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Regeneration of Ribonuclease A from the Reduced Protein. Rate-Limiting Steps†

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ABSTRACT: In the regeneration of ribonuclease A (RNase A) from the reduced protein by a mixture of reduced and oxidized glutathiones, there is a distribution of intermediates in a preequilibrium state characterized by a set of equilibrium constants. In the kinetics experiments reported here, we measured the amounts of fully regenerated RNase A produced (in >50 min) from the reduced protein at various concentrations of reduced and oxidized glutathiones at pH 8.2 and 22 °C. We relate these amounts of fully regenerated RNase A to the distributions of the intermediates in preequilibrium in order to identify the rate-limitng steps and estimate their rate constants. Several different rate-limiting steps (or pathways) were found to produce fully regenerated RNase A. Also, by starting the regeneration from the isolated intermediates 4S (two or four of whose four disulfide bonds are nonnative ones) and 3S1G1H, we were able to suggest that the reaction 4S + GSH → 3S1G1H* is a more likely rate-limiting step than is 3S1G1H → 4S* + GSH. In summary, the regeneration of RNase A with glutathiones involves oxidation of reduced RNase A to a set of intermediates; interconversions among these intermediates rapidly lead to a preequilibrium state prior to the rate-limiting steps. At the same time, fully regenerated RNase A is produced from the intermediates (that exist in this preequilibrium state prior to the rate-limiting steps) through several *different* pathways (or rate-limiting steps). The preferred pathway that is followed varies according to the distribution of intermediates, i.e., according to the relative stabilities of the intermediates and the concentrations of reduced and oxidized glutathiones.

This series of papers presents a study of the regeneration of ribonuclease A (RNase A)[1] from the reduced protein by a mixture of oxidized and reduced glutathione (GSSG and GSH, respectively), which is being carried out within the context of the general problem of determining the pathways of protein folding from an analysis of the preequilibrium, the kinetics, and the energetics of the interconversion of the intermediates. In paper 3 (Konishi et al., 1981), we showed that there is a preequilibrium among the "Intermediates"[2] on the pathways toward fully regenerated RNase A, and we estimated the equilibrium constants (some of which were obtained by appropriate extrapolation) and distributions among the various intermediates in the preequilibrium.

Such a preequilibrium treatment is applicable to protein folding pathways in general. For example, in the folding of bovine pancreatic trypsin inhibitor, Creighton (1977) showed that the reaction involving disulfide interchange from [(30–51, 5–14) + (30–51, 5–38)] to (30–51, 5–55), in his notation, is the rate-limiting step. Thus, the reduced protein and the intermediates prior to the rate-limiting step should exist in a preequilibrium, and a similar preequilibrium treatment as that reported in paper 3 would be applicable to this system. A preequilibrium also exists in the folding of proteins with intact disulfide bonds. The kinetic parameters for folding of such systems have been obtained by jumping the solution conditions from denaturing ones (e.g., 8 M urea, 6 M guanidine hydrochloride, low pH, etc.) to folding conditions (Baldwin & Creighton, 1980). Within the dead time of the jump in solution conditions, the denatured proteins undergo very rapid conformational changes to "Intermediates" (Kato et al., 1981). Then, interconversions among the "Intermediates" lead to a preequilibrium distribution of these species; at the same time, the native conformation is attained through many possible pathways (rate-limiting steps) that can involve relatively fast and/or slow folding processes.

In this paper, we obtain data for the rate of formation of fully regenerated RNase A at various concentrations of GSSG and GSH, as was done to some extent by Hantgan et al. (1974) and Ahmed et al. (1975), and show that the preequilibrium treatment provides information about the rate-limiting steps, i.e., about the folding pathway(s). In paper 5, we analyze the

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[1] Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; RNase A, bovine pancreatic ribonuclease A; BPTI, bovine pancreatic trypsin inhibitor; *l*S*m*G*n*H and *l*S*m*G*n*H*, intermediate prior to and after the rate-limiting steps, respectively, with *l* cystine residues, *m* mixed disulfide bonds between half-cystine and glutathione, and *n* free cysteine residues (if *l*, *m*, or *n* is equal to 0, *l*S, *m*G, or *n*H is omitted, e.g., 8H or 4S); Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; CMC, carboxymethylcellulose.

[2] "Intermediates" and "Intermediates*" represent the intermediates prior to and after, respectively, the rate-limiting steps in the regeneration pathway of RNase A. The fully reduced protein is included in the "Intermediates". As shown in paper 3 (Konishi et al., 1981), there are 7192 intermediates on purely statistical grounds; undoubtedly, some of them involve steric hindrance and thus cannot exist.

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energetics of the preequilibrium (Konishi et al., 1982a). Elsewhere, we discuss the general types of regeneration pathways that can be deduced from the preequilibrium, kinetic, and energetic analyses (Konishi et al., 1982b).

Experimental Procedures

The materials and methods used were those described in previous papers (Konishi & Scheraga, 1980a; Konishi et al., 1981).

