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Regeneration of Ribonuclease A from the Reduced Protein. Isolation and Identification of Intermediates, and Equilibrium Treatment†

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ABSTRACT: Reduced RNase A was reoxidized, and the incorrectly formed disulfide bonds were reshuffled to the native ones by oxidized and reduced glutathiones, as described in the first paper of this series. The intermediates in the regeneration of the disulfide bonds were trapped without any chemical modification and were fractionated on a carboxymethylcellulose column at pH 3.5 with a salt gradient. The elution curves of the partially regenerated RNase A from the carboxymethylcellulose column were obtained by measurement of the absorption at 275 nm and by determination of the SH content (of cysteine residues) and consisted of 11 fractions, G8, G7, G6, G5, G4, G3, G2, G1, G0, N, and F. Some of the fractions were isolated, and their measured molecular weights were consistent with those of monomeric RNase A. Fraction F had a molecular weight between that of the monomer and dimer, so that this fraction could not be identified. The regeneration pathway could be represented in terms of two simple reactions, RNase A(–SH) + GSSG ⇄ RNase A(–SSG) + GSH and RNase A($\frac{-SH}{-SSG}$) ⇄ RNase A(>S_2) + GSH, which produced 24 monomeric intermediates (not counting the fully reduced and the native species), which differed from each other in their amino acid composition. These 24 intermediates, plus the fully reduced protein, were assigned to fractions G8–G0 (as indicated in the last column of Table I), with the aid of data from amino acid analysis, SH content, and the elution position on the carboxymethylcellulose column chromatogram. Since the regeneration reaction rapidly reached a preequilibrium among the intermediates and the fully reduced RNase A prior to the rate-limiting steps, i.e., the relative concentrations of the intermediates and fully reduced RNase A became constant with reaction time, the populations of some of the intermediates in preequilibrium were estimated by curve fitting of the elution pattern from the carboxymethylcellulose column chromatogram. The equilibrium constants among the intermediates were calculated from their populations at preequilibrium. These equilibrium constants were "extrapolated" to other intermediates whose populations could not be estimated by curve fitting, and the relative populations of all of the possible intermediates at preequilibrium were thereby represented as a function of the concentrations of reduced and oxidized glutathiones. The regeneration process was also restarted from several of the isolated intermediates, and the resulting distribution of intermediates was consistent with that from which the equilibrium constants were determined, supporting the representation of the regeneration pathways in terms of two simple reactions. Thus, the equilibrium treatment of the regeneration pathways was useful to characterize the preequilibrium state, i.e., to identify the intermediates prior to the rate-limiting steps in the pathways and to estimate their stabilities at preequilibrium at various concentrations of reduced and oxidized glutathiones.

This series of papers is concerned with the pathways for the regeneration of ribonuclease A (RNase A)[1] from the reduced protein by a mixture of oxidized and reduced glutathiones (GSSG and GSH, respectively). The material (partially regenerated RNase A) present during the reaction consists of many different kinds of intermediates and fully regenerated RNase A.

In the first two papers of this series, we analyzed the conformations of the active site (Konishi & Scheraga, 1980a), the backbone structures, and the environments of tyrosine residues (Konishi & Scheraga, 1980a) and the environments of histidine residues (Konishi & Scheraga, 1980b) for the presence of ordered intermediates. The results showed that

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[1] Abbreviations used: RNase A, bovine pancreatic ribonuclease A; CMC, carboxymethylcellulose; GSH, reduced glutathione; GSSG, oxidized glutathione; *k*S*m*G*n*H, an intermediate with *k* cystine residues, *m* mixed disulfide bonds between half-cysteines and GSH, and *n* free cysteine residues (if *k*, *m*, or *n* is equal to 0, *k*S, *m*G, or *n*H is omitted, e.g., 8H or 4S); $\bar{M}_{w,obsd}$, weight-average molecular weight, determined by sedimentation equilibrium; M_{calcd}, molecular weight of the intermediate present in each sample, computed from the amino acid composition (ignoring the difference of 1 in residue weight between cysteine and half-cystine); RNase A(–SH), RNase A(–SSG), and RNase A(>S_2) represent a cysteine residue, a half-cystine residue involved in a mixed disulfide bond with GSH, and a cystine residue, respectively, in RNase A; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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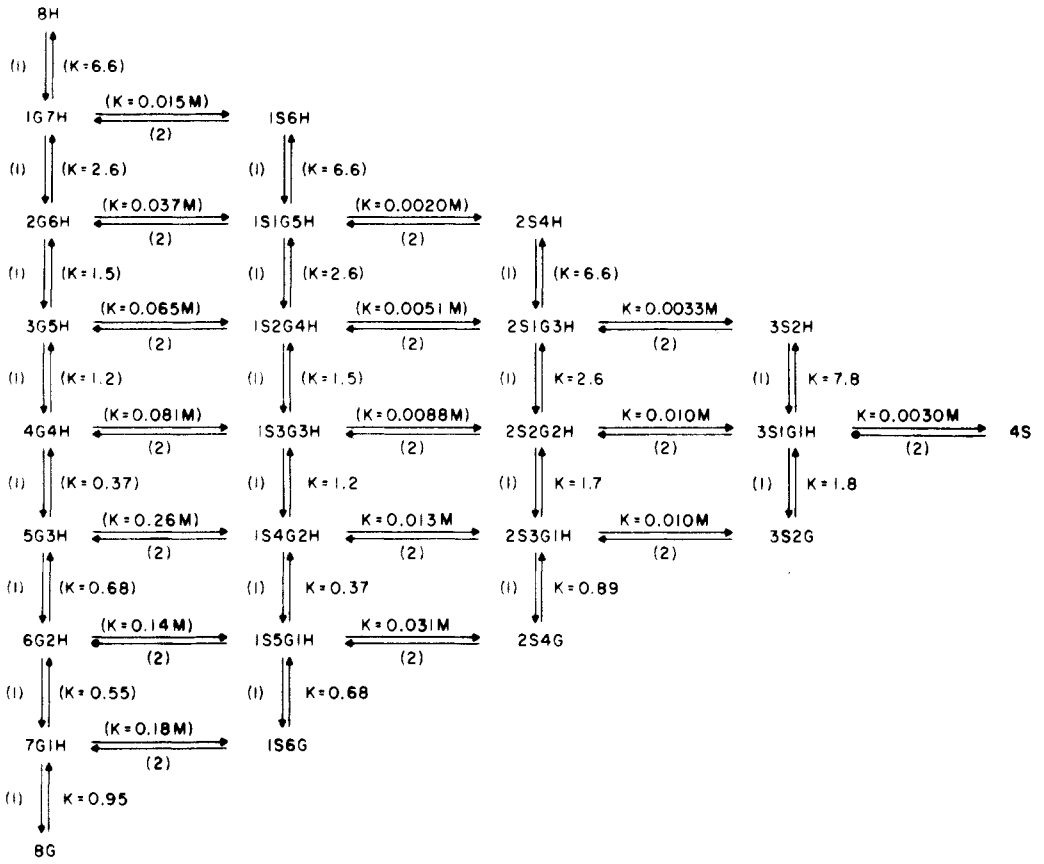


FIGURE 1: Representation of preequilibria among intermediates prior to rate-limiting steps in the regeneration of RNase A from the reduced protein, together with equilibrium constants for the various reactions at pH 7.8–8.2 and 22 °C; extrapolated equilibrium constants are enclosed in parentheses. The notation is that of footnote 1. (1) and (2) indicate that the process occurs by reactions 1 and 2, respectively, in the text.

the intermediates have predominantly disordered conformations. Although some of the data indicated the presence of specific conformations (Konishi & Scheraga, 1980a), they were present at too low concentrations to be identified. Since the presence of fully regenerated RNase A in the partially regenerated material could have influenced the results of the conformational analysis of the intermediates, it became necessary to fractionate and identify the intermediates. This fractionation is the subject of this paper. We also analyze the preequilibria among the intermediates, all of which are supposed to occur prior to the rate-limiting steps on the pathways toward fully regenerated RNase A.

In order to characterize the conformations of the intermediates, it is necessary to isolate them *without* modifying them chemically. In studying the regeneration of reduced RNase A, Creighton (1977, 1979) carboxymethylated the intermediates and fractionated and isolated them by electrophoresis and carboxymethylcellulose (CMC) column chromatography. Since carboxymethylation of the cysteine residues may, however, affect the conformation of the protein (Goto & Hamaguchi, 1979; Chavez & Scheraga, 1980b), we have developed a method to fractionate the intermediates without chemical modification.

