol of the (3S,0M) species], the concentrations of (3S,0M), (3S,-1M), and (3S,2M) can be determined by using the equilibrium expressions corresponding to the chemical reactions shown in eq 1 and 4 for GSSG/GSH and DTT^{ox}/DTT^{red}, respectively. These values are shown in Table I and will be used in the examples discussed in the following subsections.

Formation of Disulfide Bonds. In the hypothetical model involving mixed disulfides with GSSG and DTT^{ox} in the three-disulfide species, under discussion here, the major reaction for formation of disulfide bonds is assumed to involve the formation of intramolecular disulfide bonds, i.e., (3S,1M) → 4S*. The relative rates with the two redox reagents depend only on the concentration of (3S,1M), i.e., $dN/dt \propto [(3S,1M)]$. Therefore, using the concentrations of each redox couple under its own conditions of maximum regeneration [which determine the concentration of (3S,1M)], the relative rates through this type of rate-determining step can be determined from the value of [(3S,1M)] for each type of reagent in Table I. The rate is 188 times greater for regeneration with GSSG/GSH than with DTT^{ox}/DTT^{red}. This is ~6 times smaller than the value of 1040 that was predicted earlier when excessive formation of mixed-disulfide bonds was ignored. However, the difference in rate of formation of disulfide bonds with the two types of reagents is still 2 orders of magnitude greater with GSSG/GSH than with DTT^{ox}/DTT^{red}.

Reduction. In the same example system being considered here, the reductive pathways are 3S → 2S*. While differences in pK and the local environment of the different sulfhydryl groups in the protein will invariably lead to differences in the distribution of species within one subgroup from that of another subgroup, there is no way to obtain this information from our data. Therefore, we assume that there is no difference in distribution between any of the subgroups of three-disulfide species. Using this assumption, the relative rates of reduction of disulfide bonds with the two types of reagents depend solely on the concentrations of DTT^{red} and GSH. Consequently, the relative rate under the conditions of maximum regeneration with both reagents is 230 (2.3 mM/0.010 mM) times more rapid with GSSG/GSH than with DTT^{ox}/DTT^{red}.

Rearrangement. Again the assumption is made that differences in the distribution of intermediates within subgroups are not significant. There are two types of three-disulfide rearrangement that can occur. The first is a conformational rearrangement which is not coupled to a disulfide rearrangement step, e.g., proline isomerization. This type of rearrangement would depend only on the total concentration of the three-disulfide species and should, therefore, be independent of redox concentration or the type of redox reagent used, i.e., $dN/dt \propto [3S]$.

The second type of rearrangement involves rearrangement of a disulfide bond and requires at least one free thiol. There are two types of disulfide bond rearrangement that can occur; the first involves the transfer of a mixed disulfide from one sulfhydryl to another, i.e., (3S,1M) → (3S,1M)*. This is kinetically analogous to formation of disulfide bonds, and the relative rates are the same as for that process (both depend only on [(3S,1M)]).

The second type of disulfide bond rearrangement involves the rearrangement of intramolecular disulfide bonds. Therefore, both (3S,0M) and (3S,1M) can participate. A statistical factor of 2 must be included for steps involving (3S,0M) because there are two free sulfhydryl groups present. The rate, therefore, depends on the sum of the two terms, i.e., $dN/dt \propto \{2[(3S,0M)] + [(3S,1M)]\}$. Using the values in Table I under the conditions of maximum regeneration, the rate through this type of pathway would be ~2.3 $[(2 \times 99.7 + 0.26)/(2 \times 18 + 49)]$ times greater with DTT^{ox}/DTT^{red} than with GSSG/GSH.

CONCENTRATIONS AS A FUNCTION OF REDOX POTENTIAL

Thus far, only the distribution within the three-disulfide grouping has been considered, as an example. The actual

Table II: Concentrations of Intermediates at Steady-State Conditions

source	[GSSG] (mM)	[GSH] (mM)	[DTT^{ox}] (mM)	[DTT^{red}] (mM)	% of intermediates[e] R	1S	2S	3S	4S
Konishi et al.[a]	2.2	2.2			0.09	3.9	19	53	25
$K_{GSSG} = 4$[b]	2.2	2.2			1.4	19	44	31	4.3
DTT equiv[c]	2.2	2.2			7×10^{-5}	0.02	1.3	22	76
Konishi et al.[a]	0.77	2.3			0.60	9.1	18	44	29
$K_{GSSG} = 4$[b]	0.77	2.3			0.60	11	39	41	8.2
DTT equiv[c]	0.77	2.3			0.004	0.40	7.7	44	48
Rothwarf & Scheraga[d]			100	0.060	8.0	41	39	11	0.60
Rothwarf & Scheraga[d]			200	0.010	0.02	1.3	15	50	33