Regeneration of RNase A at Various Concentrations of Reduced and Oxidized Glutathiones. Fully reduced RNase A (7.3×10^{-5} M) (Konishi & Scheraga, 1980a) was (partially) regenerated at various concentrations of reduced glutathione (0.67–40 mM) and oxidized glutathione (0.40–24 mM) at 22 °C for an appropriate time (>50 min) at pH 8.2. Then, an aliquot (20 μL) of the reaction solution was added to 2 mL of cytidine 2′,3′-phosphate (0.49 mM) in 0.1 M Tris–acetate buffer (pH 5.0). The enzymatic activity of the protein was measured by following the change in the optical density at 286 nm (Crook et al., 1960; Konishi & Scheraga, 1980a) with a Union Model SM-501 spectrophotometer. Because we observed no change in the enzymatic activity of the protein during the assay at pH 5.0 for 10 min, the observed enzymatic activity quantitatively reflects the enzymatic activity in the original reaction mixture. As will be shown later, some of the "Intermediates" have a small amount of enzymatic activity, but it is negligible compared to the measured activity; hence, the activity of the "Intermediates" is ignored when using the results of the activity measurements to deduce the distribution among the "Intermediates". Further, as described elsewhere (Konishi et al., 1982b), a species (3S2H*) with a nativelike conformation and three correct disulfide bonds, which is classified as an "Intermediate*", accumulates at a *low* concentration of GSSG (i.e., 0.2 mM), with [GSH] = 0 (Creighton, 1979, 1980); it does not, however, accumulate at higher concentrations of glutathiones, i.e., at [GSH] = [GSSG] = 0.5 mM (Konishi & Scheraga, 1980a), and hence does not accumulate under our experimental conditions ([GSH] = 0.67–40 mM and [GSSG] = 0.40–24 mM). Therefore, the observed enzymatic activities represent the amount of fully regenerated RNase A in the regeneration reaction solution at the time that the reaction was stopped because almost all of the enzymatic activity in the protein arises from fully regenerated RNase A.

Regeneration from Isolated Intermediates. As will be shown below, intermediate 4S or 3S1G1H may be involved in the rate-limiting step. In order to decide whether they are, we isolated these two intermediates; this was accomplished by choosing the proper concentrations of GSH and GSSG ([GSH] = 0.55 mM and [GSSG] = 2.8 mM for 4S and [GSH] = 2.2 mM and [GSSG] = 2.6 mM for 3S1G1H) for treatment of 8H, by arresting the regeneration mixture by lowering the pH to ~3.5, then by desalting on Sephadex G-25, and finally by fractionating the mixture on a CMC column, as described previously (Konishi et al., 1981). Use of this procedure and variants of these conditions to isolate other intermediates that may be involved in rate-limiting steps was unsuccessful (Konishi et al., 1981). The purities of intermediates 4S and 3S1G1H were calculated from the apparent equilibrium constants (and associated curve-fitting technique) as 97% (3% of 3S2H impurity) and 87% (11% of 2S1G3H and 2% of 1S1G5H impurities), respectively (Konishi et al., 1981).

To check that the unidentified fraction F in paper 3 (Konishi et al., 1981) was *not* involved in a rate-limiting step, we also prepared this material. It was obtained by regeneration from 8H (7.3×10^{-5} M), with [GSH] = [GSSG] = 2.2 mM at pH 8.2 and 22 °C for 2 min, and fractionation of the protein on a CMC column.

The regeneration reaction was started from isolated intermediate 4S or 3S1G1H with [GSH] = 2.7 mM and [GSSG] = 1.9 mM at pH 8.2 and 22 °C in the presence of about 1 mM ethylenediaminetetraacetic acid and was stopped at appropriate times (≤60 s) by lowering the pH to 3–4 with 2 M acetic acid. The material was then desalted on Sephadex G-25 and lyophilized from 0.1 M acetic acid. The regain of fully regenerated RNase A was measured by its enzymatic activity; 0.1 mL of cytidine 2′,3′-phosphate (9.8 mM) in 0.1 M Tris–acetate buffer (pH 5.0) was added to 2.0 mL of the protein (3×10^{-6}–9×10^{-5} M) in 0.1 M Tris–acetate buffer (pH 5.0), and the change in the optical density at 286 nm was followed [the enzymatically active species in these samples was less than 1%, so that the enzymatically active species [$<(0.3–9) \times 10^{-7}$ M] was saturated with substrate (~0.5 mM)].

Kinetic Analysis

(*A*) *"Intermediates" and "Intermediates*".* In this paper, we define the rate-limiting step as the slowest step in the regeneration of the protein. However, the slow steps from the mostly regenerated protein to the fully regenerated protein, which may possibly occur under certain conditions, are defined as slow steps *after* the rate-limiting step. This definition is adopted because we are interested in how the entire protein molecule is regenerated but not in the local changes of conformation in the passage from the mostly regenerated species to the fully regenerated protein.

With this definition, the intermediates in the regeneration pathways can be classified into two groups. One includes the reduced protein and the intermediates that accumulate prior to the rate-limiting step (designated as "Intermediates" in this paper). These "Intermediates" exist in a preequilibrium (Konishi et al., 1981). The other includes the intermediates that appear after the rate-limiting step (designated as "Intermediates*" in this paper). These "Intermediates*" usually do not accumulate to any great extent and are rapidly converted into the fully regenerated protein [in some cases, "Intermediates*" are accumulated if the subsequent steps in the pathway are slow; see Konishi et al. (1982b)]. Since the conformations of the accumulated "Intermediates" are mostly disordered (Creighton, 1979; Konishi & Scheraga, 1980a,b; Galat et al., 1981), the observed rate-limiting steps may involve alterations in conformation in folding to the fully regenerated protein. Presumably the conformations of some "Intermediates*" are close to the native one. The "Intermediates" and "Intermediates*" are represented schematically in Figure 1. The "Intermediates" and their interconversions are the same as those of Figure 1 in paper 3 (Konishi et al., 1981). Since we have little knowledge about the "Intermediates*" because their concentrations, in general, are too low to be measured, their interconversions are expressed in a similar way as those for the "Intermediates", but with asterisks. In general, the "Intermediates" and "Intermediates*", e.g., 4S and 4S*, differ in their distributions of four disulfide bonds and/or conformation. Fully regenerated RNase A is produced from 3S1G1H*, which is the last step in all regeneration pathways according to the possible reactions among the intermediates and fully regenerated RNase A [reactions 1 and 2 in paper 3 of this series (Konishi et al., 1981)]. Furthermore, since the unfolding reactions from fully regenerated RNase A to "Intermediates" could not be induced to any significant extent by glutathiones at pH 8 and 22 °C

