Folding of Ribonuclease, S-Protein, and Des(121–124)-ribonuclease during Glutathione Oxidation of the Reduced Proteins†

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ABSTRACT: The rate of folding of reduced ribonuclease, reduced S-protein, and reduced des(121–124)-ribonuclease during the glutathione-mediated oxidation is determined by an immunological method, using purified antibodies that react specifically with segments 1–13, 31–79, and 80–124 of the native protein. The kinetics for the return of antigenic activity in each of these segments in reduced ribonuclease are consistent with the results [on the air oxidation of reduced ribonuclease, obtained by Chavez and Scheraga [Chavez, L. G., Jr., & Scheraga, H. A. (1977) *Biochemistry 16*, 1849]] which indicated that intermediate states of folding are detectable and that segment 80–124 folds first. The kinetics for reduced S-protein indicate that glutathione oxidation promotes total recovery of the antigenic determinants of the native structure, even in the absence of S-peptide, and provide further evidence that segment 80–124 folds faster than segment 31–79, which is consistent with the sequence of stages in the folding pathway proposed by Burgess and Scheraga [Burgess, A. W., & Scheraga, H. A. (1975) *J. Theor. Biol. 53*, 403], as modified by Matheson and Scheraga [Matheson, R. R., Jr., & Scheraga, H. A. (1978) *Macromolecules 11*, 819; Matheson, R. R., Jr., & Scheraga, H. A. (1979) *Biochemistry 18*, 2437]. This is strong evidence for the localization of the primary nucleation site for the folding of ribonuclease in segment 21–124. On the other hand, the folding kinetics of des(121–124)-ribonuclease demonstrate that the C-terminal tetrapeptide of ribonuclease is required for proper folding. These data are interpreted in light of several experimental and theoretical results which indicate that, whereas the primary nucleation site for the folding of ribonuclease is located in segment 80–124, most likely in the hydrophobic pocket consisting of residues 106–118 [Matheson, R. R., Jr., & Scheraga, H. A. (1978) *Macromolecules 11*, 819], residues 121–124 are needed for subsequent, but nevertheless relatively early, stages of folding.

There is well-documented evidence to support the conclusion that most of the amino acid sequence of a protein is essential to determine the unique folding of the native conformation. Taniuchi & Anfinsen (1968, 1969) proposed that staphylococcal nuclease (149 residues) must contain at least the first 126 residues before the native conformation can be formed. However, segment 1–126 does possess some (0.11%) enzymatic activity, indicating that a small amount of native structure is present and that there is a considerable restriction on the number of possible conformations that the polypeptide chain can adopt (Sachs et al., 1974). Likewise, bovine pancreatic ribonuclease ($RNase_N$)[1] requires the C-terminal tetrapeptide in order to fold to the native conformation (Taniuchi, 1970). The derivative, des(121–124)-RNase, lacking this tetrapeptide, differs somewhat from RNase in its spectrophotometric and optical rotatory properties (Sela & Anfinsen, 1957), has only a trace (0.5%) of the enzymatic activity of $RNase_N$ (Lin, 1970), and lacks the interactions for proper folding, as indicated by the formation of randomly paired disulfide bonds during the air oxidation of the reduced derivative (Taniuchi, 1970).

The requirement for the presence of the N-terminal residues 1–20 (S-peptide) of RNase for the correct folding of residues 21–124 (S-protein) is not clear. Air oxidation of reduced S-protein (with S-peptide being present only during the activity measurements but not during folding) leads to a 34% return of the enzymatic activity of air-oxidized reduced ribonuclease (Haber & Anfinsen, 1961). When air oxidation was carried out in the absence of S-peptide but in the presence of an enzyme that catalyzes disulfide exchange (De Lorenzo et al., 1966), about 20% of the S-protein was obtained (Kato & Anfinsen, 1969). It appears that the return of the native conformation to reduced S-protein depends on the conditions used for the oxidation. It is clear, however, that S-protein does possess at least a limited capacity to fold to the native structure. When air oxidation is carried out in the presence of S-peptide, the disulfide-exchange enzyme quickly promotes the return of 100% enzymatic activity (Kato & Anfinsen, 1969). By providing additional interactions, S-peptide could increase the efficiency of the folding in two ways: (1) by increasing the rate of folding and (2) by reducing the chance to form intermolecular disulfide bonds.

In this paper, we use the immunological assay of Chavez & Scheraga (1977) to examine the folding pathway(s) during the glutathione-mediated oxidation of reduced RNase, reduced S-protein, and reduced des(121–124)-RNase. As shown by Hantgan et al. (1974), the use of glutathione avoids some of the disadvantages of air oxidation, carried out either in the presence or absence of the disulfide-reshuffling enzyme. It is shown here that portions of the ribonuclease molecule can achieve their native conformation even when other portions are still unfolded. Our previous experiments on the air oxidation of RNase indicated that the pathway of folding follows the sequence of segment 80–124, then segment 1–13, and then 31–79, with the primary nucleation site residing in segment 80–124 (Chavez & Scheraga, 1977). By examining the folding of reduced S-protein and reduced des(121–124)-RNase, we can provide further information about the location of the primary nucleation site.

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[1] Abbreviations used: $RNase_N$ or simply RNase, bovine pancreatic ribonuclease A; S-peptide and S-protein, residues 1–20 and 21–124, respectively, of RNase; des(121–124)-RNase, residues 1–120 of RNase; [^{125}I]$RNase_N$, iodinated RNase; anti-X, antibody against X, where X is $RNase_N$ or a fragment thereof; K_{conf}, the equilibrium constant between the unfolded and native conformations of a protein fragment.

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Table I: Amino Acid Analyses of Derivatives of Ribonuclease[a]

amino acid residue	native RNase	des(121-124)-RNase		S-peptide		S-protein	
	theory[b]	theory[b]	found	theory[b]	found	theory[b]	found
aspartic acid	15	14	14.0	1	1.1	14	13.9
threonine	10	10	10.0	2	1.9	8	8.3
serine	15	14	12.5	3	2.7	12	11.6
glutamic acid	12	12	12.3	3	2.9	9	9.8
proline	4	4	4.3			4	4.3
glycine	3	3	3.6			3	3.2
alanine	12	11	10.7	5	4.7	7	8.4
valine	9	8	7.8			9	9.2
$^1/_2$-cystine	8	8	8.5			8	6.9
methionine	4	4	3.6	1	1.0	3	3.0
isoleucine	3	3	2.7			3	2.6
leucine	2	2	2.0			2	1.8
tyrosine	6	6	6.3			6	6.0
phenylalanine	3	3	3.1	1	1.0	2	2.2
histidine	4	4	3.9	1	1.0	3	2.9
lysine	10	10	10.7	2	2.0	8	9.0
arginine	4	4	4.0	1	1.0	3	3.2

[a] Hydrolysis was in 6 N HCl at 105 °C for 48 h under vacuum. Corrected for hydrolysis losses (Rupley & Scheraga, 1963) and given as moles of amino acid per mole of peptide. All values not recorded were less than 0.2 mol of amino acid per mol of peptide. [b] Calculated from the sequence of bovine pancreatic ribonuclease determined by Smyth et al. (1963).