All possible chemical reactions between reduced RNase A and oxidized and reduced glutathiones would lead to 7191 different intermediates (see Appendix). For example, there are 104 different intermediates with four intramolecular disulfide bonds (Sela & Lifson, 1959). The number 7191 is so large that it is expedient to divide the intermediates into groups. Creighton (1979) classified the intermediates by their average numbers of intramolecular disulfide bonds, i.e., as I, II, III, and IV. It is, however, possible to group them in an experimentally feasible manner which conveys more information about the nature of the intermediates, viz., on the basis of the *numbers* of intramolecular disulfide bonds, cysteine residues, and intermolecular disulfide bonds with glutathione. According to this classification, there are 24 different intermediates, as shown in Figure 1 (Y. Isogai, private communication); this is a more manageable number than 7191 to deal with experimentally.

A major goal in this research is the determination of the relative amounts of these 24 intermediates in the rapid preequilibria that exist prior to the rate-limiting steps in the formation of fully regenerated RNase A. The populations of these 24 intermediates are close to zero after the rate-limiting steps because they are formed slowly in the rate-limiting steps and are converted rapidly to fully regenerated RNase A. Thus, the intermediates after the rate-limiting steps are not studied in this paper.[2]

The regeneration pathway of RNase A from the reduced protein has been analyzed kinetically, by following the appearance of enzymatic activity, the change in OD_{287}, the change in fluorescence, the appearance of native disulfide bonds, the appearance of the native conformation, and the appearance of fractionated intermediates, as a function of regeneration time (Hantgan et al., 1974; Ahmed et al., 1975; Schaffer et al., 1975; Creighton, 1979; Chavez & Scheraga, 1980a; Konishi & Scheraga, 1980a,b). Creighton (1979), in particular, studied the early stages of the regeneration pathway and found that the pairing of half-cystines was primarily random in those intermediates that contained one intramo-

[2] After the rate-limiting steps, there is an analogue of Figure 1, but the compositions and relative amounts of the components of these intermediates differ; e.g., for intermediate 4S, there is a different distribution among the 104 possibilities before and after the rate-limiting step.

lecular disulfide bond and that the pairing was nonrandom in those intermediates that contained three or four disulfide bonds. Creighton (1977) also found that the relative amounts of the intermediates rapidly approach a preequilibrium. We, therefore, have developed a treatment of these preequilibria in this paper in order to characterize the distributions of species along the regeneration pathway.

Experimental Procedures

Materials. The same materials were used as in the first paper of this series (Konishi & Scheraga, 1980a).

Instruments. A Zeiss Model PMQII, a Cary Model 14, or a Union Model SM-501 spectrophotometer was used for absorption measurements. A PRIME 350 and a FACOM M160 AD computer were used for curve fitting and data analysis.

Partially Regenerated RNase A. Partially regenerated RNase A was prepared as described in the previous paper (Konishi & Scheraga, 1980a) except for the following change. Reduced RNase A was treated with *various* concentrations of GSH and GSSG in order to study the effect of the concentrations of glutathiones on the regeneration process at pH 7.8–8.2 and 22 °C. Regeneration reactions, starting from isolated intermediates, were carried out for 15 s under oxidative conditions, viz., [GSH] = 2.7 mM and [GSSG] = 1.9 mM at pH 7.8–8.2 and 22 °C.

Fractionation of Partially Regenerated RNase A. Since partially regenerated RNase A is a mixture of various kinds of intermediates and fully regenerated RNase A (Hantgan et al., 1974; Creighton, 1975, 1979; Konishi & Scheraga, 1980a,b), the material was fractionated, and the various components were isolated on a CMC column as follows. The pH of the regeneration reaction mixture was lowered from 7.8–8.2 to 3–4 by addition of glacial acetic acid in order to stop the reaction. This procedure does not disturb the distribution of cysteine or cystine residues or of mixed disulfide bonds with glutathione or the conformations in the intermediates (Konishi & Scheraga, 1980a). The solution was then desalted on a Sephadex G-25 column (equilibrated with 0.6% acetic acid) and lyophilized. About 20 mg of the lyophilized material was dissolved completely in 3.0 mL of 6 mM acetic acid, and then 2.0 mL of a solution (6 mM in acetic acid and 0.40 M in NaCl) was added to adjust the salt concentration to 0.16 M (pH 3.5) [in some cases, clear solutions could not be obtained by dissolving the lyophilized protein *directly* into 0.16 M NaCl/acetic acid (pH 3.5)]. The concentration of protein was determined by measurement of OD_{275}, using ϵ_{275} = 9300 M^{-1} cm^{-1}, because 275 nm is the isosbestic point for the intermediates and native RNase A (Hantgan et al., 1974; Konishi & Scheraga, 1980a).

The protein solution was premixed with 2–3 cm^3 of CMC resin (in order to avoid a local high concentration of protein on the CMC column because of the low solubility of partially regenerated RNase A in 0.16 M NaCl) and applied to the CMC column (1.1 × 30 cm), which had been equilibrated with 0.16 M NaCl/acetic acid (pH 3.50). Fractionation was achieved with a slightly sigmoidal salt gradient between 250 mL of 0.16 M NaCl/acetic acid (pH 3.50) and 250 mL of 0.40 M NaCl/acetic acid (pH 3.46), at a flow rate of ~0.5 mL/min. In some cases, the salt gradient was maintained between 0.12 and 0.36 M NaCl. The total yield of fractionated protein was 95 ± 4%. Therefore, it can be assumed that all intermediates and fully regenerated RNase A were eluted quantitatively from the column. In some cases, the intermediates were refractionated on the same CMC column.

SH Determination. The average number of sulfhydryl groups in each fraction was determined spectrophotometrically with Ellman's reagent (Ellman, 1959; Taniuchi, 1970). The analysis was performed at pH 8.0 by establishing the base line at 412 nm with 2 mL of 0.1 M (or 0.2 M) Tris-HCl buffer (pH 8.0) containing ~0.1 mM EDTA, which prevents Cu^{2+} ion catalysis of air oxidation in the sample and reference cells. Then 250 μL (or 1.0 mL) of 0.16 or 0.12 M NaCl/acetic acid (pH 3.5) was added to the pH 8 buffer in the reference cell, and 250 μL (or 1.0 mL) of each protein fraction (within 1 h of its elution from the CMC column) was added to the pH 8 buffer in the sample cell. The average number of SH groups in each fraction was estimated from the change in OD_{412}, before and after the addition of the RNase A solution, by using ϵ_{412} = 13 600 M^{-1} cm^{-1} for reduced 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959).

Amino Acid Analysis. The amino acid composition of each fraction [expressed as the number of residues per mole of RNase A, relative to alanine whose content was set equal to 12.00 (Smyth et al., 1963)] was determined with a Technicon TSM amino acid autoanalyzer as follows. About 0.5–1.0 mg of each lyophilized fraction was dissolved in 2 mL of 6 N HCl, degassed, and hydrolyzed at 110 °C for 24 h in vacuo. The amount of "amino acid" contamination was estimated by carrying a blank (containing all reagents except the RNase A derivatives) through the hydrolysis procedure. The loss of amino acids during hydrolysis was estimated from the hydrolysis of a sample of native or fully regenerated RNase A. Since bound glutathione contains one residue each of glutamic acid, glycine, and half-cystine, the number of glutathione molecules bound to each intermediate (i.e., fraction) was determined from the average of the excess numbers of these three residues over their content in native RNase A, after correcting for contamination and hydrolysis losses. The results in Table I are the averages of three or six measurements.

Molecular Weight Measurements. The weight-average molecular weights, $\bar{M}_{w,obsd}$, of the intermediates were determined by the conventional sedimentation equilibrium method using a Spinco Model E ultracentrifuge with interference optics. Each fraction was dissolved in 0.6% acetic acid, and a solution of 0.6% acetic acid/1 M KCl was added to obtain a final KCl concentration of 0.1 M. The protein solution was then dialyzed overnight at 4 °C against 0.6% acetic acid/0.1 M KCl (pH 2.78) to define the activities of the dialyzable components (Eisenberg, 1976). The dialyzate was used as the reference solvent in the ultracentrifuge cell for the measurements of molecular weight. The concentrations of the intermediates were 0.05–0.1% (w/v), and the measurements were made at 20 °C at a rotor speed of 24 630 rpm over a period of 24 h. A value of $\bar{v}$ = 0.682 mL/g for performic acid oxidized RNase A (Harrington & Schellman, 1956) was used for all of the intermediates.