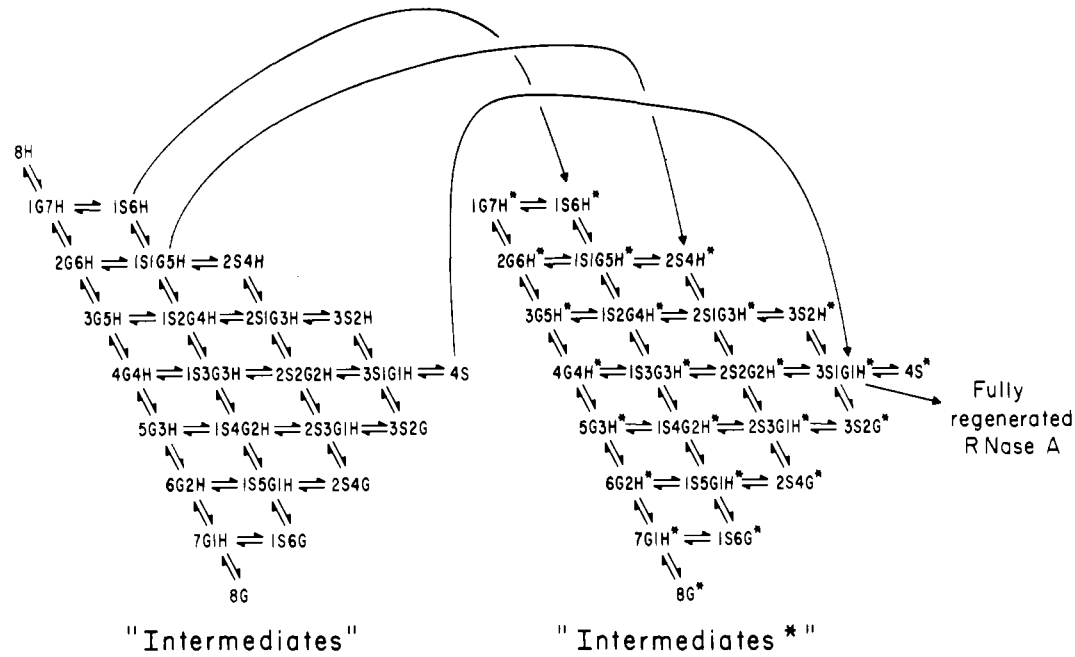

FIGURE 1: Representation of the "Intermediates" prior to the rate-limiting steps (left side) and the "Intermediates*" (designated by asterisks) after the rate-limiting steps (right side). All possible interconversions among the intermediates are represented and correspond to reaction 1 or 2 in paper 3 (vertical and horizontal arrows, respectively). Corresponding intermediates, with and without asterisks, differ in the location of the *l* disulfide bonds, the *m* intermolecular disulfide bonds with glutathione, and the *n* free sulfhydryl groups. Three examples of pathways are shown, with the connection of the two species in each pathway corresponding to the rate-limiting steps. Species 8H appears only before, and not after, the rate-limiting step. All species must go to fully regenerated RNase A through intermediate 3S1G1H* by means of reaction 2 of paper 3.

[i.e., incubation of native RNase A (7.3×10^{-5} M) with an excess of GSH (44 mM) at pH 8 and 22 °C for 2 weeks[3] led to no detectable reduced protein], we ignore the back reactions from the "Intermediates*" to the "Intermediates". In other words, each pathway contains a single rate-limiting step from an "Intermediate" to an "Intermediate*". Three examples of rate-limiting steps are shown in Figure 1. One is accompanied by a reshuffling of a disulfide bond (1S6H → 1S6H*), a second involves the formation of a disulfide bond (1S1G5H → 2S4H* + GSH), and a third involves the cleavage of a disulfide bond (4S + GSH → 3S1G1H*).

(*B*) *Equilibrium and Kinetic Constants.* There is a very large number of possible reactions among the "Intermediates". For example, in the interconversions

$$1G7H \rightarrow 1S6H + GSH \quad (1)$$

and

$$1S6H + GSH \rightarrow 1G7H \quad (2)$$

there are 8 possible species 1G7H, each of which has a mixed disulfide bond between a different half-cystine and glutathione, and 28 possible species 1S6H. Each 1G7H species produces seven possible kinds of 1S6H, and each 1S6H species produces two possible kinds of 1G7H. Therefore, the above interconversions involve an ensemble of 56 (=7 × 8 or 2 × 28) possible reactions (of the types of reactions 1 and 2 above), and the *apparent* equilibrium constant is

$$K_{1G7H:1S6H} = [1S6H][GSH]/[1G7H] \quad (3)$$

There is also a very large number of possible rate-limiting steps, i.e., possible reactions from each "Intermediate" to each "Intermediate*". For example, in the reaction

$$1G7H \rightarrow 1S6H^* + GSH \quad (4)$$

the eight possible species 1G7H can each produce seven possible species 1S6H*. Thus, reaction 4 represents an ensemble of 56 (=8 × 7) possible reactions. Therefore, it is necessary to express the rate-limiting steps in a simpler form. The rate of formation of fully regenerated RNase A, i.e., N, through rate-limiting step 4 is