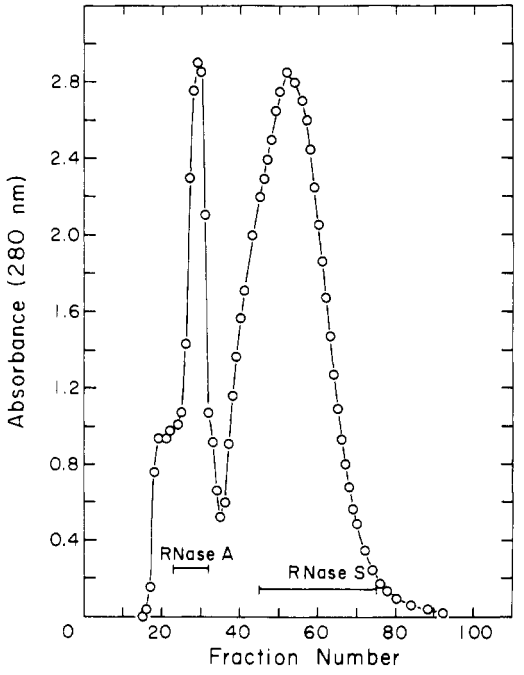

FIGURE 1: Separation of RNase S from RNase A on a column (1.5 × 50 cm) of sulfopropyl-Sephadex-C25 equilibrated and eluted in 0.13 M sodium phosphate buffer, pH 6.55; 2-mL fractions were collected. The appropriate fractions were pooled as indicated.

Methods

Preparation of Ribonuclease and Derivatives. Bovine pancreatic ribonuclease A (Sigma Chemical Co. Type II-A) was purified further by the method of Taborsky (1959) on carboxymethylcellulose (Whatman CM-52). Fractions corresponding to Taborsky's Fraction D were pooled, lyophilized, desalted on Sephadex G-15 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) in 0.1 M acetic acid, and lyophilized again.

RNase S was prepared by the procedure of Richards & Vithayathil (1959). Separation of RNase S from undigested RNase A and more extensively digested derivatives was accomplished by chromatography of 200 mg of the lyophilized protein digest in 5 mL of 0.13 M sodium phosphate buffer, pH 6.55, on a column of sulfopropyl-Sephadex-C25 (Sigma) (1.5 × 50 cm) equilibrated in 0.13 M sodium phosphate buffer

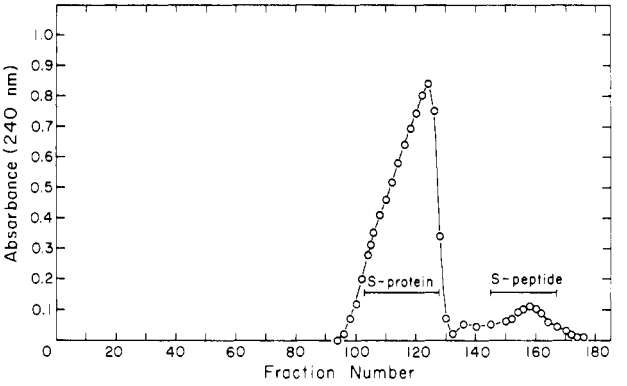

FIGURE 2: Separation of S-protein and S-peptide on a column (2 × 150 cm) of Sephadex G-50 (superfine) equilibrated and eluted in 5% acetic acid. RNase S (200 mg) was dissolved in 5 mL of the elution solvent, and the pH of the solution was adjusted to 2.0 with 6 N HCl. The effluent was collected in 5-mL fractions. The skewing of the S-protein peak was also observed by Richards & Vithayathil (1959). The fractions were pooled as indicated.

at the same pH (Figure 1). RNase S eluted as the major peak after undigested RNase A. The appropriate fractions were pooled, lyophilized, and then dissolved in 5 mL of 5% acetic acid. This solution was applied to a column (2 × 150 cm) of Sephadex G-50 (superfine) (Pharmacia Fine Chemicals) equilibrated in 5% acetic acid in order to separate S-peptide (residues 1–20) from S-protein (residues 21–124) (Figure 2). S-Peptide eluted after a skewed S-protein peak. Amino acid analysis (Table I) was used to identify the material corresponding to these peaks. Each pooled peak was lyophilized and stored as a powder at 4 °C. We found that the column used to obtain the data of Figure 1 gave better and more rapid separation than could be obtained on IRC-50 (Richards & Vithayathil, 1959).

Des(121–124)-RNase was prepared by the procedure of Anfinsen (1956), as modified by Lin et al. (1968) and Lin (1970), in order to remove the C-terminal tetrapeptide and to separate des(121–124)-RNase, undigested RNase, and the tetrapeptide. The appropriate fractions were pooled, lyophilized, desalted on Sephadex G-15 in 0.1 M acetic acid, and lyophilized again.

The reduction of RNase and its derivatives was carried out with the method of Anfinsen et al. (1961), as modified by

Teale & Benjamin (1976). The proteins were reduced by adding 2-mercaptoethanol (to a final concentration of 0.3 M) to a protein solution (5–20 mg/mL) in 8.0 M urea and 0.1 M Tris-HCl, pH 8.0. The solution was allowed to stand for 15 h under nitrogen and then desalted on Sephadex G-25 in 0.1 M acetic acid. This reduction and desalting procedure was carried out immediately before use in the oxidation (folding) experiments. By use of a sulfhydryl group titration (see below), it was found that no oxidation occurs at low pH (0.1 M acetic acid) over a period of 2 weeks at room temperature (Chavez & Scheraga, 1977); also, no oxidation was found to occur over short periods of time (~4 h) at pH ≤6.0. While 2-mercaptoethanol is not as strong a reducing agent as, say, dithiothreitol (Cleland, 1964), nevertheless the sulfhydryl titration and related data of Garel (1977) indicate that, under the conditions used here, all disulfide bonds were reduced completely [Garel (1977) found that 0.075–0.1 disulfide bond per molecule, corresponding to 0.15–0.2 sulfhydryl group, remained after 1-h reduction at a mole ratio of 40 of mercaptoethanol to $RNase_N$ and that no detectable disulfide bonds remained after a second treatment at a mole ratio of 100; since the mole ratio in our experiments was >200, we can conclude that reduction was complete]. Complete reduction of RNase is also implied by the values of K_{conf}, which are lowered significantly when the sulfhydryl groups of reduced RNase are carboxymethylated (Chavez & Scheraga, 1980).