Results

The oxidation of the cysteine residues of reduced RNase A by GSSG may be expressed schematically by two types of reactions

$$\text{RNase A(-SH)} + \text{GSSG} \rightleftharpoons \text{RNase A(-SSG)} + \text{GSH} \quad (1)$$

$$\text{RNase A}\begin{pmatrix}\text{-SH}\\ \text{-SSG}\end{pmatrix} \rightleftharpoons \text{RNase A}\begin{pmatrix}\text{-S}\\ \text{-S}\end{pmatrix}^{3} + \text{GSH} \quad (2)$$

where RNase A(–SH), RNase A(–SSG), and RNase A(>S_2) represent a cysteine residue, a half-cystine residue involved in a mixed disulfide bond with GSH, and a cystine residue, respectively, in the protein (Saxena & Wetlaufer, 1970;

[3] Represented as RNase A(>S_2) in the body of the paper.

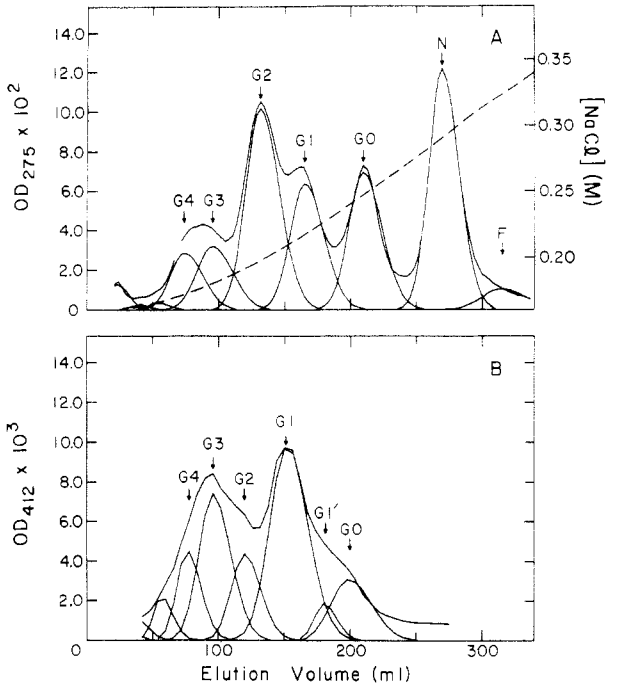


FIGURE 2: Elution curves of partially regenerated RNase A from a CMC column with a salt gradient from 0.16 to 0.40 M NaCl at pH 3.5 and room temperature, illustrating the curve fitting of each peak. The protein was regenerated for 60 min at 22 °C and pH 7.75. The initial concentrations of the reagents are [reduced RNase A] = 7.3 × 10^{-5} M, [GSH] = 2.2 mM, and [GSSG] = 2.6 mM. The elution of the protein was followed by OD_{275} (A) or SH content (B). Peak G1′ was added to optimize the curve fitting. The dashed line represents the salt concentration at the top of the column, in order to provide a rough idea of the salt concentration required to elute each fraction; e.g., after 100 mL of solution has been eluted, the salt concentration at the top of the column is 0.182 M.

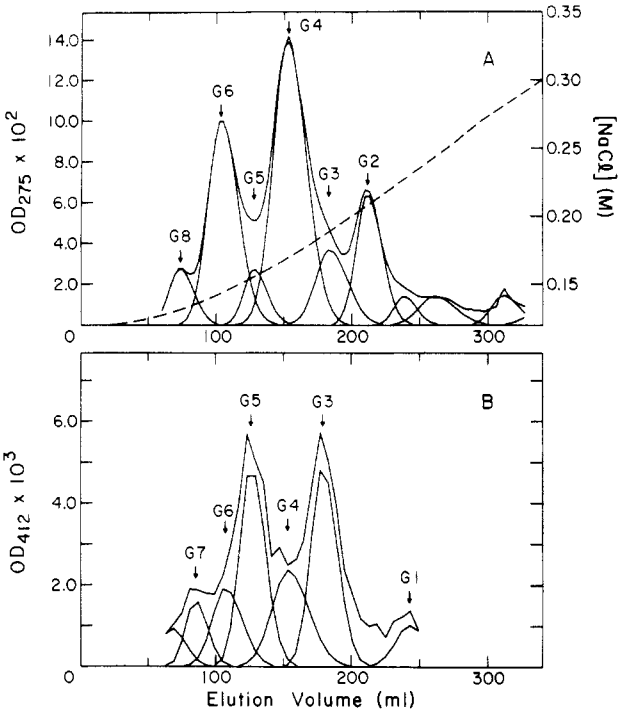


FIGURE 3: Elution curves of partially regenerated RNase A from a CMC column with a salt gradient from 0.12 to 0.36 M NaCl at pH 3.5 and room temperature, illustrating the curve fitting of each peak. The protein was regenerated for 120 min at 22 °C and pH 7.75. The initial concentrations of the reagents are [reduced RNase A] = 7.3 × 10^{-5} M, [GSH] = 2.2 mM, and [GSSG] = 39.6 mM. The elution of the protein was followed by OD_{275} (A) or SH content (B). The dashed line represents the salt concentration at the top of the column, as described in Figure 2A.

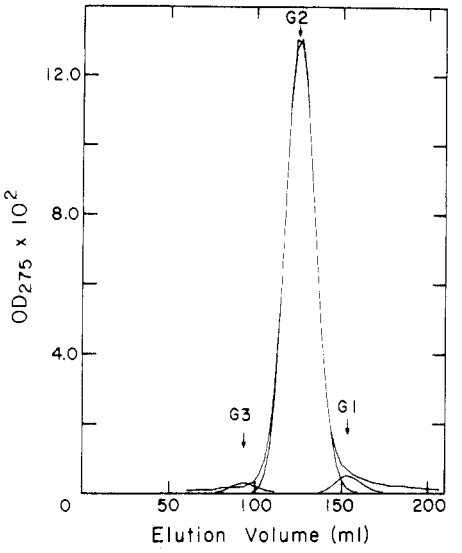


FIGURE 4: Elution curve (followed by OD_{275}) of refractionated fraction G2 from a CMC column with a salt gradient from 0.16 to 0.40 M NaCl at pH 3.5 and room temperature. The elution profile was fitted with three fractions (G3, G2, and G1). The salt concentration is the same as that described in Figure 2A.

Hantgan et al., 1974). Reactions 1 and 2 suffice to represent all possible intermediates in the regeneration process, as shown in Figure 1.

If a glutathione molecule is bound to an –SH group of reduced RNase A, then (at pH 3.5) the positive charge of the protein molecule will be reduced by ~0.5 negative charges since the p*K*s of the two COOH groups of *free* GSH are 2.12 and 3.53 (Pirie & Pinhey, 1929), and GSH also contains one amino group. Thus, the cation-exchanging CMC column (with the aid of a salt gradient), which serves to separate the intermediates according to their net charge, can fractionate them according to the number of bound GSH moieties. In addition, fully regenerated RNase A has some abnormally ionizing carboxyl groups (Hermans & Scheraga, 1961). Therefore, since nonnative intermediate 4S can be expected to have a different conformation than fully regenerated RNase A and hence possibly different carboxyl p*K*s, we can expect these two species to be separable on the CMC column.

Some typical elution curves (followed by measurement of OD_{275} or by SH content), and the curve fitting of each peak (to be described below), obtained from the fractionation of partially regenerated RNase A by CMC column chromatography, are shown in Figures 2 and 3. It should be emphasized that many regeneration experiments were carried out at various concentrations of GSH and GSSG, and Figures 2 and 3 are only two examples. In these figures, there appear to be 11 peaks and shoulders, arbitrarily designated as G8–G0, N, and F, in their order of elution. Corresponding peaks in Figures 2 and 3 were identified by combining them (e.g., fractions G2) and demonstrating that the combined material eluted as a single peak when reapplied to the CMC column and refractionated (see Figure 4). The elution profiles of Figures 2 and 3 differ because different concentrations of GSSG were used in both regeneration reactions; i.e., since the 24 intermediates of Figure 1 are related by eq 1 and 2, their relative populations would depend on the concentrations of GSH and GSSG.