$$dN/dt = \sum_{i=1}^{56} k_i[1G7H_i] \quad (5)$$

where k_i is the rate constant for reaction of the ith species. We simplify this equation as

$$dN/dt = k_{1G7H:1S6H^*}[1G7H] \quad (6)$$

where [1G7H] is the total concentration of the eight possible 1G7H species and $k_{1G7H:1S6H^*}$ ($=\sum_{i=1}^{56} k_i[1G7H_i]/[1G7H]$) is the *apparent* rate constant for rate-limiting step 4. The rate of formation of fully regenerated RNase A from all of the possible rate-limiting steps can then be expressed as a sum of 90 terms like the right-hand side of eq 6 (the rate-limiting step 1G7H + GSH → 8H* + GSSG is excluded, because 8H is the starting material and is a single species as is native RNase A). The 90 possible rate-limiting steps are all indicated by arrows in Figure 2; each arrow represents a reaction from an "Intermediate" to the corresponding "Intermediate*".

In order to simplify the analysis even further, we reduce the 90 terms to 27, as follows. Consider, as an example, the rate constants for the following five rate-limiting steps.

$$2S2G2H \rightarrow 2S2G2H^* \quad (7)$$

$$2S2G2H \rightarrow 3S1G1H^* + GSH \quad (8)$$

$$3S1G1H + GSH \rightarrow 2S2G2H^* \quad (9)$$

$$3S2H + GSSG \rightarrow 3S1G1H^* + GSH \quad (10)$$

$$3S1G1H + GSH \rightarrow 3S2H^* + GSSG \quad (11)$$

With the help of the equilibrium constants in Figure 1 of paper

[3] Even though native RNase A is thermodynamically unstable in excess GSH, as will be seen in Figure 4 of paper 5 (Konishi et al., 1982a), the *rate* of reduction is too slow to detect any reduced protein in 2 weeks.

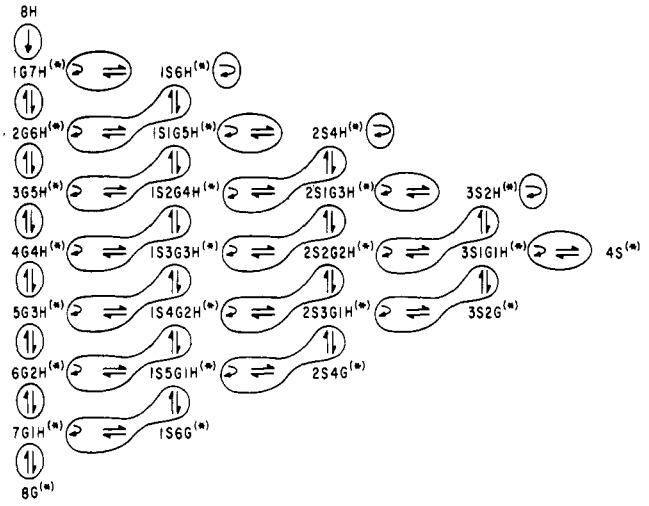

FIGURE 2: The 90 possible rate-limiting steps. For example, 1G7H(*) $\rightleftharpoons$ 1S6H(*) represents the two possible reactions, 1G7H $\rightarrow$ 1S6H* + GSH and 1S6H + GSH $\rightarrow$ 1G7H*, and the curved arrows represent reshuffling reactions *within* the given species. Each closed contour, which may surround one, two, three, or five possible rate-limiting steps, corresponds to one term on the right-hand side of eq 19; there are 27 such closed contours.

3 (Konishi et al., 1981), the individual steps and their rates may be written as

$$dN/dt = k_{2S2G2H:2S2G2H^*}[2S2G2H] = k_{2S2G2H:2S2G2H^*}K_{3S1G1H:2S2G2H}[3S1G1H][GSH] \quad (12)$$

for reaction 7 because

$$K_{3S1G1H:2S2G2H} = [2S2G2H]/([3S1G1H][GSH]) \quad (13)$$

at preequilibrium. Similarly, for reaction 8

$$dN/dt = k_{2S2G2H:3S1G1H^*}[2S2G2H] = k_{2S2G2H:3S1G1H^*}K_{3S1G1H:2S2G2H}[3S1G1H][GSH] \quad (14)$$

For reaction 9

$$dN/dt = k_{3S1G1H:2S2G2H^*}[3S1G1H][GSH] \quad (15)$$

For reaction 10

$$dN/dt = k_{3S2H:3S1G1H^*}[3S2H][GSSG] = k_{3S2H:3S1G1H^*}K_{3S1G1H:3S2H}[3S1G1H][GSH] \quad (16)$$

For reaction 11

$$dN/dt = k_{3S1G1H:3S2H^*}[3S1G1H][GSH] \quad (17)$$

Therefore, the formation of fully regenerated RNase A through the five pathways having the rate-limiting steps 7–11 is

$$dN/dt = (k_{2S2G2H:2S2G2H^*}K_{3S1G1H:2S2G2H} + k_{2S2G2H:3S1G1H^*}K_{3S1G1H:2S2G2H} + k_{3S1G1H:2S2G2H^*} + k_{3S2H:3S1G1H^*}K_{3S1G1H:3S2H} + k_{3S1G1H:3S2H^*})[3S1G1H][GSH] = k_{3S1G1H}[3S1G1H][GSH] \quad (18)$$

where k_{3S1G1H} is defined as the sum of the constants in the parentheses in the previous equation, and we regard it as an apparent rate constant in this paper. Thus, the constants for five reactions have been reduced to a single one. In some cases, the constants for three reactions are reduced to a single one. In summary, the constants for 90 reactions are reduced to 27, as shown by closed contours in Figure 2, and the formation of fully regenerated RNase A is expressed as