Amino Acid Analysis. The amino acid composition of each derivative was determined by hydrolyzing in 6 N HCl for 48 h at 105 °C, followed by chromatography with a Technicon TSM automated amino acid analyzer. The results are presented in Table I.

C-Terminal Analysis. When S-protein and des(121–124)-RNase were analyzed for their C-terminal amino acids with carboxypeptidase A at pH 7.2 for 24 h at 45 °C, approximately equimolar quantities of valine, serine, and alanine and slightly less amounts of aspartic acid, phenylalanine, and histidine were found for the former, and only phenylalanine and histidine were found for the latter.

Preparation of Specific Antibodies. The preparation of rabbit anti-$RNase_N$ serum and the fractionation of antibodies on columns to which peptide fragments 1–13, 31–79, and 80–124 were attached were described previously (Chavez & Scheraga, 1977, 1979). No cross-reaction was observed among these fragments when tested at high molar ratios ($>10^4$-fold) of each fragment (relative to the fragment for which the antibody was specific).

Oxidation of Reduced Proteins. In an earlier report (Chavez & Scheraga, 1977), air was used to oxidize reduced ribonuclease. However, further experimentation showed that this method gave less reproducible results than glutathione-mediated oxidation (Hantgan et al., 1974). Our results demonstrated that the air-oxidized protein was unstable, i.e., the amount of nativelike structure and enzymatic activity in the completely oxidized protein decreased from 100% over extended periods of time (>4 h). Also, it has been shown that formation of intermolecular disulfide bonds occurs as a primary step in air oxidation, especially at high concentrations of reduced ribonuclease (Epstein et al., 1962). Both of these difficulties can be avoided by glutathione oxidation of reduced ribonuclease (Hantgan et al., 1974; Creighton, 1977; Y. Konishi and H. A. Scheraga, work in progress). In the presence of glutathione, both oxidation of sulfhydryl groups and reshuffling of disulfide bonds occur.

The kinetics of oxidation of reduced RNase, reduced S-protein, and reduced des(121–124)-RNase were studied as follows. Enough reduced protein (which was in 0.1 M acetic acid) was added to a solution of 1.0 M Tris-HCl (the *final* conditions being as follows: pH 8.0, 10^{-3} M EDTA, 5.0×10^{-4} M of both reduced and oxidized glutathione, and a protein concentration of 3.65×10^{-5} M in a total volume of 3.0 mL at 24 °C). We have found by titration of sulfhydryl groups that this concentration of EDTA is sufficient to inhibit metal ion catalyzed air oxidation completely; hence, the only oxidation that can occur is that due to glutathione. These conditions were very similar to those used by Hantgan et al. (1974), except that 1.0 M Tris-HCl was substituted for 0.1 M Tris-HCl to increase the buffer capacity. At various times, 100-μL aliquots of this solution were diluted into different volumes of a solution of 0.1 M acetic acid, which had been titrated to pH 5.0 with 2.0 M Tris base, to inhibit oxidation (and glutathione-catalyzed disulfide interchange). The final concentrations in these diluted solutions were 1.56×10^{-7}, 3.12×10^{-6}, and 3.12×10^{-7} M for RNase, S-protein, and des-(121–124)-RNase, respectively.

Inhibition Assay for Degree of Folding. The degree of folding was assessed by determining how well the partially folded protein competed with [^{125}I]$RNase_N$ for each of the purified antibodies against segments 1–13, 31–79, and 80–124, respectively. For this purpose, purified RNase A was trace iodinated by the Chloramine-T method described previously by McConahey & Dixon (1966) and Chavez & Scheraga (1977). Samples (100 μL) of the dilute arrested-oxidation solution described in the previous paragraph were used in the inhibition assay.

The inhibition assay was that of Farr (1958), as modified by Teale & Benjamin (1976) and Chavez & Scheraga (1977), except for the following changes. The [^{125}I]$RNase_N$ and the inhibiting protein from the arrested-oxidation solution were used at only one-tenth the previous concentration (Chavez & Scheraga, 1977), i.e., 0.125 μg of [^{125}I]$RNase_N$ and 0.125 μg of partially refolded RNase per tube, and the final pH in the assay tubes was 6.0 instead of 7.2. The lower concentration enabled a 10-fold reduction to be made in the amount of antibody used in the assay, and the lower pH served to inhibit air oxidation during the assay period. The reaction between antibody and either the oxidized product or [^{125}I]$RNase_N$ is completed in less than 1 min (Chavez & Scheraga, 1977). Therefore, any oxidation of reduced proteins during the assay period would have no effect on the determination of the amount of protein that refolded prior to the assay. The results are expressed as the return of antigenic activity (percent of theoretical), calculated as follows:

$$\text{return of antigenic activity (\% of theoretical)} = \frac{\text{\% inhibition}}{\text{MI}} \times 100 \quad (1)$$

where MI (maximum inhibition) is the percent inhibition obtained by an equivalent amount of native protein (Chavez & Scheraga, 1977).

Sulfhydryl Group Titration. The titer of unoxidized sulfhydryl groups in partially oxidized proteins was determined by the method of Taniuchi (1970). Twenty microliters of a solution containing 39.6 mg of 5,5′-dithiobis(2-nitrobenzoic acid) (Aldrich Chemical Co.) in 10 mL of 0.1 M sodium phosphate buffer, pH 7.0, was added to 3 mL of the oxidation mixture and allowed to incubate for 10 min at room temperature. Then the absorbance at 412 nm was measured. This same method was used to demonstrate that no oxidation of free sulfhydryl groups occurred during the incubation of reduced RNase with antibody in 1.0 M Tris-HCl (pH 8), containing 10^{-3} M EDTA. The initial titer of 8.1 SH groups per

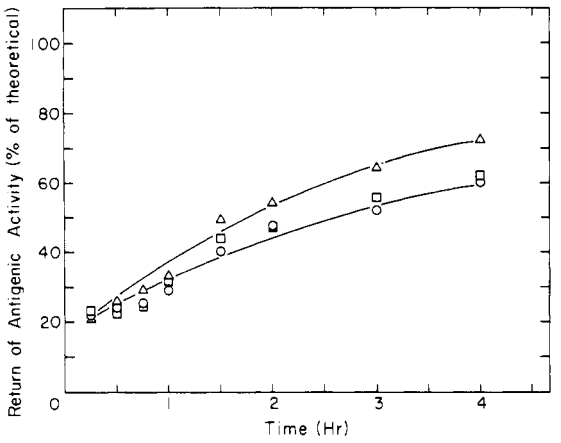

FIGURE 3: Kinetics of return of native antigenic activity, i.e., $y(t)$, during the glutathione oxidation of reduced RNase at pH 7.0 and 23 °C, as determined with the purified antibodies against fragments of ribonuclease. The oxidation conditions were those described in the text. The curves represent the points from the best fit least-squares line on a plot of ln $[y(\infty) - y(t)]$ vs. t, according to eq 2. The lower curve pertains to the folding of both segments 31–79 and 1–13. Refolding was measured by anti-$(1-13)_N$ (○); anti-$(31-79)_N$ (□), and anti-$(80-124)_N$ (△). The correlation coefficients [r, where r = (slope)σ_x/σ_y for input data points (x_i, y_i) for i = 1, 2, ..., N] for each curve were 0.987, 0.990, and 0.995, respectively.

molecule of reduced RNase decreased to 7.8 SH groups per molecule after 24 h.