Molecular Weight. The molecular weights of seven of the fractions were determined by sedimentation equilibrium. As shown in Table I, all except fraction F have the same molecular weight as monomeric RNase A, demonstrating that no *inter*molecular disulfide bonds between RNase A molecules are involved in these intermediates. Fraction F exhibited an elution

Table I: Identification of Intermediates

fraction[a]	$\bar{M}_{w,obsd}$[b] ($\times 10^{-4}$)	M_{calcd}[c] ($\times 10^{-4}$)	no. of bound glutathione molecules/molecule of RNase A[d]	characterization of intermediate[e]
G8				*8G*[f]
G7				*7G1H*
G6	1.50	1.55	6.11 ± 0.85	6G2H, *1S6G*
G5				5G3H, *1S5G1H*
G4	1.30	1.49	4.28 ± 0.69	4G4H, 1S4G2H, *2S4G*
G3	1.38	1.46	2.84 ± 0.84	3G5H, 1S3G3H, *2S3G1H*
G2	1.24	1.43	2.16 ± 0.48	2G6H, 1S2G4H, 2S2G2H, *3S2G*
G1	1.55	1.40	1.20 ± 0.50	1G7H, 1S1G5H, 2S1G3H, *3S1G1H*
G0	1.27	1.37	0.02 ± 0.61	8H, 1S6H, 2S4H, 3S2H, *4S*
N		1.37	0.0	fully regenerated RNase A
F	1.81		0.85 ± 0.42	

[a] Peaks of the elution curve from the CMC column chromatogram. [b] Weight-average molecular weight measured by sedimentation equilibrium. [c] Calculated molecular weight. The difference of 1 in residue weight between cysteine and half-cystine is ignored; thus, e.g., 6G2H and 1S6G in fraction G6 have the "same" calculated molecular weight. [d] Estimated from amino acid analysis. [e] According to the scheme of Figure 1, the symbols in each line of this column represent all possible species having a given number of bound glutathiones. The relative amounts of the various species in each fraction are discussed in the text. These relative amounts (e.g., the relative amounts of 4G4H, 1S4G2H, and 2S4G in fraction G4) depend on the concentrations of GSH and GSSG used in the regeneration reaction, but, despite variations in these relative amounts, all three species always appear in fraction G4 because the intermediates are fractionated according to the number of bound glutathiones. Because of this variation in the relative amounts of the species in a given fraction, the average free -SH content per fraction also varies. [f] Italics indicates the most highly oxidized species (within each fraction) that are expected to predominate under the regeneration conditions used for Figures 2 and 3.

curve of about twice the breadth of the other fractions, and its molecular weight was intermediate between that of monomeric and dimeric RNase A. These data suggest that fraction F is a mixture of several species (monomer, dimer, etc), and it could not be identified.

Fractions G8, G7, and G5 were not obtained in sufficiently large quantities for measurements of their molecular weights. Since fraction N was indistinguishable from native RNase A in its elution position on CMC column chromatography, in enzymatic activity toward cytidine cyclic 2',3'-phosphate (Konishi & Scheraga, 1980a), and in its absorption spectrum, this fraction was identified as fully regenerated RNase A and its molecular weight was not determined.

Amino Acid Composition. Table I also gives the number of bound glutathione molecules per molecule of RNase A (determined from the amino acid analysis) for the same fractions analyzed in the ultracentrifuge, plus fraction N. Fractions G6, G4, G3, G2, G1, and G0 have an average of six, four, three, two, one, and zero bound glutathiones, respectively. This is a reasonable result since the intermediates with the larger number of bound glutathiones have a lower net positive charge and would be expected to elute earlier.

SH Content. The average number of free -SH groups in each fraction was determined spectrophotometrically with Ellman's reagent, and two examples of the results are shown in Figures 2B and 3B. It can be seen that the fractions that contain an even number of glutathiones (e.g., G6, G4, G2, and G0) contain a small number (less than one) of free -SH groups and those with an odd number of bound glutathiones contain a larger number (greater than one). When the regeneration reaction was carried out under oxidative conditions, such as those of Figures 2 and 3, the predominant species in each fraction (see footnote *f* in Table I) contained zero or one -SH group for an even or odd number, respectively, of glutathiones. For example, fractions G3 and G1 are rich in free -SH groups (see footnote *e* of Table I). Thus, even though we could not obtain a sufficient amount of material in fraction G5 for amino acid analysis, it is possible to identify this fraction as one with five bound glutathiones because it is rich in free -SH groups (compared to fractions G6 and G4 with even numbers of bound glutathiones) and elutes between fractions G6 and G4. Similarly, fractions G8 and G7 may be assumed to have eight and seven bound glutathiones, respectively, because fraction G8 has a lower -SH content than fraction G7, and they both elute prior to G6. Therefore, fractions G8, G7, and G5 are reasonably identified as the ensembles of intermediates with eight, seven, and five bound glutathiones, respectively.

Since we observed no change in the number of free -SH groups of fraction G3 upon standing in 0.18 M NaCl/acetic acid (pH 3.5) for up to 20 h at and below 15 °C, it can be concluded that there is no oxidation of -SH groups in the isolated intermediates, under our experimental conditions.

Fractions G0–G8 are, therefore, identified as the ensembles of intermediates with zero to eight bound glutathiones, respectively. The 24 possible monomeric intermediates (plus reduced and native RNase A), which are illustrated in Figure 1, are then assigned to each fraction according to the number of bound glutathiones (last column of Table I; also see footnote *e* of Table I).

Curve Fitting. In the analysis of the original elution profile, four parameters were involved: (1) the number of fractions, (2) the elution volume of each fraction, (3) the half-width of each peak, and (4) the peak height. Since it was difficult to make an initial estimate of the half-widths in the original elution profile, several of the fractions of Figures 2 and 3 (G2–G0, N, and F) were refractionated on the same CMC column with a salt gradient from 0.16 to 0.40 M NaCl at pH 3.5 and room temperature (Figure 4). As can be seen for fraction G2 in Figure 4, the elution curves of the refractionated materials were not symmetric. Hence, different half-widths (σ and σ^*) on each side of the maximum peak ordinate (i.e., a modified Gaussian function) were used in the curve fitting, where σ is the half-width on the faster elution side. In fitting the curves of the refractionated material, the initial parameters were estimated from the appearance of the peaks. The computer program of Dennis & Mei (1975) was used to minimize the deviation between the calculated and experimental optical density by a least-squares curve fitting procedure in which the experimental optical density was used as a weighting factor (i.e., greater weight was given to the points of higher optical density). The elution profile of Figure 4 was fitted with three peaks (G3, G2, and G1), thereby providing an estimate of the contamination by G3 and G1 in refractionated G2. The fitted elution volumes were 93, 124, and 153 mL, the half-widths,

σ, were 15.5, 17.6, and 15.5 mL (σ^* was 1.3σ), and the peak heights were 0.0030, 0.132, and 0.0050 OD units for G3, G2, and G1, respectively. The relative areas were used to estimate the purity of G2 as 95%, with contaminations of 2.0 and 3.0% from G3 and G1, respectively. Similar curve fitting was carried out for the elution profiles for refractionated G1, G0, N, and F. The average best fit for these five refractionated fractions was obtained with $\sigma^* = 1.3\sigma$. These refractionated materials (as well as refractionated G4 and G6) were used for the molecular weight measurements and glutathione analyses of Table I.

In order to then analyze the original elution profiles of Figures 2 and 3, we estimated the number of fractions initially by counting the apparent number of peaks and shoulders. In a similar way, an initial estimate was made of the elution volume and peak height of each fraction from the appearance of the elution profile. The *initial* estimates of the half-widths of fractions G2–G0, N, and F were taken from their refractionated profiles (similar to those of Figure 4), and similar values (15–18 mL) were assigned to the other fractions, with σ^* fixed at 1.3σ. With these initial estimates, the elution profiles of Figures 2 and 3 were fit by the same procedure used to analyze the profile of Figure 4. In this fitting procedure, the number of fractions was altered from the initial estimate if the use of an additional peak appeared to improve the fit of the rest of the elution profile (e.g., the introduction of peak G1′ in Figure 2B).[4]

Each fraction contains one to five intermediates, with 8H being included in fraction G0 (see Table I). The intermediates *within* a given fraction are related by equations of the following type (written, as an example, for two intermediates of fraction G0):

$$3S2H + GSSG \rightleftarrows 4S + 2GSH \qquad (3)$$

This demonstrates that the relative amounts of the intermediates in a given fraction depend on the concentrations of reduced and oxidized glutathiones and on the redox potential of the 3S2H/4S couple. For example, in fraction G0, the most reduced species, 8H, would be the dominant one when the concentration of GSH is high and the concentration of GSSG is low, and the most oxidized intermediate, 4S, would be the dominant one when the concentration of GSSG is high and the concentration of GSH is low. *Under the oxidizing conditions of Figure 2*, fraction G0, for example, contains only 0.35 free cysteine residue, which was estimated from tubes taken from peak G0 of the original elution profile (Figure 2B); hence, since all species but 4S contain free –SH groups, the dominant intermediate in fraction G0 must be 4S. Under such oxidizing conditions, the populations of the highly reduced intermediates (1S6H and 2S4H) and 8H should be negligible (see footnote *f* of Table I). Thus, we can expect that most of the free –SH groups of fraction G0, under the oxidative conditions of Figure 2, would arise from the intermediates 3S2H. Similarly, only the two oxidized intermediates in each fraction were considered to be present under the oxidative reaction conditions of Figure 2, and the amounts of the other highly reduced intermediates were considered to be very small and ignored, because the –SH contents of the fractions with even or odd numbers of bound glutathiones were found to be close to zero or one, respectively. It should again be emphasized that the relative amounts of the various species in each fraction depend on the relative amounts of GSSG and GSH, and the data quoted in the text (for the experiments in Figures 2 and 3) correspond to two different sets of concentrations of GSSG and GSH.