$$dN/dt = \sum_{i=1}^{24} k_i[I_i][GSH] + \sum_{i=1}^{3} k_i[I_i] \quad (19)$$

where k_i and I_i are the rate constant and "Intermediate", respectively, for the rate-limiting step in the ith pathway. The last term on the right-hand side of eq 19 corresponds to the intramolecular reshuffling of disulfide bonds from 1S6H, 2S4H, and 3S2H to 1S6H*, 2S4H*, and 3S2H*, respectively. The remaining 24 closed contours in Figure 2 correspond to the first term on the right-hand side of eq 19; e.g., eq 18 is a component of eq 19, and k_{3S1G1H} and 3S1G1H correspond to k_i and I_i, respectively (even though GSSG appears in reactions such as 10, it is eliminated from eq 16 and hence does not appear in eq 19).

Since the concentrations of the "Intermediates*" are close to zero, $[I_i]$ may be expressed as $f_i(1 - N)$, where f_i is the fraction of "Intermediate" I_i among the total "Intermediates" (and is independent of the regeneration time at preequilibrium) and N is the fraction of fully regenerated RNase A in the *whole* protein mixture; in other words, $1 - N$ is the fraction of total "Intermediates" in the whole protein mixture. Therefore

$$dN/dt = \sum_{i=1}^{24} k_i f_i(1 - N)[GSH] + \sum_{i=1}^{3} k_i f_i(1 - N) \quad (20)$$

There are no cross terms in these sums because the back reactions from "Intermediates*" to "Intermediates" are neglected. Since the preequilibrium state is attained rapidly (Konishi et al., 1981) and the small dependence of [GSH] on the regeneration reaction time after the preequilibrium condition is achieved can be neglected, we may integrate to obtain

$$-\ln(1 - N) = \sum_{i=1}^{24} k_i f_i[GSH]t + \sum_{i=1}^{3} k_i f_i t \quad (21)$$

The fraction f_i of an "Intermediate" *l*S*m*G*n*H can be expressed in terms of the equilibrium constants and the mole fractions of glutathiones at preequilibrium (which is attained rapidly in the regeneration) as follows.

$$8H + (l + m)GSSG \rightleftarrows lSmGnH + (2l + m)GSH \quad (22)$$

$$K_i = ([lSmGnH][GSH]^{2l+m})/([8H][GSSG]^{l+m}) \quad (23)$$

$$f_i = [lSmGnH]/[\text{"Intermediates"}] = K_i[8H][GSSG]^{l+m}/([\text{"Intermediates"}][GSH]^{2l+m}) = K_i f_{8H}[GSH]^{-(2l+m)}[GSSG]^{l+m} \quad (24)$$

where $f_{8H} = [8H]/[\text{"Intermediates"}]$, there being 25 such "Intermediates" including fully reduced RNase A (see Figure 1). When the f_i's are normalized so that $\sum_{i=1}^{25} f_i = 1.0$, f_{8H} may be expressed in terms of the equilibrium constants and the concentrations of GSH and GSSG.

Results

Starting with fully reduced RNase A, we carried out 193 regeneration experiments, each with different concentrations of reduced and oxidized glutathiones, as described under Experimental Procedures. The amounts of fully regenerated RNase A produced at a certain time (>50 min) of regeneration were measured and used to estimate the apparent kinetic constants and apparent equilibrium constants, as follows.

In one type of calculation, the equilibrium constants were fixed at those derived from Figure 1 of paper 3 (Konishi et al., 1981), viz., as redefined in eq 23, and the 27 values of k_i were obtained by minimizing the difference between the observed and calculated values of $-\ln(1 - N)$ (see Experimental Procedures and eq 21). We tried 20 different sets of initial estimates of the 27 values of k_i. Some of these sets led to different solutions (local minima) because of the multiple-minima problem. However, the average deviation of the fit

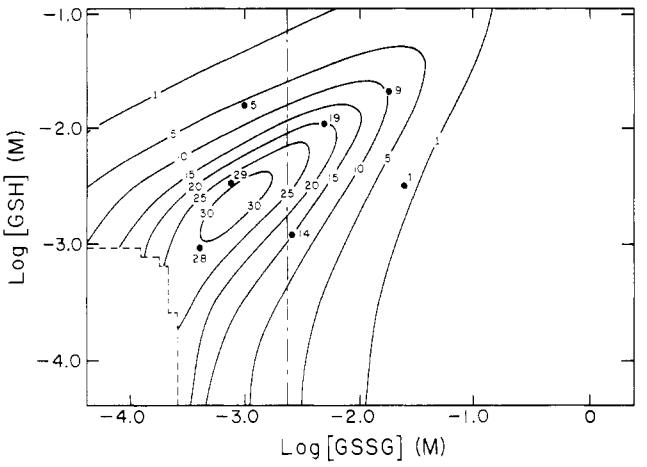

FIGURE 3: The curves represent the fraction (%) of fully regenerated RNase A produced by the reaction of reduced RNase A (7.3×10^{-5} M) with initial concentrations of GSH of 4.2×10^{-5}–0.11 M and of GSSG of 4.2×10^{-5}–2.3 M at pH 8.2 and 22 °C for 60 min, as calculated (with eq 21) from the relative populations of the intermediates in preequilibrium (Konishi et al., 1981) and the apparent kinetic constants of all possible rate-limiting steps (Table I) (those apparent kinetic constants not listed in Table I are too small to make any significant contribution). The total amount of RNase A (the "Intermediates" and fully regenerated species) was taken as 100%. The filled circles represent some of the experimental data (sampled arbitrarily from 193 experiments), i.e., observed enzymatic activity (%) relative to that of native RNase A at the given values of [GSH] and [GSSG]. In the region surrounded by the dashed lines (low values of [GSH] and [GSSG]), the distributions of intermediates at preequilibrium could not be obtained with the computer (Konishi et al., 1981). The line (---) of constant [GSSG] is the basis for drawing Figure 4.