[a] Calculated using the preequilibrium constants in Figure 1 of Konishi et al. (1981). [b] Calculated from the estimate of $K_{GSSG} = 4$ coupled with the experimentally determined equilibrium constants between intermediates from column 2 of Table I of Rothwarf and Scheraga (1993a) and the experimentally determined value of 229 M for the glutathione–dithiothreitol equilibrium constant (Rothwarf & Scheraga, 1992). [c] Concentrations are those that would be observed with DTT^{ox}/DTT^{red} at the redox potential given by the concentrations of GSSG and GSH, calculated using the experimentally determined equilibrium constants between intermediates from column 2 of Table I of Rothwarf and Scheraga (1993a) and the experimentally determined value of 229 M for the glutathione–dithiothreitol equilibrium constant (Rothwarf & Scheraga, 1992). [d] Calculated using the experimentally determined equilibrium constants between intermediates from column 2 of Table I of Rothwarf and Scheraga (1993a). [e] Using the procedures described in footnote 3, we have directly measured the relative concentrations of each intermediate at 2.2 mM GSH, 2.2 mM GSSG, 25 °C, pH 8.0: [R] = 2.7; [1S] = 16; [2S] = 17; [3S] = 45; [4S] = 19.

concentration of three-disulfide species varies as a function of the disulfide/thiol ratio of the redox reagent and *not* the redox potential as will be shown in this section. In the case of DTT^{ox}/DTT^{red}, the thiol/disulfide ratio and the redox potential are the same. In the case of GSSG/GSH, they are not (the redox potential depends on [GSSG]/[GSH]^2), and the absolute values of the concentrations of the reagents as well as the concentration ratio are, therefore, important. This point has been developed thoroughly in the literature (Konishi et al., 1982a; Wetlaufer et al., 1987), but it has been suggested recently that the regeneration of RNase A depends on the redox potential used and not on the type of reagent or the absolute concentration of reagents used (Wearne & Creighton, 1988).

As shown in the previous section and in eq 1, the distribution of mixed disulfides with glutathione within any disulfide grouping depends on the thiol/disulfide ratio of the redox reagent and not on the redox potential. The concentration of any particular grouping of intermediates is the sum of all the individual species most of which are involved in mixed disulfides. Furthermore, since less oxidized groupings have more free sulfhydryls available for formation of mixed disulfide bonds, at a given redox potential, a thiol/disulfide ratio which favors formation of mixed-disulfide bonds leads to a greater population under steady-state conditions of species with fewer intramolecular disulfides. This point is illustrated in Table II. For each of the two redox conditions with glutathione that were considered earlier, the distribution of groupings has been calculated in three ways. The first distribution, labeled "Konishi et al.", was calculated by using the preequilibrium constants determined experimentally by Konishi et al. (1981). The second was calculated by using the estimate of $K_{GSSG} = 4$ determined above. The third and most illuminating set of values, because it allows comparison between the two types of reagents at an equivalent redox potential, is labeled "DTT equivalent" and was determined by using DTT^{ox}/DTT^{red} at a redox ratio equivalent to the redox potential defined by the concentrations of GSH and GSSG.

As shown in Table II, at equivalent redox potentials, the distribution of intermediates is very different for GSSG/GSH and DTT^{ox}/DTT^{red}. In particular, the concentrations of species with greater numbers of intramolecular disulfide bonds, i.e., 3S and 4S, are favored with dTT^{ox}/DTT^{red}. Clearly then, given the very different distribution of species expected with the two types of reagents at the *same* redox potential, there is no reason to suppose that regeneration rates would have any simple dependence on the redox potential, and this has been shown experimentally with RNase A (Konishi et al., 1982a; Wetlaufer et al., 1987) and lysozyme (Wetlaufer et al., 1987). It is important to recognize that it is possible for the regeneration rate to be the same at a given redox potential with both DTT^{ox}/DTT^{red} and GSSG/GSH, but such a result would simply be coincidental. As shown in Table II (rows 4–6), at the redox potential that corresponds to 0.77 mM GSSG, 2.3 mM GSH, the concentrations of three-disulfide species are independent of the redox reagent used. Therefore, if the rate-determining step involved only the three-disulfide species, it is conceivable that the regeneration rate would be the same for both types of reagents. However, such a result is purely coincidental, because there is an infinite number of concentrations of GSSG and GSH that would provide the same redox potential but a very different distribution of species (same [GSH]^2/[GSSG] but different [GSH]/[GSSG]).