The areas under the fitted curves of Figure 2A correspond to the relative amounts (mole fractions) of the intermediates; e.g. [because 275 nm is an isosbestic point for the intermediates and native RNase A (Hantgan et al., 1974; Konishi & Scheraga, 1980a)], the relative area of peak G1 is 0.152 (=[3S1G1H] + [2S1G3H]). In addition, the areas under the fitted curves of Figure 2B correspond to the relative amounts (mole fractions) of cysteine residues in the intermediates; e.g., the summed area of peaks G1 and G1′ is 0.186 (=[3S1G1H] + 3[2S1G3H]), where fraction G1′ was added to improve the curve fitting.[4] Thus, from Figure 2A,B, the relative populations of the intermediates were estimated to be [4S] = 0.133, [3S2H] = 0.028, [3S1G1H] = 0.135, [2S1G3H] = 0.017, [3S2G] = 0.211, [2S2G2H] = 0.029, [2S3G1H] = 0.066, [1S3G3H] = 0.013, [2S4G] = 0.040, [1S4G2H] = 0.025, [intermediate F] = 0.050, and [fully regenerated RNase A] = 0.253. The relative amounts of fractions G5, G6, G7, and G8 were too small to estimate. Similarly, the curve fitting of the elution profiles in Figure 3 also enabled us to estimate the relative amounts of the more oxidized intermediates under a different set of oxidizing conditions, i.e., [3S1G1H] = 0.0061, [2S1G3H] $\simeq$ 0.0, [3S2G] = 0.148, [2S2G2H] = 0.0049, [2S3G1H] = 0.025, [2S4G] = 0.428, [1S4G2H] = 0.0081, [1S5G1H] = 0.0237, [1S6G] = 0.280, [6G2H] = 0.0054, [7G1H] = 0.0066, and [8G] = 0.0649. The long tailing after 240 mL (elution volume) in Figure 3A was arbitrarily fitted with four curves. The amounts of the more highly reduced intermediates were ignored under the highly oxidizing conditions of Figure 3, as they were in Figure 2. Since peaks G3 and G5 were submerged in the profile of Figure 3A, it was difficult to estimate their relative amounts accurately. Hence, they were estimated from Figure 3B, under the assumption that fractions G3 and G5 in Figure 3 were attributable only to 2S3G1H and 1S5G1H, respectively, and that the contributions of other more reduced intermediates in G3 and G5 could be ignored under the highly oxidizing conditions of Figure 3.

Since interconversion among the intermediates can be expressed by eq 1 and 2, the relative amounts of the intermediates depend on the concentrations of GSH and GSSG. This is the reason why the relative populations of the intermediates in Figures 2 and 3, which were prepared with different concentrations of GSH and GSSG, are different. The data of Figures 2 and 3 (and data from other similar experiments) were combined in order to obtain the equilibrium constants given in Figure 1 (see below).

Regeneration from Isolated Intermediates. The data of Figures 2 and 3 reflect the distribution of the intermediates at preequilibrium prior to the rate-limiting steps along the pathways from reduced RNase A to the native protein. Additional information about this pathway is obtainable by restarting the regeneration reaction, not from the fully reduced protein, but from one or more isolated intermediates, and following the regeneration reaction for a *short* time. In such an experiment, the adjacent intermediates along the pathway would be expected to be the dominant ones produced. For this purpose, refractionated fractions G6, G4, G3, G2, G1, G0, and F were each used as starting materials in the regeneration

[4] Since the values of OD_{412} in part B of Figure 2 are $\sim$10 times smaller than those of OD_{275} in part A, the data of part B are less accurate than those of part A. For example, the value of OD_{412} for peak G1′ is only $\sim$0.002 OD unit. Thus, it is difficult to identify G1′. Hence, G1′ is arbitrarily assigned to G1 rather than G0, because the former peak is larger and a small deviation in the SH content of G1 could lead to G1′. The deviation in SH content would have to be much larger to assign G1′ to G0.

Table II: Mole Fractions of Fractions Produced in 15-s Regeneration Reactions from Isolated Fractions

	G6[a]	G4	G3	G2	G1	G0	F
G6[b]	(0.80)[c]	0.08	0.01	0.00	0.00	0.00	0.00
G4	0.16	(0.72)	0.14	0.02	0.01	0.00	0.00
G3	0.02	0.11	(0.52)	0.12	0.04	0.01	0.01
G2	0.01	0.08	0.22	(0.69)	0.36	0.06	0.03
G1	0.01	0.01	0.03	0.10	(0.23)	0.31	0.04
G0	0.00	0.00	0.07	0.05	0.19	(0.61)	0.04
N	0.00	0.00	0.01	0.01	0.17[d]	0.01	0.04
F	0.00	0.00	0.00	0.01		0.00	(0.84)

[a] The regeneration reactions were restarted from the refractionated fractions given on this line. (See text for regeneration conditions.) The purity of the isolated fractions was >99% (estimated from curve fitting of the refractionated fractions, as in Figure 4) because material from only the middle portion of the refractionated peaks was used in these experiments. [b] The intermediates in this column were produced from the isolated fractions in a reaction time of 15 s. [c] The mole fractions of the fractions, from which the regeneration was started, are given in parentheses after the 15-s regeneration reaction. [d] Since the large peak F overlapped peak N, we could not estimate the amount of peak N by curve fitting. Besides, we believe that most of this peak pertains to fraction F because the formation of fully regenerated RNase A is very slow and the amount of N in the 15-s regeneration reaction time would be negligibly small.

reactions at a concentration of intermediates of 1.4×10^{-5}–7.6×10^{-5} M, under oxidative conditions ([GSH] = 2.7 mM, [GSSG] = 1.9 mM at pH 7.8–8.2 and 22 °C for 15-s reaction time). Too little of fractions G8, G7, and G5 was available for similar experiments with these fractions.

The regeneration reactions were stopped in the usual manner, and the proteins were fractionated by CMC column chromatography, as before. From a curve-fitting analysis of the elution curves, the data of Table II were obtained.

From Figure 1, we would expect fraction G1 to be the first one produced from fraction G0, in a single reduction or oxidation reaction. Other fractions (G8–G2) would require two or more reduction or oxidation steps to be formed from G0 and would appear later in time than G1. Similarly, fractions G0 and G2 should be the first ones formed from G1, fractions G1 and G3 from G2, fractions G2 and G4 from G3, fractions G3 and G6 from G4, and fraction G4 from G6 (because fractions G5 and G7 are at too low a concentration to detect). The results shown in Table II support these expectations and hence the general validity of the scheme in Figure 1.

The unidentified fraction F was produced mainly from G1 (see Table II). Also, the number of bound glutathiones is close to that of G1 (see Table I). Thus, some of the protein in fraction F may be dimerized G1.

Preequilibrium. The relative populations of the various fractions were measured as a function of reaction time in the regeneration process. Figure 5A shows that the percent of fully regenerated RNase A increases continuously with reaction time, as observed by Hantgan et al. (1974). The percent of all other fractions (except G8, G7, and G5, which could not be detected at this concentration of GSH/GSSG), however, increased in the early stages of the reaction and then decreased slowly with reaction time (the initial increase in the percent of G1 occurred too rapidly to be observed).

Since native RNase A is extremely stable to reduction by GSH at neutral pH and room temperature (Davidson & Hird, 1967), the formation of fully regenerated RNase A may be considered to be an irreversible process at room temperature and neutral pH. Hence, *after* the rate-limiting step occurs, we would not expect to find a state of preequilibrium among the intermediates, and hence the concentrations of such intermediates would be extremely small.[2] On the other hand, *prior* to the rate-limiting step, the intermediates present would be expected to exist in preequilibria in which their relative populations would not change with reaction time. Therefore, the data of Figure 5A are replotted in Figure 5B as percents of intermediates among the total population of intermediates prior to the rate-limiting step, i.e., by subtracting the amount of fully regenerated RNase A from the total population. The percents of all of the intermediates become constant at $t \geq 20$ min, demonstrating that a preequilibrium is achieved at an early stage in the reaction ($t \geq 20$ min) [implied by the data of Creighton (1977)]. Since the relative populations of the intermediates in preequilibrium (such as those shown in Figures 2 and 3) may be considered to be very close to those at equilibrium, we can compute approximate equilibrium constants for the various equilibria. For a reaction of the type of eq 1, e.g.