Protein Concentration. The concentrations of native and reduced RNase were determined with a Zeiss Model PMQII spectrophotometer, by using extinction coefficients of 9800 M^{-1} cm^{-1} at 277.5 nm (Harrington & Schellman, 1956) and 9390 M^{-1} cm^{-1} at 276 nm (White, 1961), respectively. The concentrations of S-protein, des(121–124)-RNase, and their reduced derivatives were determined by micro-Kjeldahl nitrogen analysis (Lang, 1958; Noel & Hambleton, 1976a,b).

Results

Residual Structure in Reduced RNase. In our previous report (Chavez & Scheraga, 1977), we used ultraviolet derivative and circular dichroism spectra to try to detect residual structure in reduced RNase. Since these techniques were not sensitive enough, we have examined this question further by determining K_{conf} for reduced RNase.

When unfractionated anti-$RNase_N$ and low concentrations of reduced RNase (1:1 mole ratio to [^{125}I]$RNase_N$) were used to detect residual structure in reduced RNase, none was found (Chavez & Scheraga, 1977). In contrast, however, when the fractionated antibodies and higher concentrations of reduced RNase (400:1 mole ratio of [^{125}I]$RNase_N$) were used here, residual structure was found in all antigenic regions. The values of K_{conf} for segments 1–13, 31–79, and 80–124 were 0.073, 0.029, and 0.078, respectively (Chavez & Scheraga, 1980). If these data pertain to the entire molecule, approximately 6% of the native structure is retained in the absence of disulfide bonds in ribonuclease at pH 8.3 and 4 °C. Since the value of K_{conf} for a region of random coil would be in the range of 10^{-5}–10^{-6} (Anfinsen & Scheraga, 1975), the initial reduced ribonuclease, prior to oxidation, possesses a significant amount of native structure which must be taken into account in interpreting the data from the kinetic studies described below. This conclusion is supported by the finding of Takahashi et al. (1977) that reduced RNase possesses 14% α helix and 25% β structure (on the basis of curve-fitting of the circular dichroic spectrum) and by that of Garel (1978), who found that reduced RNase has an enzymatic activity of 0.04–1.5% toward cytidine cyclic 2′,3′-phosphate.

Table II: Kinetic Parameters for Oxidation of Reduced Ribonuclease at 23 °C and pH 7.0

antibody	$y(0)^a$ (%)	$k_2 \times 10^3$ (min^{-1})^b
anti-$(1-13)_N$	17.0	3.9
anti-$(31-79)_N$	16.1	4.4
anti-$(80-124)_N$	13.6	5.9

[a] Defined in eq 2. [b] Defined in eq 2. The corresponding average value of Hantgan et al. (1974) was 7.3×10^{-3} min^{-1} for RNase at the same concentration of glutathione, pH 7.89, and 25 °C. Since the reaction is faster at pH 7.89 than at pH 7.0 (L. G. Chavez and H. A. Scheraga, unpublished observations; Ahmed et al., 1975), the corresponding value of Hantgan et al. (1974) would be $<7.3 \times 10^{-3}$ min^{-1} at pH 7.0.

Reduced RNase. The rate of folding of reduced RNase during oxidation by glutathione is shown in Figure 3. The oxidation was performed at pH 7.0 (at 23 °C) instead of pH 8.0 in order to reduce the initial folding rates in each region and to provide better separation between each kinetic curve. By use of a least-squares analysis, the data were fit to a first-order rate equation of the form

$$y(\infty) - y(t) = [y(\infty) - y(0)]e^{-k_2 t} \quad (2)$$

where $y(t)$, $y(0)$, and $y(\infty)$ are the percent of regenerated protein (as measured by return of antigenic activity) at times t, 0, and infinite time, respectively, and the first-order rate constant is designated as k_2 for comparison with the corresponding rate constant of Hantgan et al. (1974). The values of $y(\infty)$ for segments 1–13, 31–79, and 80–124 were determined after 24 h of oxidation to be 89, 88, and 92%, respectively. Hantgan et al. (1974) found a similar value for the maximum return of enzymatic activity, 87 ± 8%.

Equation 2 is a limiting law since at least one rapid process precedes the one corresponding to the data of Figure 3. The reduced protein, which possesses some native structure, undergoes conformational fluctuations, and its cysteine residues are oxidized rapidly by oxidized glutathione, resulting in the formation of a mixture of species with wrongly paired disulfide bonds [half-time of less than 1 min at pH 7.65, 23–24 °C (Hantgan et al., 1974)]. This is followed by a disulfide exchange reaction among these wrongly paired species, with a half-time of about 5 min at pH 8.7, 25 °C (Creighton, 1977); the rate constant k_1 of Hantgan et al. (1974) presumably pertains to this rapid process. The last process, with a rate constant k_2 and a half-time of 95 min at pH 7.65, 25 °C, consists of the (slower) reshuffling of the wrongly paired disulfide bonds to the native pairing (Hantgan et al., 1974). The process whose rate is being measured here is the last reshuffling reaction which produces native antigenic activity. The residual structure in reduced RNase and the rapid process with rate constant k_1 contributes to $y(0)$. The residual native structure of reduced RNase contributes $K_{conf}/(1 + K_{conf})$, or 6.8, 2.8, and 7.2% for segments 1–13, 31–79, and 80–124, respectively (Chavez & Scheraga, 1980), and the rapid process with rate constant k_1 (producing some degree of native structure recognized by the antibodies) contributes the balance to $y(0)$. The values of $y(0)$ and k_2 for each segment were obtained from a logarithmic plot of eq 2, weighting the points at longer times more than those at earlier times. The intercept is ln $[y(\infty) - y(0)]$ which, together with the known value of $y(\infty)$, gives $y(0)$, and the slope is $-k_2$. The values of $y(0)$ and k_2 are shown in Table II. The values of k_2 for the folding of the antigenic regions of RNase indicate that segment 80–124 folds faster than segments 31–79 and 1–13.