COMPARISONS TO BPTI

It is of interest to apply the foregoing analysis to literature data on the folding pathways of BPTI. Using the definition of a regeneration pathway that we adopted in the previous paper (Rothwarf & Scheraga, 1993b), a pathway is specified by the intermediates and type of process involved in the rate-determining step, and we find that BPTI, like RNase A, regenerates through multiple pathways. In the regeneration of BPTI, only one of the three two-disulfide species containing native disulfide pairings is able to form the final disulfide bond rapidly. However, this species, [30–51; 5–55], forms more slowly than the other two species with native disulfide pairings, [5–55; 14–38] and [30–51; 14–38] (Creighton & Goldenberg, 1984; Weissman & Kim, 1991). All three of these species have completely native structure (Staley & Kim, 1992). The major regeneration pathway is believed to involve a disulfide rearrangement in which [30–51; 5–55] is formed from [30–51; 14–38] (Creighton, 1992; Weissman & Kim, 1991, 1992), or 2S → 2S* in our nomenclature. However, this rearrangement must involve the formation of a two-disulfide species with one nonnative disulfide pairing, and there is still some controversy as to the identity of that species (Creighton, 1992; Weissman & Kim, 1992). Since there are eight possible intermediates that could form, it is quite likely that this process, by our definition, involves multiple pathways.

However, of greater interest within the scope of this paper is a second major regeneration pathway involving disulfide

bond formation. This second pathway involves the oxidation of either [5–55] or [30–51] to form [30–51; 5–55], or 1S → 2S* in our nomenclature. This pathway was originally identified through studies of a derivative of BPTI in which Cys–14 and Cys–38 were blocked irreversibly by carboxymethylation (Creighton, 1977a) and characterized further on a mutant of BPTI in which Cys-14 and Cys-38 had been replaced by serine (Goldenberg, 1988). The rate constant for the intramolecular step of the reactions that involve formation of the 2S* species (from the 1S species) in this pathway (i.e., k_{intra}) is similar to the rate constant for the regeneration pathway involving rearrangement of a two-disulfide species (Creighton, 1977a; Goldenberg, 1988; Weissman & Kim, 1992).

Therefore, on the basis of the analysis provided in this paper, that has shown the differences in the relative rates of disulfide bond formation, reduction, and rearrangement, the relative populations of the two pathways (1S → 2S* and 2S → 2S*) can be discussed. By direct analogy to the relative rates of regeneration processes involving RNase A, since the formation of intramolecular disulfide bonds is much slower with DTT^{ox}/DTT^{red} than intramolecular disulfide bond rearrangement, we would expect that the rate of the 2S → 2S* pathway would be at least 2 orders of magnitude faster than that of the 1S → 2S* pathway during regeneration with DTT^{ox}/DTT^{red}. Therefore, the 1S → 2S* pathway should not be experimentally detectable when the regeneration is carried out with DTT^{ox}/DTT^{red}. However, during regeneration with GSSG/GSH, the relative population through the two pathways should depend primarily on the concentrations of the two species, i.e., [1S] and [2S], and both pathways should be populated provided that 1S and 2S are of reasonable population. Studies of the mutant lacking Cys-14 and Cys-38 support these conclusions. The mutant regenerates at a rate that is ~3-fold slower than that of wild-type BPTI with GSSG/GSH but fails to regenerate with DTT^{ox}/DTT^{red} (as expected from our analysis) (Marks et al., 1987; Goldenberg, 1988). Therefore, we concentrate our analysis on the regeneration pathways with GSSG/GSH.

The value of k_{intra} for the formation of [30–51; 5–55] from the grouping of one-disulfide species, 1S, has been estimated as 1.6×10^{-3} s^{-1} (Goldenberg, 1988) and 5×10^{-3} s^{-1} (Creighton, 1977a). The value of k_{EX_G} ($\approx k_{EX_P}$) at pH 8.7, the pH under which all the measurements on BPTI that we are discussing were carried out, is ~13 M^{-1} s^{-1} (Creighton, 1975a; Rothwarf & Scheraga, 1992). Therefore, even at millimolar concentrations of GSSG, the rate-limiting step (see eq 1) in the 1S → 2S* pathway is the k_{intra} step [(4×13 M^{-1} s^{-1})[GSSG] is larger than k_{intra}, when [GSSG] > 10^{-4} M]. The rate equation for that pathway is given by eq 11. The equation for the relative rates for the two pathways is

$$\frac{\text{rate through 1S} \rightarrow \text{2S*}}{\text{rate through 2S} \rightarrow \text{2S*}} = \frac{K_{GSSG} k_{intra}^{1S} ([GSSG]/[GSH])[1S]}{k_{intra}^{2S}[30\text{–}51; 14\text{–}38]} \quad (17)$$

where k_{intra}^{1S} is the value of k_{intra} for the 1S → 2S* pathway, and k_{intra}^{2S} is the value of k_{intra} for the 2S → 2S* pathway.