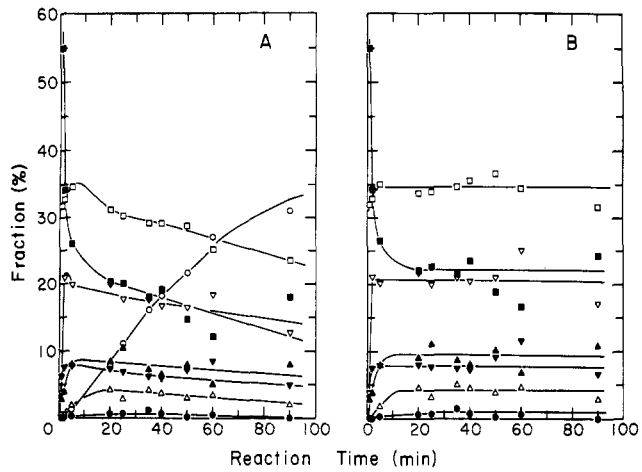


FIGURE 5: Mole percent of each of fractions G6 (●), G4 (△), G3 (▲), G2 (□), G1 (■), G0 (▽), N (○), and F (▼) in the whole sample of the *partially regenerated RNase A* (A) and in the portion of the sample containing the *intermediates* and the fully reduced protein (B). The protein was regenerated at 22 °C and pH 7.75 for a reaction time of 2–90 min. The initial concentrations of the reagents are [reduced RNase A] = 7.3×10^{-5} M, [GSH] = 2.2 mM, and [GSSG] = 2.2 mM.

$$3S1G1H + GSSG \rightleftharpoons 3S2G + GSH \quad (4)$$

the equilibrium constant among the species in preequilibrium may be written as

$$K_{3S1G1H:3S2G} = \frac{[3S2G][GSH]}{[3S1G1H][GSSG]} \quad (5)$$

Since we measured the relative concentrations [3S1G1H] and [3S2G] by using different sets of values of the concentrations [GSH] and [GSSG], the estimated values of $K_{3S1G1H:3S2G}$ are averaged as

$$(K_{3S1G1H:3S2G})_{av} = (1/N)\sum_{i=1}^{n} f_{w,i}(K_{3S1G1H:3S2G})_i \quad (6)$$

with

$$N = \sum_{i=1}^{n} f_{w,i} \quad (7)$$

where the subscripts i and av pertain to the equilibrium constant from the ith experiment and from the average over n (=8) experiments, respectively. The weighting factors $f_{w,i}$ were taken to be proportional to the accuracy of the data. The accuracy of the concentrations [GSH] and [GSSG] was fairly constant over the range of concentrations used. The accuracy of the concentrations of the intermediates, however, is greater

for the larger peaks obtained in the curve-fitting procedure. Thus, the relative concentrations of the intermediates were taken as the weighting factor, i.e.

$$f_{w,i} = [3S1G1H]_i[3S2G]_i \quad (8)$$

The estimated equilibrium constant from eq 6 and 8 is 1.8 and is listed in Figure 1. Other equilibrium constants were estimated in the same manner. By this procedure, nine values of K_1 and six values of K_2 were determined, i.e., those values *without* parentheses in Figure 1. The standard deviations of the averaged equilibrium constants in the eight experiments were 10–45%. Actually, these 15 equilibrium constants were obtained (with the aid of a computer) as the solutions of a set of simultaneous equations, having in common the same values of [GSH] and [GSSG] in a given set of experiments. The values of K_1 and K_2 in parentheses were estimated by the procedure described under Discussion; they were not estimated by the above procedure because the fractionations were successful only for the intermediates produced under oxidative reaction conditions such as those in Figures 2 and 3. A reaction time of ≥60 min was allowed for the system to reach its preequilibrium state.

Discussion

Potential Isolation of Intermediates. In order to calculate the equilibrium constants between those intermediates that could not be detected experimentally, we make the assumption that the equilibrium constant for the reaction in which one cystine residue is formed from two cysteine residues (i.e., the combined reactions 1 and 2) depends on the number of cystine residues in any of the intermediates and is independent of the number of bound glutathiones. For example, for the reactions

$$2S1G3H + GSSG \rightleftharpoons 3S1G1H + 2GSH \quad (9)$$

$$K = 2.6 \times 10^{-2}\ M$$

$$2S2G2H + GSSG \rightleftharpoons 3S2G + 2GSH \quad (10)$$

$$K = 1.8 \times 10^{-2}\ M$$

where the values of K are the products of the appropriate values of K_1 and K_2 of Figure 1. The average of these two values of K is $(2.2 \pm 0.4) \times 10^{-2}$ M. For the reactions

$$1S3G3H + GSSG \rightleftharpoons 2S3G1H + 2GSH \quad (11)$$

$$K = 1.5 \times 10^{-2}\ M$$

$$1S4G2H + GSSG \rightleftharpoons 2S4G + 2GSH \quad (12)$$

$$K = 1.1 \times 10^{-2}\ M$$

the average of these two values of K is $(1.3 \pm 0.2) \times 10^{-2}$ M. Thus, the above assumption is roughly reasonable, in light of these experimental results, and such "extrapolated" equilibrium constants are listed in parentheses in Figure 1. The following example illustrates how the values in parentheses in Figure 1 were obtained.

$$2S4H + GSSG \rightleftharpoons 3S2H + 2GSH \quad (13)$$

$$K = 2.2 \times 10^{-2}\ M \text{ (av value of } K)$$

$$2S1G3H \rightleftharpoons 3S2H + GSH \quad (14)$$

$$K = 0.0033\ M \text{ (reaction 2, Figure 1)}$$

Then

$$2S4H + GSSG \rightleftharpoons 2S1G3H + GSH \quad (15)$$

$$K = \frac{2.2 \times 10^{-2}}{0.0033} = 6.6 \text{ (reaction 1, Figure 1)}$$

The equilibrium constants in Figure 1 can be used to calculate the mole fractions of the intermediates in preequilibrium as a function of the concentrations of reduced and oxidized glutathiones. Since the concentrations of GSH and GSSG appear in the expressions for the equilibrium constants (eq 1 and 2), we started the computation from the *initial* concentrations of GSH and GSSG and estimated the concentrations of the intermediates at preequilibrium by treating all of the equilibria of Figure 1 simultaneously, i.e., as a combined set of multiple equilibria. Then the concentrations of glutathiones were recalculated according to the estimated extent of oxidation of reduced RNase A. In a second step, the recalculated concentrations of glutathiones were used to reestimate the concentrations of the intermediates. These calculations were repeated until the change of the concentrations of glutathiones was <0.01% between successive steps or until 100 iterations had been carried out.

Figure 6 shows the mole fractions (percent) of several (illustrative) intermediates in preequilibrium in the range of *initial* concentrations of reduced glutathione from 5.0×10^{-5} to 0.11 M and oxidized glutathione from 5.0×10^{-5} to 2.3 M. The region bounded by two solid (contour) lines (e.g., −1− and −10−) is that in which the population of the intermediate in preequilibrium is expected to be between 1 and 10%, where the populations were normalized by dividing by the total amount of reduced RNase A plus all intermediates in preequilibrium prior to the rate-limiting step. In Figure 6, we could not obtain the concentrations of glutathiones in preequilibrium at low initial concentrations of both GSH and GSSG (i.e., the region surrounded by dashed lines) within 100 iterations.

For isolation of a particular intermediate experimentally, it should be the dominant one in the fraction isolated by CMC column chromatography. The singly and doubly diagonally lined regions in Figure 6d, for example, indicate that the summed populations of 1G7H, 1S1G5H, and 2S1G3H in fraction G1 (which are estimated from the equilibrium constants of Figure 1) are between 1 and 10% and <1%, respectively. In other words, the purity of intermediate 3S1G1H in the isolated fraction G1 is >90% (but <99%) in the singly diagonally lined region and is >99% in the doubly diagonally lined region.[5] Similarly, the diagonally lined regions in the other parts of Figure 6 show the concentrations of GSH and GSSG that are appropriate for the isolation of the particular intermediates.