$(\sum|\ln(1-N)_{\text{exptl}} - \ln(1-N)_{\text{eq 21}}|/193$, where the sum is taken over the 193 sets of data) was in the range of 0.00162–0.00163 for the four best fitted minimizations. Since further trials failed to reduce this deviation below 0.00162–0.00163, the amounts of fully regenerated RNase A at various concentrations of GSH and GSSG at pH 8.2 and 22 °C were calculated from eq 21 by using one of the four best fitted sets of values of k_i's and are shown in Figure 3, in which the regeneration time is taken as 60 min. The optimum production of fully regenerated RNase A (i.e., 32%) at a 60-min regeneration time under the above conditions is obtained when reduced RNase A (7.3×10^{-5} M) is oxidized by [GSH] = 2.3 mM and [GSSG] = 0.77 mM at pH 8.2 and 22 °C.

The amount of fully regenerated RNase A produced in 60 min through the ith pathway, N_i, was computed from eq 20 as

$$N_i = N\frac{(dN/dt)_i}{(dN/dt)_{\text{total}}} = N\frac{k_i f_i[\text{GSH}] \text{ or } k_i f_i}{\sum_{i=1}^{24} k_i f_i[\text{GSH}] + \sum_{i=1}^{3} k_i f_i} \quad (25)$$

where $(dN/dt)_i$ ($=k_i f_i[\text{GSH}]$ or $k_i f_i$) and $(dN/dt)_{\text{total}}$ (=right side of eq 20) are the rates of formation of fully regenerated RNase A through the ith pathway and all possible pathways, respectively. It should be noted that N represents the fully regenerated, and *not* the total, protein. Some of these results are shown in Figure 4, in which the initial concentration of GSSG is fixed at 2.3 mM and the value of N_i at t = 60 min is plotted as a function of [GSH]. Six possible pathways, "1S6H", "1S1G5H", "2S1G3H", "2S2G2H", "3S2H", and "3S1G1H", each of which produces more than 0.1% of fully regenerated RNase A in a 60-min regeneration time, are shown. These pathways are also observed at other concentrations of GSH (4.2×10^{-5}–0.11 M) and GSSG (4.2×10^{-5}–2.3 M). Figure 5 shows the values of N_i for some of the

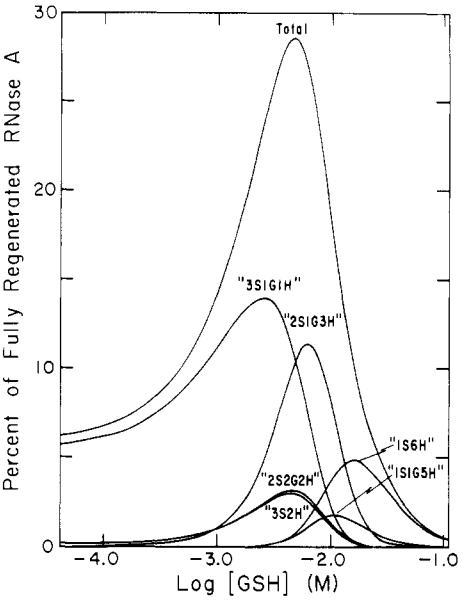

FIGURE 4: Calculated fraction (%) of fully regenerated RNase A produced by reaction of reduced RNase A (7.3×10^{-5} M) with initial concentrations of GSH of 4.2×10^{-5}–0.11 M and fixed concentration of GSSG (=2.3 mM) at pH 8.2 and 22 °C for 60 min. The curve labeled total pertains to the line (---) of Figure 3. The other curves labeled "1S6H", "1S1G5H", "2S1G3H", "2S2G2H", "3S2H", and "3S1G1H" pertain to the amount of fully regenerated RNase A produced in the specific pathways described in Table I.

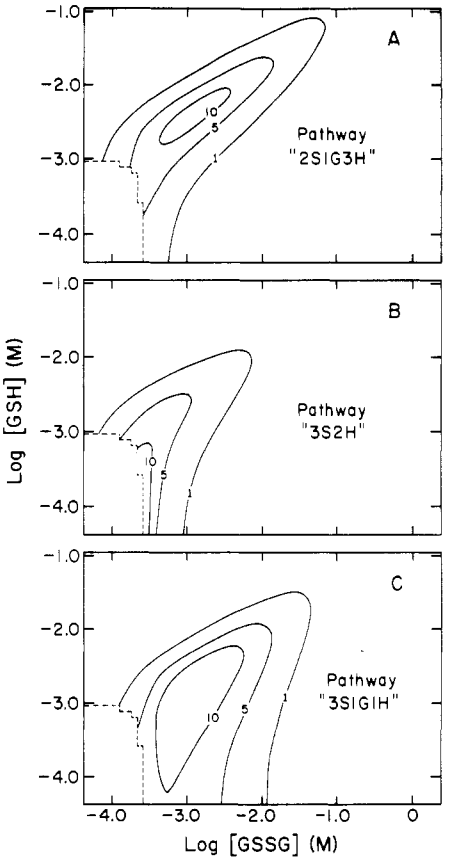

FIGURE 5: Calculated fraction (%) of fully regenerated RNase A produced through three different pathways under the same reaction conditions as those in Figure 3. The dashed lines have the same meaning as that in Figure 3.