In order to compute the ratio in eq 17, the numerical values for the individual terms must be determined. Since the 1S species and [30–51; 14–38] are in rapid equilibrium (Creighton & Goldenberg, 1984), the value of [1S]/[30–51; 14–38] in eq 17 can be expressed in terms of the forward and reverse rate constants (k_f and k_r, respectively) that describe the interconversion between the intermediates:

$$\frac{[1S]}{[30\text{–}51; 14-38]} = \frac{k_r[GSH]^2}{k_f[GSSG]} \quad (18)$$

The values of k_f and k_r have been determined as 90 M^{-1} s^{-1} and 600 M^{-2} s^{-1}, respectively (Creighton & Goldenberg, 1984). The value of k_{intra}^{2S} has been determined (Creighton & Goldenberg, 1984) as 5×10^{-3} s^{-1}. For the value of k_{intra}^{1S}, we choose the more recently determined value of 1.6×10^{-3} s^{-1} (Goldenberg, 1988). We again make the assumption that $K_{GSSG} = 4$. This is a particularly good estimate in the case of BPTI, since the pK's of Cys-14 and Cys-38 have been determined to be 8.80 (Creighton, 1975a) and the pK's of all six sulfhydryls have been shown to be similar (Creighton, 1975b), and a value of 4 for K_{GSSG} was used by Goldenberg (1988) to determine k_{intra}^{1S}. Substitution of these values and eq 18 into eq 17 yields

$$\frac{\text{rate through 1S} \rightarrow \text{2S*}}{\text{rate through 2S} \rightarrow \text{2S*}} = 8.5[GSH] \quad (19)$$

This equation can be rewritten in terms of the percent of the total regeneration of native protein that arises through the 1S → 2S* pathway:

$$\%\ \text{regeneration through 1S} \rightarrow \text{2S*} = \frac{8.5[GSH]}{8.5[GSH] + 1} \quad (20)$$

In deriving eq 19 and 20 by using eq 11, we have assumed that excessive mixed-disulfide formation does not occur. Furthermore, the values of k_f and k_r determined by Creighton and Goldenberg (1984) were derived under conditions where the concentration of GSH was much greater than that of GSSG, and effects due to the formation of mixed disulfides were ignored. Therefore, eq 18, 19, and 20 are valid only for conditions under which [GSH] ≫ [GSSG].

The highest concentration of GSH used in the earlier studies is 20 mM (Creighton & Goldenberg, 1984). Insertion of that value into eq 20 (since the concentration of GSSG used was only 0.15 mM, eq 20 is valid) leads to the result that, under those conditions, ~15% of the observed native protein should have been regenerated through the 1S → 2S* pathway. This actually may represent a lower limit because, had we used the value of 5×10^{-3} s^{-1} for k_{intra}^{1S}, determined by Creighton (1977a), then our estimate of the percentage of protein regenerated through the 1S → 2S* pathway at 20 mM GSH would be 35%. Regardless of which of the two values of k_{intra} that we use, a significant percentage of protein should regenerate through the 1S → 2S* pathway, and at significantly high enough concentrations of GSH, the major regeneration pathway should change from 2S → 2S* to 1S → 2S*.

Unfortunately, there is no direct experimental evidence for the existence of multiple pathways in unmodified wild-type BPTI. However, given the conclusions drawn here, it is our belief that reexamination of the regeneration processes of BPTI will reveal that the major regeneration pathway changes as a function of the concentration of GSH and that the 1S → 2S* pathway accounts for a significant percentage of BPTI regenerated in earlier studies (Creighton & Goldenberg, 1984) using GSSG/GSH.

Finally, it is interesting to note that the two regeneration pathways of BPTI lead to the formation of a common species (2S*) as a result of kinetic and not thermodynamic factors. This situation is similar to the proposal made in an accompanying paper (Rothwarf & Scheraga, 1993b) in which it was suggested that the regeneration of RNase A with DTT^{ox}/

DTT^{red} might require the formation of a particular three-disulfide species with native disulfide pairings.

CONCLUSION

The discussion presented here has shown that the type of redox reagent used can strongly influence the regeneration pathways of a protein. Regeneration with a dithiol reagent, e.g., DTT^{ox}/DTT^{red}, as compared to a monothiol reagent, e.g., GSSG/GSH, results in fewer types of pathways, favors rearrangement pathways as opposed to pathways involving oxidation or reduction, and in the steady state at a given redox potential favors the formation of species containing more intramolecular protein disulfide bonds.

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