Figures (similar to those of Figure 6) for the other intermediates indicate that only the most oxidized intermediates in each fraction can be isolated, viz., 4S in G0, 3S1G1H in G1, 3S2G in G2, 2S3G1H in G3, 2S4G in G4, 1S5G1H in G5, 1S6G in G6, 7G1H in G7, and 8G in G8. It may be very difficult, however, to isolate 2S3G1H and 1S5G1H because their populations are very small at the appropriate concentrations of GSH and GSSG required for their isolation. Furthermore, even though Figure 6b shows that it is possible to isolate the intermediate 1G7H, its peak overlaps that of reduced RNase A in the CMC chromatogram so that separation of these two species is not possible by this technique. For reasons that we do not understand, it is not possible to fractionate highly reduced intermediates by this method.

The optimum initial concentrations of GSH and GSSG to produce each intermediate in the range of [GSH] = 5 ×

[5] The relative populations of 2S1G3H of fraction G1 at various sets of [GSH] and [GSSG] are not shown in Figure 6; the other intermediates (1G7H, 1S1G5H, and 3S1G1H) in fraction G1 are shown in Figure 6b–d. These relative populations were used in determining the representation of the singly and doubly diagonally lined regions.

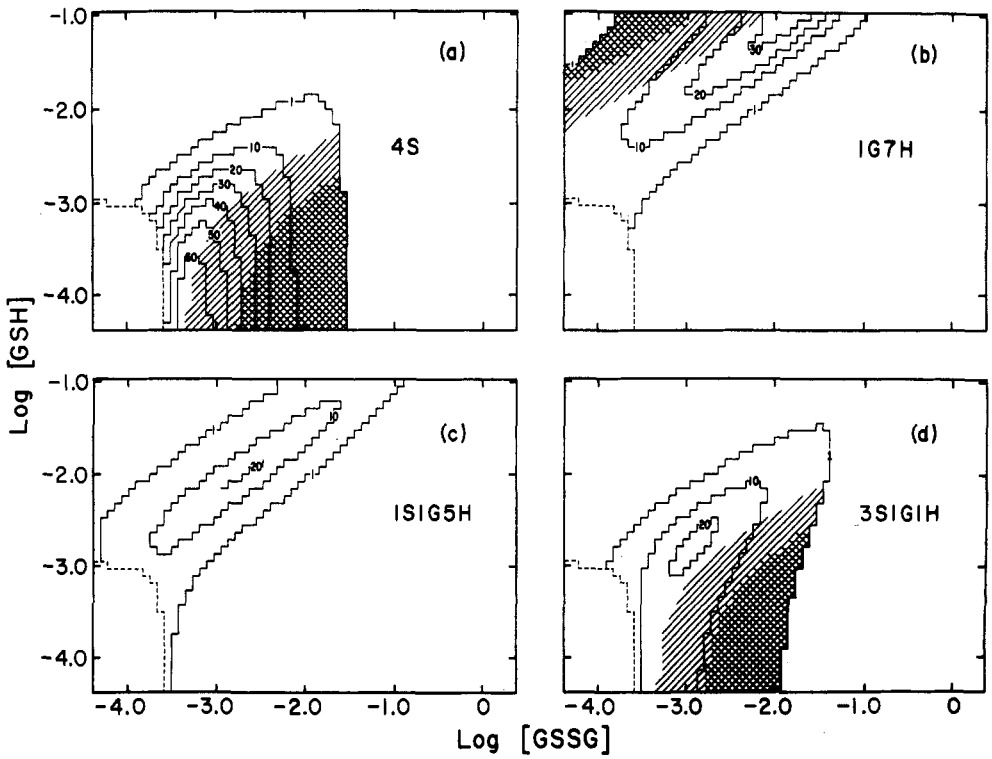


FIGURE 6: Mole percents of each of several illustrated intermediates, 4S (a), 1G7H (b), 1S1G5H (c), and 3S1G1H (d), at preequilibrium in the portion of the sample containing the intermediates and 8H (excluding fraction F), are represented at various *initial* concentrations of reduced and oxidized glutathiones. The initial concentration of reduced RNase A was taken as 7.3×10^{-5} M. When [GSH] and [GSSG] are in the singly diagonally lined region of a particular intermediate, then the intermediate isolated from the appropriate fraction is 90–99% pure. Similarly, when [GSH] and [GSSG] are in the doubly diagonally lined region of a particular intermediate, then the intermediate isolated from the appropriate fraction is >99% pure. For example, in (d), "10" means that 10% of the whole reaction mixture (except for N and F) contains 10% of 3S1G1H; after fractionation, fraction G1 (which contains 3S1G1H) is 90–99% in 3S1G1H in the single diagonally lined region. In the range of very low concentrations of GSH and GSSG (surrounded by dashed lines), the final distributions of the intermediates in preequilibrium could not be obtained with the computer within 100 iterations. (c) contains no singly or doubly diagonally lined regions because 1S1G5H could not be isolated in a high enough state of purity.

10^{-5}–0.11 M and [GSSG] = 5×10^{-5}–2.3 M are shown in Figure 7. The intermediates without bound glutathiones (8H, 1S6H, 2S4H, 3S2H, and 4S of fraction G0) are the dominant species at low concentrations of GSSG. The intermediates with no free –SH groups (8G, 1S6G, 2S4G, and 4S) are the dominant species at low concentrations of GSH. The intermediates without S–S bonds (7G1H, 6G2H, 5G3H, 4G4H, 3G5H, 2G2H, and 1G7H, except for 8G and 8H) are the dominant species at high concentrations of both GSH and GSSG. The intermediates with S–S bonds, free –SH groups, and bound glutathiones (1S1G5H, 2S1G3H, 3S1G1H, 1S2G4H, 2S2G2H, 1S3G3H, 2S3G1H, 1S4G2H, and 1S5G1H) are the dominant species at intermediate concentrations of GSH and GSSG. Thus, the influence of GSH and GSSG on the stabilities of intermediates can be described as follows. Reduced glutathione stabilizes –SH groups and oxidized glutathione stabilizes half-cystine residues with mixed disulfide bonds with glutathione; S–S bonds in the intermediates are destabilized by both reduced and oxidized glutathiones.

Reshuffling of Disulfide Bonds during Assay. Although a *spontaneous* reshuffling between disulfides related to cystine was not observed in the pH range of 1–6.7 (Schöberl & Gräfje, 1958; Spackman et al., 1960), Eager & Savige (1963) reported that reshuffling of disulfide bonds could be initiated by addition of a small amount of a thiol or by irradiation with ultraviolet light at pH 1–6. Since thiol is present in some of our intermediates (as cysteine residues) and our experiments were performed in a room with fluorescent lights, it was necessary to check whether reshuffling of disulfide bonds in the protein occurs after the regeneration reaction is allegedly quenched,

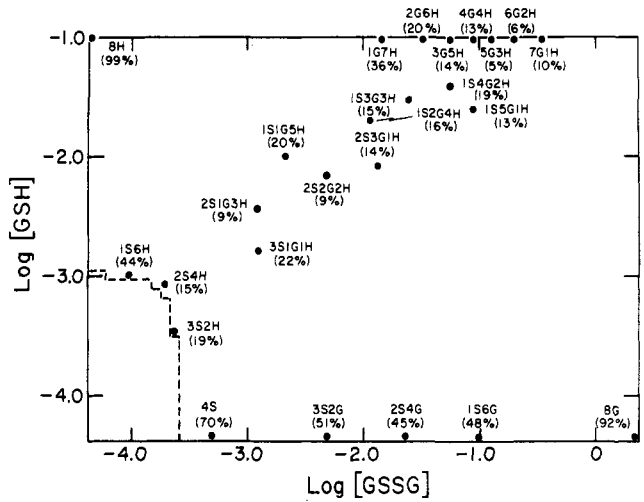


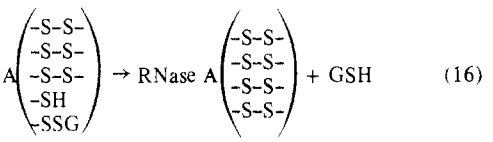
FIGURE 7: Distributions of intermediates, and reduced RNase A, in preequilibrium at *initial* concentrations of GSH in the range of 5.0×10^{-5}–0.11 M and of GSSG in the range of 5.0×10^{-5}–2.3 M; the initial concentration of reduced RNase A was taken as 7.3×10^{-5} M. The largest percent of each intermediate (i.e., the maxima in analogues of Figure 6) is given in parentheses over each symbol (●). The equilibrium constants in Figure 1 were used to estimate this distribution. The dashed lines have the same meaning as in Figure 6.

and whether such reshuffling affects our results (if it occurs).