pathways ("2S1G3H", "3S2H", and "3S1G1H") at t = 60 min under the same conditions as in Figure 3. No pathways, other than those shown in Table I, were found in this analysis to

Table 1: Regeneration Pathways

pathway[a]	rate-limiting reactions[b]	apparent rate constant (s^{-1})
"1S6H"	1S6H → 1S6H*	7.7×10^{-5} [c]
"1S1G5H"	1S1G5H → 1S1G5H* 1S1G5H → 2S4H* + GSH 2S4H + GSH → 1S1G5H*	0.72[d]
"2S1G3H"	2S1G3H → 2S1G3H* 2S1G3H → 3S2H* + GSH 3S2H + GSH → 2S1G3H*	7.1[d]
"2S2G2H"	2S2G2H → 2S2G2H* 2S2G2H → 3S1G1H* + GSH 3S1G1H + GSH → 2S2G2H* 3S1G1H + GSH → 3S2H* + GSSG 3S2H + GSSG → 3S1G1H* + GSH	0.69[d]
"3S2H"	3S2H → 3S2H*	1.9×10^{-4} [c]
"3S1G1H"	3S1G1H → 3S1G1H* 3S1G1H → 4S* + GSH 4S + GSH → 3S1G1H*	4.0[d]

[a] Each pathway is expressed in terms of the most reduced "Intermediate" involved in the rate-limiting steps. [b] Possible rate-limiting reactions in each pathway. [c] The rate constant corresponds to that in the second term on the right-hand side of eq 21. [d] The rate constant corresponds to that in the first term on the right-hand side of eq 21. The concentration unit of GSH does not appear because it is expressed as mole fraction.

produce significant amounts (i.e., more than 0.1%) of fully regenerated RNase A in a 60-min regeneration time at pH 8.2 and 22 °C with GSH (4.2×10^{-5}–0.11 M) and GSSG (4.2×10^{-5}–2.3 M); i.e., the values of k_i for these other pathways were smaller than those shown in Table I and are not listed (the values for these very small k_i's being less reliable than the larger ones). The values of k_i in Table I are approximate because of the multiple-minima problem in the minimization (and it was not possible to estimate the errors in the computed rate constants), but each of the pathways listed in Table I produced fully regenerated RNase A in most of the 20 minimizations.

We also tried a second type of minimization in which the 27 k_i's and 24 K_i's (which relate 25 "Intermediates", as shown in eq 23) were parameters to be fitted, because some of the K_i's had been obtained by extrapolation in paper 3 (Konishi et al., 1981). The regeneration data could not be fitted by any *single* pathway, indicating that RNase A is regenerated from the reduced protein through many pathways. Although we could not obtain the same minimized results in 20 trials with different sets of initial values of k_i and K_i because of the multiple-minima problem in this second type of minimization, the 27 fitted values of k_i and 24 fitted values of K_i were approximately the same as those in Table I and in Figure 1 of paper 3, respectively.

Equation 25 shows that the preference for a given pathway depends on k_i, the apparent rate constant of the rate-limiting step (Table I), on f_i, which reflects the relative stability of the intermediate taking part in the rate-limiting step, and on the concentrations of glutathiones. In other words, none of these factors, by itself, determines the dominant pathway. Thus, pathway "2S1G3H" with the highest apparent rate constant (Table I) is not the dominant one under some solution conditions. Figure 4 shows that pathway "1S6H" would predominate under reductive conditions ([GSH] > ~14 mM), pathway "2S1G3H" would predominate under relatively oxidative conditions ([GSH] = 5–14 mM), and pathway "3S1G1H" would be the dominant one under highly oxidative conditions ([GSH] < 5 mM), when [GSSG] is fixed at 2.3 mM. Reductive and oxidative conditions are defined by the concentrations of GSH and GSSG, e.g., high and low values of [GSH] corresponding to reductive and oxidative conditions, respectively, at a fixed concentration of GSSG (Figure 4).

In order to confirm these conclusions about the dominant pathways, we isolated intermediates 3S1G1H and 4S in pure form (87% and 97%, respectively), as described under Experimental Procedures. The regeneration reaction was started from the isolated 3S1G1H or 4S with [GSH] = 2.7 mM and [GSSG] = 1.7 mM (for ≤60 s). If regeneration is started from an isolated "Intermediate" that takes part in a rate-limiting step, two simultaneous processes occur, viz., (1) the rate-limiting reaction to regenerate the native protein and (2) the set of reactions leading to other "Intermediates" to achieve the preequilibrium condition. As a result, regeneration of the native protein would be rapid in the early stages of the reaction, because of the high concentration of the intermediate that takes part in the rate-limiting step, and then would slow down gradually, because of the decrease in the concentration of the intermediate as it forms other "Intermediates". On the contrary, when regeneration is started from an isolated "Intermediate" that does not take part in the rate-limiting step, the regeneration of RNase A would be slow in the initial stages of the reaction, because of the absence of the intermediate that takes part in the rate-limiting step, and would gradually become more rapid because of the formation of the intermediate (that takes part in the rate-limiting step) from the starting intermediate; i.e., the time course of the regeneration of RNase A would be sigmoidal.

The regeneration reaction (starting from 3S1G1H or 4S) was followed by measurements of enzymatic activity. Some isolated intermediates showed small amounts of enzymatic activity against cytidine 2′,3′-phosphate; i.e., the measured enzymatic activities of isolated 4S, 3S1G1H, and 3S2G[4] were 0.080%, 0.33%, and 0.14%, respectively, relative to that of native RNase A.[5] By ignoring the enzymatic activities of the intermediates with one or two disulfide bonds, and arbitrarily

[4] 3S2G was isolated according to the procedure of paper 3 (Konishi et al., 1981), i.e., with [GSH] = 2.2 mM and [GSSG] = 2.6 mM. The purity of intermediate 3S2G was calculated from the apparent equilibrium constants (and associated curve-fitting technique) as 98% (2% of 3S1G1H impurity) (Konishi et al., 1981). Since it was not involved in the rate-limiting steps (Table I), it was not used in these studies of the dominant pathways.