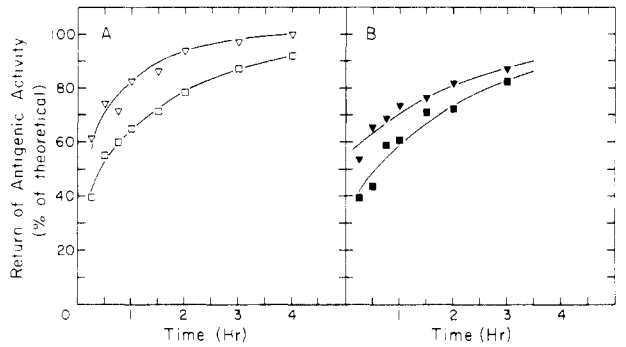

FIGURE 4: Kinetics of return of native antigenic activity during the glutathione oxidation of reduced S-protein at pH 8.0 and 23 °C, as determined with the purified antibodies used in Figure 3. The oxidation conditions were those described in the text. The curves represent only an approximation to the data. (A) Oxidation of reduced S-protein alone; refolding was measured by anti-$(31-79)_N$ (□) and anti-(80–124) (▽). (B) Oxidation of reduced S-protein in the presence of an equimolar concentration of S-peptide; refolding was measured by anti-(31–79) (■) and anti-(80–124) (▼).

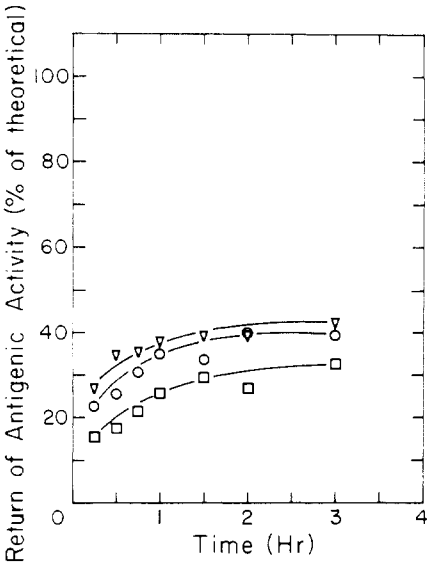

FIGURE 5: Kinetics of return of native antigenic activity during the glutathione oxidation of reduced des(121–124)-RNase at pH 8.0 and 23 °C, as determined the purified antibodies used in Figure 3. The oxidation conditio... ..ere those described in the text. The curves represent only an approximation to the data. Refolding was measured by anti-$(1-13)_N$ (○), anti-$(31-79)_N$ (□), and anti-$(80-124)_N$ (▽).

The data of Figure 3 also demonstrate that approximately 20% of the native antigenic activity has returned to all segments very early during folding. During the glutathione oxidation of RNase, all segments appear to begin with a low level of native structure (~6%) and attain ~20% native structure after 15 min. This is in contrast to the amount of native structure deduced from the enzymatic activity (8% after 17 min) in reoxidized ribonuclease at pH 7.65, 23 °C (Hantgan et al., 1974). The latter technique appears to underestimate the amount of native structure. We can conclude from our data, in conjunction with those of Hantgan et al., that a significant amount of native structure, at least in the regions around the antigenic determinants, exists in the intermediates. This observation differs from the suggestion of Takahashi et al. (1977) that the disulfide bonds form prior to stable, regular backbone structures.

Reduced S-Protein. If the primary nucleation site does reside in segment 80–124 and is uninfluenced by segment 1–13, then the oxidation of a reduced derivative that lacks segment 1–13 (reduced S-protein) should result in a significant return of native antigenic activity. Tsong et al. (1970) have demonstrated that S-protein is much less stable (to thermal denaturation) than native RNase; hence, it would be expected that higher concentrations of S-protein would be required in the inhibition assay in order to detect the return of native structure. Experiments with "native" S-protein did indeed bear out this expectation since a 20-fold increase in the concentration of S-protein (compared to native RNase) was required to achieve the same degree (50%) of inhibition of the binding of unfractionated anti-$RNase_N$ antibody to [^{125}I]RNase [see Figure 6 of Chavez & Scheraga (1979)]. The decreased stability of S-protein relative to native RNase is also indicated by a low value of K_{conf} (average of ~0.06) (Chavez & Scheraga, 1980). Therefore, the concentration of regenerated S-protein in the inhibition assay was increased 20-fold in order to compensate for the lower stability. Complete conversion of reduced S-protein to "native" S-protein would lead to 50% inhibition, because of the relative concentrations chosen for S-protein and [^{125}I]$RNase_N$. [As shown previously (Chavez & Scheraga, 1979), excess "native" S-protein (i.e., much more than 20-fold) leads to 100% inhibition of the binding of purified anti-$(31-79)_N$ and anti-$(80-124)_N$ to [^{125}I]$RNase_N$.]

The rate of folding of reduced S-protein, in the absence and presence of S-peptide, is shown in parts A and B of Figure 4, respectively. In contrast to the folding of reduced RNase, the process here is more complicated, involving not only similar types of consecutive reactions but also the folding/unfolding equilibrium of "native" S-protein, i.e., a reverse reaction which arises from the significant amount of unfolding of S-protein as compared to native RNase. For $RNase_N$, the folding equilibrium is completely in the direction of the native structure. Therefore, instead of presenting a quantitative analysis of the data in Figure 4, we interpret them qualitatively.

"Native" S-protein is folded to an extent given by $K_{conf}/(1 + K_{conf})$ = 7.9 and 3.5% for segments 31–79 and 80–124, respectively (Chavez & Scheraga, 1980). The data of Figure 4 have been normalized by designating these two values as 100% for each of the two segments. It can be seen in Figure 4A that segment 80–124 achieves its degree of "native" folding faster than does segment 31–79 and that both segments approach 100% of the folding of "native" S-protein after 4 h of oxidation. Addition of S-peptide at an equimolar concentration to reduced S-protein, at the start of the oxidation reaction, decreased the rate of folding of segment 80–124 significantly but had little effect on the rate of folding of segment 31–79 (see Figure 4B). The rapid folding of segment 80–124 supports the hypothesis that this segment contains the primary nucleation site in RNase. The ability of S-protein to fold without S-peptide suggests that S-peptide does not play a major role in the initial stages of folding, except possibly to supply some competing interactions that slow down the folding of segment 80–124.