One check for the effect of irradiation with ultraviolet light was performed by incubating intermediate 4S at pH 6 for ~20 h at 22 °C in a glass vessel under a fluorescent lamp (the use of glass vessels in all of our experiments would have provided

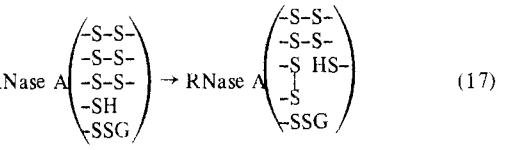
some degree of protection from ultraviolet light). No reshuffling of the incorrect disulfide bonds to the correct ones was observed in the elution profile of the CMC chromatogram, i.e., reaction 4S → N did not occur (the limit of detection of N formed in this reaction was ~0.5%).

A check for the effect of thiol was performed with fraction G1, which contains one or more thiol groups per molecule of RNase A. The reshuffling of intermediate 3S1G1H, for example, in fraction G1 can occur as follows:



(16)

or



(17)

If reaction 16 occurs, then an intermediate from fraction G1 (i.e., 3S1G1H) would change to one from fraction G0 (i.e., 4S). The elution curve of refractionated fraction G1 was similar to that of Figure 4. From the curve fitting, it was estimated that there were 5.2 and 6.7% of G2 and G0, respectively, as contaminants and that fraction G1 was 88.1% pure. From an examination of the elution profile of Figure 2A, in which fractions G1 and G0 overlap to some extent, one would expect to find a few percent of G0 in the profile of refractionated G1. Thus, at most, some portion of this small percent of G0 could have arisen by reshuffling during one cycle (~2 days) of fractionation on the CMC column. This cycle included all the steps normally used in the fractionation procedure. This amount of possible reshuffling is so small that it can be ignored under our experimental conditions.

If intermediate 3S1G1H underwent reshuffling by reaction 17, it would be undetectable by our method, but none of the results in this paper would be affected. The reason for this is that each intermediate is itself an ensemble of many species which have the same numbers of S–S bonds, bound glutathiones, and –SH groups, with these groups occurring at different places in the amino acid sequence in each component of the ensemble. Our analysis in this paper does not distinguish among the various components in each ensemble.

Therefore, we may conclude that the relative amounts of the various intermediates (ensembles) determined from the elution profiles of CMC chromatograms are not affected by possible reshuffling and hence correspond quantitatively to those present in partially regenerated RNase A at the time that the regeneration reaction was stopped.

Conclusion

We have developed an equilibrium treatment of the pathway for regeneration of RNase A from the reduced protein by glutathiones. This process has been represented by two simple reactions. This has enabled us to specify and to relate all possible intermediates (Figure 1). The experiments reported here demonstrate that a preequilibrium state exists and that the intermediates at preequilibrium could be separated and identified, thereby providing information about the relations among the intermediates. Since the regeneration process rapidly reaches a preequilibrium state, the equilibrium constants between the intermediates prior to the rate-limiting step were estimated approximately from the mole fractions of the intermediates at preequilibrium. With these equilibrium constants, we estimated the relative populations of the intermediates prior to the rate-limiting step at any concentrations of GSH and GSSG.

The regeneration pathways can be described in terms of three steps. The first one is the transformation from reduced RNase A to the intermediates prior to the rate-limiting steps (attainment of the preequilibrium state). The second one consists of the rate-limiting steps, and the third one comprises processes in which the native structure is formed after the rate-limiting steps. The significance of the results in this paper is, as described above, the characterization of the first step or preequilibrium state, i.e., the identification of the intermediates prior to the rate-limiting steps and the estimation of their distributions at various concentrations of GSH and GSSG. These results are applied to an analysis of the second, or rate-limiting, steps, which will be presented in the following paper of this series.

Acknowledgments

We are indebted to T. W. Thannhauser for carrying out the amino acid analysis, to M. Okada for the molecular weight measurements, and to J. R. McQuie for developing the program involving the modified Gaussian function used in the curve fitting. We are also indebted to Drs. E. R. Stimson, Y. Isogai, and K. Nishikawa for helpful discussions and to J. B. Denton, R. A. Fredrickson, C. A. McWherter, G. T. Montelione, and T. W. Thannhauser for helpful comments on this manuscript.

Appendix

Degeneracy among Intermediates. Let ν_{kmn} be the number of species in intermediate kSmGnH.[1] In a protein with $2N$ half-cystine residues

$$\nu_{kmn} = ({}_{2N}C_m)({}_{2N-m}C_n)[(2N - m - n - 1)!!] \quad \text{(A-1)}$$

where ${}_{2N}C_m$ is the number of ways to attach m glutathiones to $2N$ half-cystine residues, ${}_{2N-m}C_n$ is the number of ways to obtain n free SH groups from the $2N - m$ half-cystine residues that are not bonded to glutathione, and $(2N - m - n - 1)!!$ is the number of ways to form disulfide bonds among the $2N - m - n$ remaining half-cystine residues. The symbol $\mu!!$ is defined as

$$\begin{aligned} \mu!! &= \mu(\mu - 2)(\mu - 4) \ldots 1 \quad \text{for odd } \mu \\ &= \mu(\mu - 2)(\mu - 4) \ldots 2 \quad \text{for even } \mu \end{aligned} \quad \text{(A-2)}$$

For example, for $2N = 8$, $k = 4$, $m = 0$, and $n = 0$

$$\nu_{4,0,0} = \frac{8!}{0!8!}\frac{8!}{0!8!}7!! = 105$$

as found by Sela & Lifson (1959).

The total number of species, T_{2N}, in a protein with $2N$ half-cystine residues is

$$T_{2N} = \sum_k \sum_m \sum_n \nu_{kmn} \quad \text{(A-3)}$$

with the constraint

$$2k + m + n = 2N \quad \text{(A-4)}$$

Using eq A-4, eq A-3 becomes

$$T_{2N} = \sum_{k=0}^{N} \sum_{m=0}^{2N-2k} \nu_{kmn} \quad \text{(A-5)}$$

Substituting eq A-1 into eq A-5 and rearranging, we obtain

$$T_{2N} = (2N)! \sum_{k=0}^{N} \frac{(2k-1)!!}{(2k)!} \sum_{m=0}^{2N-2k} \frac{1}{m![(2N-2k-m)!]} \quad \text{(A-6)}$$

For RNase A, with $2N = 8$, $T_{2N} = 7193$, i.e., 7191 intermediates plus the native and fully reduced species. We are indebted to Y. Isogai for this derivation and to R. A. Fredrickson for a similar one.

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Effect of Hydrostatic Pressure on Lysozyme and Chymotrypsinogen Detected by Fluorescence Polarization†

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ABSTRACT: The effect of hydrostatic pressure upon solutions of chymotrypsinogen and lysozyme at room temperature has been followed by employing a new technique [Chryssomallis, G. S., Drickamer, H. G., & Weber, G. (1978) *J. Appl. Phys. 49*, 3084] that permits the measurement of fluorescence polarization at pressures of up to 10 kbar. Lysozyme shows a stable, reversible 60% increase in apparent volume when the pressure is raised to 9 kbar. This can be given a simple interpretation in terms of solvent penetration of the structure at higher pressures. In contrast, the results with chymotrypsinogen are time dependent and only partially reversible on release of the pressure. They involve conversion ($t_{1/e} = 5$ min) to a form with a lower rotational rate at approximately 6 kbar and return to a fast-rotating form at higher pressure. This latter form persists on pressure release. The possibility of generating what are clearly metastable conformations, not only in chymotrypsinogen but also in flavodoxins [Visser, A. J. W. G., Li, T. M., Drickamer, H. G., & Weber, G. (1977) *Biochemistry 16*, 4879], indicates that there are unresolved questions about the relative stability of protein conformations which can be profitably investigated by high-pressure experiments.

The perturbation of protein structure due to hydrostatic pressure has been under investigation for several years by various methods, including absorption spectroscopy (Brandts et al., 1970; Hawley, 1971; Zipp & Kauzmann, 1973), electrophoresis (Hawley & Mitchell, 1975), and fluorescence spectroscopy, either of the intrinsic tryptophans or of added ligands (Heremans et al., 1974; Li et al., 1976a,b; Visser et al., 1977). These investigations have demonstrated that pressure can alter the affinity of proteins for small molecules, either increasing or decreasing it, depending on the nature of the binding site (Togerson et al., 1979). They also have shown that pressure perturbs the environment of tryptophan, increasing its exposure to the solvent as compared to the native protein in the case of lysozyme (Li et al., 1976b). All these data point to the appearance of large changes in the protein structure at the higher pressures, and by analogy with the denaturation reactions (Tanford, 1968), one must expect considerable changes in the hydrodynamic properties. No data on this point have been recorded, but the recent development

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