[5] The enzymatic activity of these intermediates is not due to contamination by fully regenerated RNase A because the isolation procedure on the CMC column was repeated twice to eliminate native RNase A. Creighton (1979) observed enzymatic activities of 0.13–0.16% for his peak III material with three disulfide bonds and 5.1% for his peak IV material in which the major components were the intermediates with four disulfide bonds (i.e., 4S). The high enzymatic activity of the peak IV material was probably due to the appearance of species III_n with a nativelike conformation (Creighton, 1979) in peak IV. This overlap of III_n and IV was due to two different factors that decelerated and accelerated, respectively, the movement of III_n on the CMC column. The first factor, the nativelike conformation of III_n, delayed its elution compared to that of peak III (similarly, native RNase A elutes after 4S because of the difference in conformation). However, while III_n eluted (later) with peak IV, it did not elute still later with native RNase A because of the second factor; i.e., the two extra negative charges on carboxymethylated cysteine residues led to earlier elution (compared to that of native RNase A) (Konishi et al., 1981). Since we did not carboxymethylate our intermediates, the mobility of our species 3S2H*, corresponding to Creighton's III_n, on CMC was not influenced by the second factor; thus, 3S2H* is separable from 4S. Actually, a low (intrinsic) enzymatic activity (0.08%) was observed for the isolated 4S, but it was not due to III_n. We did not use Creighton's conditions to increase the concentration of 3S2H* because it would have eluted with (and hence not be separable from) native RNase A. Under our conditions, essentially no 3S2H* accumulated; hence, the peak corresponding to native RNase A was not contaminated with 3S2H*.

assigning an enzymatic activity of 0.47% (the sum of the activities of 3S1G1H and 3S2G) to 3S2H, which could not be isolated, the enzymatic activity inherent in the intermediates was subtracted from the observed enzymatic activity of the reaction mixture (<1%) by using the kinetic data of Table II in paper 3 (Konishi et al., 1981). When the reaction was started from 4S, the regeneration of RNase A was initially fast and then slowed down; when the reaction was started from 3S1G1H, the time course of the regeneration of RNase A was sigmoidal. In light of the discussion in the previous paragraph, these results suggest that intermediate 4S takes part in the rate-limiting step and that intermediate 3S1G1H probably does not. Therefore, the rate-limiting steps in Table I are likely to be 2S2G2H → 2S2G2H*, 2S2G2H → 3S1G1H* + GSH or 3S2H + GSSG → 3S1G1H* + GSH and 4S + GSH → 3S1G1H* for pathways "2S2G2H" and "3S1G1H", respectively. Similar experiments could not be carried out for pathways "1S6H", "1S1G5H", "2S1G3H", and "3S2H" because those intermediates could not be isolated by CMC column chromatography (Konishi et al., 1981).

As a check on this result, an experiment was carried out under similar oxidative conditions for 60 s but starting with isolated fraction F. The enzymatic activity of this starting material was 0.17%, and it did not *increase* in the 60-s regeneration time. Hence, the unidentified intermediate F is not involved in a rate-limiting step in the regeneration pathways.

Discussion

The pathway for regeneration of a native protein would be expected to follow the potential energy surface from the denatured form to the native form. Since many factors such as temperature, pressure, pH, ionic strength, neutral ions, urea, etc. may influence the potential energies of the intermediates along the pathway, the regeneration pathway is probably not a unique one. Instead, there may be many pathways depending on the solution conditions (Scheraga, 1980). Each step in the pathway for regeneration of RNase A from the reduced protein by glutathiones was expressed in terms of two types of chemical reactions (eq 1 and 2 in paper 3) and, thereby, all possible regeneration pathways could be expressed in terms of these two types of reactions. The contributions of each pathway to produce fully regenerated RNase A have been analyzed. The results are consistent with a multipathway treatment and demonstrate that all the possible pathways are not equivalent and that RNase A is regenerated through some preferred pathways such as those given in Table I. We have found that intermediates with a large number of bound glutathiones are not involved in the rate-limiting steps. This may be due to the disturbance of the conformational folding of the protein by the bulkiness of many bound glutathiones.

A multipathway regeneration mechanism has also been reported for other protein folding systems. In BPTI, both of the intermediates, (30–51, 5–14) and (30–51, 5–38), take part in the rate-limiting step; i.e., this is a two-pathway system (Creighton, 1978). When reduced lysozyme is reoxidized by air, three intermediates are accumulated (Acharya & Taniuchi, 1976). They contain three correct disulfide bonds and two free cysteine residues (Cys-6 and Cys-127, Cys-76 and Cys-94, and Cys-64 and Cys-80). These intermediates show enzymatic activity of 40–50% of that of native lysozyme, indicating that they are "Intermediates*" (Konishi et al., 1982b). It seems reasonable to interpret these observations to mean that the intermediates are formed through at least three independent pathways rather than being formed one by one through a single pathway. Denatured proteins with intact disulfide bonds generally contain two kinds of species, i.e., slow-folding species and fast-folding species (Baldwin & Creighton, 1980), and their folding is interpreted elsewhere in terms of two types of pathways (Konishi et al., 1982b). In summary, proteins can generally be regenerated through multiple pathways.

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