Reduced Des(121–124)-RNase. In studying the folding of this reduced protein, its concentration was increased twofold to account for its decreased stability compared to that of RNase [see Figure 5 of Chavez & Scheraga (1979)]. As can be seen in Figure 5 of the present paper, each antigenic region folded to a maximum extent of approximately 35%. This value remained constant up to 24 h, and no perceptible precipitation (indicating no extensive aggregation) occurred during this period. This degree of folding is larger than that found by Taniuchi (1970) for air oxidation. Taniuchi (1970) has shown that wrongly paired intramolecular disulfide bonds form preferentially during air oxidation of des(121–124)-RNase. Only 1% of the air-oxidized des(121–124)-RNase molecules

possess the native disulfide pairings; i.e., there appears to be a random distribution among the 105 theoretically possible pairs of 4 disulfide bonds. Mispairing of disulfide bonds is likely ot occur also during the early stages of glutathione oxidation of des(121–124)-RNase. The question then arises as to whether our higher value arises from cross-reaction between the product with mispaired disulfides and the native protein.

The extent to which the return of antigenic activity represents the return of native structure in regenerated des(121–124)-RNase and the other RNase derivatives must be considered in light of the results of Mills & Haber (1963). They found that reduced RNase which was oxidized in the presence of urea (and possesses randomly paired disulfide bonds; Haber & Anfinsen, 1962) cross-reacted with native RNase to the extent of about 10% using anti-$RNase_N$ that had been induced with complete Freund's adjuvant [as was the case in our experiments; see Chavez & Scheraga (1977, 1979)]. This amount of cross-reaction can explain some of the competitive inhibition seen in Figures 3–5, especially during the initial stages of oxidation (<15 min), but would not affect our computed values of k_2 since these initial folding reactions are much faster than the one to which k_2 applies. The *increase* in antigenic activity afterward, however, cannot be due to cross-reaction but must be considered to be due to folding in which the protein approaches the native conformation to an increasing extent. [It would be interesting to determine the disulfide bond arrangements of regenerated des(121–124)-RNase after glutathione oxidation to see if there is more native disulfide pairing than random.] Therefore, des(121–124)-RNase appears to be capable of folding to the native conformation to some extent. Also, since a limited, but significant, amount of native folding does occur in des(121–124)-RNase, i.e., in the absence of the C-terminal tetrapeptide, this tetrapeptide cannot be involved in the nucleation site. It must, however, supply interactions that are essential for the stabilization of the native conformation.

Discussion

It is difficult to distinguish between the residues of RNase that are required for nucleation and those that are necessary for later stages of folding. Identification of possible nucleation sites has been attempted by determining (1) which residues participate in the earliest detectable kinetic step in the folding process (Chavez & Scheraga, 1977), (2) which residues remain the most resistant to denaturation (Burgess & Scheraga, 1975; Matheson & Scheraga, 1979), (3) which residues participate in the strongest short-range interactions in the folded protein, assuming that residue contacts forming a tight grouping near the diagonal of a triangular map are those that arise first during folding (Takahashi & Ooi, 1976; Tanaka & Scheraga, 1977), (4) which residues participate in the strongest hydrophobic interactions in the unfolded protein (Matheson & Scheraga, 1978), and (5) which residues can be eliminated from the nucleation region because they have no effect on the initiation of folding. We have used procedures 1, 4, and 5 to demonstrate the existence of a nucleation site and to identify its location.

Studies of the folding of reduced RNase, as detected by the return of antigenic activity in segments 1–13, 31–79, and 80–124, indicate that segment 80–124 folds before the other segments during both air (Chavez & Scheraga, 1977) and glutathione oxidation. But there are differences between the two processes which reflect the differences between air and glutathione oxidation pointed out by Hantgan et al. (1974) and Chavez & Scheraga (1977). The fast initial return of antigenic activity in all segments (Figure 3; <15 min) is consistent with the rapid initial decrease in SH titer and the immediate return of enzymatic activity during glutathione oxidation; in contrast, there is a lag period for the return of enzymatic activity during air oxidation (Anfinsen et al., 1961) which is consistent with the slow return of antigenic activity in segment 31–79 (Chavez & Scheraga, 1977). By demonstrating the existence of a sequential regeneration of native antigenic determinants, these results confirm the hypothesis (Burgess & Scheraga, 1975) that there is a definite folding pathway with detectable intermediate conformational states.

While the data of Figure 3 indicate that segment 80–124 folds faster than the others, the data are not accurate enough to determine whether a species with segment 80–124 folded, but with the other segments unfolded (and with wrongly paired disulfide bonds), is on the direct folding pathway or is on an unproductive bypass. Nevertheless, even if such a species were on a bypass (and had to have its disulfide bonds reshuffled to return to the correct folding pathway), the rapid formation of antigenic activity in segment 80–124 is indicative of the intrinsic character of the RNase molecule, viz., the existence of strong interactions (presumably the hydrophobic bonds in the nucleation site, segment 106–118) that lead to rapid formation of the antigenic site in this portion of the molecule.

Creighton (1979) has recently questioned the existence of intermediates in the folding pathway, because the folding process is allegedly a two-state one. Indeed, the folding process is a two-state one in the sense that the intermediates are always at low concentration. If, however, one uses a method (such as the present immunological technique) that can detect such intermediates, then information *is gained* about the pathway from the unfolded to the folded form. One possible interpretation for the existence of the wrongly paired disulfide species identified by Creighton is that they represent trapping of species that are *not* on the folding pathway; such trapping could occur simply when the reduced protein, in undergoing conformational fluctuations near that of the native structure, encounters the oxidizing agent (with an attendant rapid formation of many wrongly paired disulfide species). As Matheson & Scheraga (1978) pointed out, "once wrong disulfide bonds are formed, the normal folding pathway is blocked ... and ... species with incorrect pairings must wait for a free SH to aid in breaking wrong disulfide bonds and permit S–S reshuffling and refolding"; i.e., the correct pathway of folding [involving nucleation by a mechanism such as that proposed by Matheson & Scheraga (1978)] could be a bypass (from various stages) of the one shown by Creighton—and local interactions in the polypeptide chain (not disulfide pairings) could direct the folding. If the concentrations of correctly folded intermediates in Creighton's trapped species were too low to be detected by his procedures, they would not be recognized by his kinetic data (Creighton, 1977).

There are some experimental problems that could influence the nature of the species identified by Creighton. His use of carboxymethylation to identify intermediates perturbs the system being studied. We find values of K_{conf} of 0.06 and 0.0013 for reduced RNase and carboxymethylated RNase, respectively (Chavez & Scheraga, 1980); i.e., carboxymethylation of the free SH groups reduced K_{conf} by a factor of 50, presumably because of the charges on the added carboxymethyl groups. In addition, Spackman et al. (1960) have found that disulfide bond interchange occurs between oxidized glutathione and cystine during *extended periods* (30 h) of incubation at pH 6.7, 40 °C, in the presence of 1 mM EDTA, conditions similar to those used by Creighton to isolate

his intermediates; i.e., Creighton's intermediates may not have been stable against disulfide reshuffling. Further, the techniques used by Creighton (activity measurements and spectrophotometric analysis) are not as sensitive as the immunological one used here to detect intermediates. Finally, our results are *not* due to "variable extents of formation of refolded RNase during the measurements", as Creighton suggested, since our results are *independent* of the time required for the assay (between 5 and 30 min) because of the rapidity of the reaction between antibody and [^{125}I]RNase$_N$ and the stability of the immune complex (Chavez & Scheraga, 1977). Hence, our kinetic data do pertain to the rate of folding under the conditions of our experiments.

The high initial amount of folding during glutathione oxidation, i.e., the large values of $y(0)$, indicated by the immunological methods employed here, as compared to the lower initial enzymatic activity found by Hantgan et al. (1974), could be explained by the following reasons. (1) Each segment does show significant native structure and antigenic activity even in the reduced protein ($\sim$6%) (Chavez & Scheraga, 1980). (2) A significant number of the 105 theoretically possible disulfide intermediates, that would result from a random pairing of half-cystines, have been shown to cross-react with antibodies against RNase (Mills & Haber, 1963). If disulfide pairing occurred randomly, about 14% of the antigenic activity of RNase$_N$ would be expected to return to molecules having at least one native disulfide (Sela & Lifson, 1959); thus, the observed contribution of $\sim$10% to $y(0)$ is not unreasonable. Although such intermediates interconvert to other species, the antibodies can recognize, and react with, the native-like conformations therein. These intermediates affect the measured value of the return of antigenic activity primarily during the initial stage of folding because the number of mispaired disulfide intermediates will be greatest during this period. Successive intermediates should become more constrained to the native arrangement of disulfide bonds during the reshuffling process. Therefore, the amount of native structure, which is on the pathway to complete native folding, is overestimated by the return of antigenic activity. The overestimation is largest initially and decreases progressively to a level that corresponds to the maximum return of native structure ($\sim$87%) determined from the enzymatic activity (Hantgan et al., 1974). The cross-reaction between native RNase and the folding intermediates would affect the kinetic data obtained early (<15 min) in the folding process because the antibody may recognize intermediates (with native determinants) that are not on the folding pathway. The high initial amount of folding is consistent with unpublished results of Y. Konishi and H. A. Scheraga wherein the concentrations of a number of isolatable intermediates increase rapidly initially and then decrease with a concomitant increase in the concentration of native RNase. We do not observe a *decrease* in antigenic activity, presumably because it is masked by a continuous increase in the concentration of intermediates (and ultimately of RNase$_N$) possessing the native structure. The return of antigenic activity would then represent several differently folded forms and not a single one, as implied by eq 2, during the *initial* stages of folding. The data obtained later in the run, which were weighted more heavily, do lead to more correct values of $y(0)$ and k_2.

The fair agreement of k_2 determined here (Table II) for each antigenic region and by the spectrophotometric methods of Hantgan et al. (1974) indicates that both methods observe the same reshuffling-dependent reaction. The initially high antigenic activity also seems to indicate that there is a great reduction in the number of conformations which are present in the early stages of folding and argues against the presence of an initial *totally* random pairing of the disulfides of RNase.

Our results for the folding of S-protein during glutathione oxidation, which demonstrated complete return of the antigenic activity of "native" S-protein, differ from those of Kato & Anfinsen (1969), who used the disulfide interchange enzyme of DeLorenzo et al. (1966) during air oxidation and showed that approximately 20% of the enzymatic activity returned to S-protein (S-peptide was used only to assay for enzymatic activity and not during oxidation). The inclusion of S-peptide increased the efficiency (rate and extent) of the return of enzymatic activity in their experiments but had no such effect on the return of antigenic activity in similar experiments presented here.

These differences probably do not arise because of differences in the methods of oxidation since both methods have been shown to be capable of producing native protein from scrambled disulfide bonds in RNase (Kato & Anfinsen, 1969; unpublished observations in our laboratory). Hence, differences in results must depend on the methods used to assay for the return of native structure. It has been demonstrated previously that some antigenic cross-reaction ($\sim$10%) occurs between randomly scrambled RNase and native RNase (Mills & Haber, 1963) even when no enzymatic activity was demonstrable in the nonnative protein. Hence, a greater retention of native conformation is required for enzymatic activity than for antigenic determinants. The product obtained in the oxidation of S-protein in the absence of S-peptide possesses the antigenic behavior of "native" protein. This observation is consistent with the finding that S-protein (in the early stages of oxidation where many wrongly paired disulfide bonds form) contains a significant amount of correctly paired disulfide bonds (Kato & Anfinsen, 1969). Therefore, a significant amount of cross-reaction would also be expected between native RNase and regenerated S-protein. The low level of enzymatic activity, however, that they found during the oxidation of S-protein in the absence of S-peptide definitely appears to be due to some molecular distortion caused by wrongly paired disulfide bonds since the addition of S-peptide for assay purposes did not yield 100% enzymatic activity (Kato & Anfinsen, 1969). S-Peptide does appear to increase the stability of some of the native disulfide bonds, since "one of the native S–S bonds was entirely missing", according to Kato & Anfinsen (1969). But, it is clear from our results that nucleation and native folding can occur in the absence of S-peptide. The level of return of enzymatic activity to S-protein may be anomalously low when S-peptide is added for assay purposes because it may be difficult for S-peptide (which contains the active-site residue His-12) to bind to a distorted but significantly native structure in the regenerated material.

S-Peptide is required for complete native folding but it hinders the folding process as seen from Figure 4 (compare the rates of folding for region 80–124 in S-protein in the presence and absence of S-peptide). The following are possible explanations for this behavior, based on the nucleation model of Matheson & Scheraga (1978). Nucleation at residues 106–118 may promote the folding of the β structure in residues 96–110 [or the antigenic site in 98–104 (Chavez & Scheraga, 1979)]. Phenylalanine-8, however, which is included in the same hydrophobic pocket as residues 106–118 in native RNase, may compete for the interactions with residues 106–118 and, thus, may prevent the stabilizing interactions between the nucleation residues and the antigenic determinant in residues 98–104. These competing reactions are plausible in terms of

the folding model of Burgess & Scheraga (1975), as modified by Matheson & Scheraga (1979). The folding of residues 81–102 precedes or overlaps that of residues 1–16, making interactions between these two sets of residues quite likely.

Similarly, our results for the glutathione oxidation of des-(121–124)-RNase differ somewhat from those of Taniuchi (1970), who used air oxidation. Whereas we find some degree of return of native antigenic activity (see Figure 5), Taniuchi found no return of the circular dichroic properties of the "native" protein. The most significant difference between these two results is in the methods of oxidation. Although we did not measure the sulfhydryl titer during glutathione oxidation of reduced des(121–124)-RNase, one would expect that the number of sulfhydryl groups would decrease to a level of ~1 SH group per molecule within the first 5 min of oxidation since we used the same experimental conditions as Hantgan et al. (1974) used for RNase. In contrast, under the conditions used for air oxidation of des(121–124)-RNase, the process is much slower, taking 4–5 h to achieve the same degree of oxidation (Taniuchi, 1970). Also, for intact RNase, the initial products of oxidation differ, being primarily intermolecular aggregates during air oxidation but intramolecular monomers during oxidation with a sulfhydryl reagent (Epstein et al., 1962). These results indicate that these two methods of oxidation involve different mechanisms. Furthermore, disulfide rearrangement during glutathione oxidation appears to be more efficient since there is a lag period for regain of enzymatic activity during air oxidation (Anfinsen et al., 1961) but there is none during glutathione oxidation (Hantgan et al., 1974). Our own results have demonstrated that, while glutathione-oxidized RNase is stable after reaching maximal levels of enzymatic and antigenic activities, the air-oxidized protein is unstable in that the enzymatic activity and antigenic activity of the completely oxidized protein decreases from maximum values over extended periods of time (>4 h) (L. G. Chavez, Y. Konishi, and H. A. Scheraga, unpublished observations). These observations suggest that products of air oxidation of reduced des(121–124)-RNase may not be native. In contrast, glutathione oxidation can produce stable, monomeric molecules which retain at least some nativelike structural characteristics (viz., antigenic determinants).

In a previous paper (Chavez & Scheraga, 1979), the four antigenic determinants of RNase have been located in segments 1–10, 40–61 (around residues 49–52), 63–75 (probably containing the β bend at residues 66–68), and 87–104 (probably at residues 98–104). These data, and those reported in this paper, can be used to examine the validity of the folding pathway proposed by Burgess & Scheraga (1975), as modified by Matheson & Scheraga (1979). The revised form of the Burgess–Scheraga pathway for the folding of RNase (with disulfide bonds intact) consists of the following six stages (the primes referring to folding, whereas the unprimed stages of Burgess and Scheraga refer to unfolding): I′, residues 35–50, 62–74, and 81–102 fold; II′, residues 1–12 fold; III′, residues 51–60 and 104–124 fold; IV′, residues 27–34 and 75–80 fold; V′, residues 13–25 fold and residues 1–12 become less exposed to solvent; VI′, residue 92 folds to its native conformation. This pathway was proposed on the basis of experimental data on the thermal denaturation of RNase and has been supported by subsequent studies (Burgess et al., 1975; Chen & Lord, 1976; Matheson et al., 1977; Howarth, 1979; Matheson & Scheraga, 1979). Nevertheless, it may not be directly applicable to the folding that accompanies oxidation of sulfhydryl groups. Our data indicate, however, that stage I′ can be further subdivided. The folding of the determinant in segment 87–104 precedes the folding of the determinants in segments 40–61, 63–75, and 1–10. This conclusion assumes that both of the determinants in the segment 31–79 fold at comparable rates. Thus, the Burgess–Scheraga pathway must be modified to show that residues 81–102 fold prior to residues 62–74 and 35–50 and, on the basis of the data of Figure 3, that residues 1–10 closely parallel the folding of these last two segments. It is reasonable that the folding of residues 62–74, 35–50, and 1–12 would overlap since some destabilization of the native structure in residues 35–50 and 62–74 would be expected when the disulfide bonds at half-cystines-40, -65, and -72 are not present during the initial stages of folding. This results in the merging of stages I′ and II′, the overlap of which already appeared in the Burgess–Scheraga pathway.

Residues 104–124 were proposed to fold during stage III′. Evaluation of the evidence for incorporation of these residues in this stage shows that it is based only on the folding of residues His-105 and -119 (deduced from NMR analysis of thermal denaturation; Roberts & Benz, 1973) and residues 121–124 (analyzed by exposure to carboxypeptidase A during thermal denaturation; Burgess et al., 1975), all of which are outside of the proposed nucleation region. No direct evidence supports the folding of residues 106–120 in stage III′. Therefore, this region cannot be excluded as a nucleation site.

Since the formation of antigenic determinants in des(121–124)-RNase occurs to some extent (see Figure 5), it appears that residues 121–124 are not necessary for the initial stage of folding. Matheson & Scheraga (1978) have suggested that residues 106–118 contain the nucleation site for RNase. This suggestion was made on the basis of the strong hydrophobic interactions which would be the driving force for folding of this segment. Thus, stage III′ would be modified to include only residues 51–60 and 121–124, and the folding of residues 106–118 (which is interior in the protein and, hence, not immunogenic or antigenic) must precede the folding of residues 81–102. This result explains the finding that the antigenic determinant in segment 87–104 folds before the other antigenic determinants. The most likely location of this determinant is in segment 98–104 (Chavez & Scheraga, 1979) which is at one edge of the hydrophobic pocket in segment 106–118.

In summary, the folding pathway [consistent with our results and those reported by Burgess & Scheraga (1975) and Matheson & Scheraga (1978, 1979)] during the oxidation of reduced RNase consists of the following stages. The primary nucleation site is in the hydrophobic core at residues 106–118. This nucleated region then induces the folding of the antigenic determinant in residues 87–104 (Chavez & Scheraga, 1979), perhaps by interaction with residues 120–124. The antiparallel β pleated sheet formed from residues 81–87 and 96–110, with the intervening β bend at residues 92–94, is a structural component in this folded region. As folding continues, the polypeptide chain decreases the exposure of the primary nucleation site (residues 106–118) to the solvent. After residues 63–75 are in place, the β bend at residues 66–68 returns the chain around the hydrophobic core again and deposits the last section of β sheet in residues 40–48. According to the order in which antigenic activity returns, segments 63–75 and 40–48 could fold sequentially, as suggested here, or simultaneously. Residues 1–12 also appear to fold at this stage, probably through stabilizing interactions with the C-terminal residues. At this point (i.e., after the folding of residues 1–12), most of the essential structural characteristics of RNase have been established. The hydrophobic core has been buried, and the remaining residues pack in order to decrease the overall free energy. From our results with S-

protein and des(121–124)-RNase, we see that residues 1–12 and 121–124 are not necessary for nucleation of the folding of RNase. But, segment 1–13 does contain His-12, which is required for enzymatic activity, and Phe-8, which interacts with the interior of the protein, very close to the initial hydrophobic core. It may be that the burial of Phe-8 and the positioning of His-12 in the active site shift the hydrophobic contacts slightly from residues 106–118 in the postulated nucleation site to where they occur in the native structure, viz., residues 106–120; such a shift is allowed for in the nucleation model of Matheson & Scheraga (1978).

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