mine procedure introduced by van der Westhuyzen and von Holt (1971), and, finally, our procedure should be readily applicable to isolation of other DNA-binding proteins, including the nonhistone chromatin proteins that are dissociated by NaCl.

Acknowledgments

We thank Dr. David J. Cox for stimulating discussions throughout the course of this work and for his critical reading of the manuscript. In addition we thank Dr. A. W. Burgstahler of the University of Kansas for generously allowing us to use the circular dichroism instrument.

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Immunological Determination of the Order of Folding of Portions of the Molecule during Air Oxidation of Reduced Ribonuclease†

Lloyd G. Chavez, Jr.,‡ and Harold A. Scheraga*

ABSTRACT: An immunological method is used to follow the folding of different portions of the reduced bovine pancreatic ribonuclease molecule during air oxidation. Antibodies that react specifically with segments 1-13, 31-79, and 80-124 of native ribonuclease, as they are folded, were purified by affinity chromatography, using antiserum to native ribonuclease and columns to which the ribonuclease fragments were attached. The kinetics of reaction between these purified antibodies and refolded portions that are produced when reduced ribonuclease is oxidized by air demonstrate the presence of intermediate states of folding, and are consistent with folding of the antigenic determinants in the order 80-124, 1-13, and 31-79. The relative stabilities of each of these segments to thermal denaturation in the native protein provide additional evidence that the native conformation of region 80-124 is a very stable one in the intact molecule. On the basis of these two types of evidence, it appears that segment 80-124 contains a nucleation site for the folding of the protein molecule.

Several methods are being used to determine the pathway of folding of either thermally denatured or reduced bovine pancreatic ribonuclease A. These include calorimetric measurements (Tsong et al., 1970), kinetic measurements (Tsong et al., 1971; Hantgan et al., 1974; Garel and Baldwin, 1975; Garel et al., 1976), measurements of the sequence of formation of disulfide bonds (Hantgan et al., 1974), nuclear magnetic resonance (NMR) and proteolytic digestion studies (summarized by Burgess and Scheraga, 1975; Burgess et al., 1975; Benz and Roberts, 1973), laser Raman spectroscopy (Chen and Lord, 1976), flash photolytic labeling of exposed segments of the molecule (Matheson et al., 1977b), and measurement of correlation times of electron spin resonance (ESR) labels attached to various segments of the molecule (Matheson et al., 1977a). In this paper, we apply the immunological technique of Teale and Benjamin (1976a,b) and Chavez and Benjamin (manuscript in preparation) to determine the order in which the various portions of the molecule fold during air oxidation of reduced ribonuclease. Purified antibodies, which are specific for different antigenic sites of the native ribonuclease molecule, are used in kinetic experiments to detect the rates at which the

† From the Department of Chemistry, Cornell University, Ithaca, New York 14853. *Received January 13, 1977.* This work was supported by research grants from the National Science Foundation (PCM75-08691) and from the National Institute of General Medical Sciences, National Institutes of Health, U.S. Public Health Service (GM-14312).

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TABLE I: Amino Acid Analyses of Cyanogen Bromide Fragments[a] (Moles of Amino Acid/Mole of Peptide).

Amino Acid Residue	Fragment 1–13	Residues 1–13[b]	Fragment 31–79	Residues 31–79[b]	Fragment 80–124	Residues 80–124[b]
Lysine	2.1	2	4.5	5	3.4	3
Histidine	0.8	1	0.9	1	1.6	2
Arginine	1.0	1	2.0	2	1.3	1
S-Carboxymethylcysteine			4.1		3.0	
Aspartic acid			7.0	7	5.2	5
Threonine	0.9	1	4.2	4	3.6	4
Serine			4.7	5	3.9	4
Glutamic acid	3.0	3	5.9	5	3.4	3
Proline			1.1	1	2.9	3
Glycine			1.2	1	2.0	2
Alanine	2.6	3	3.3	3	4.1	4
Valine			4.7	5	3.1[e]	4
Methionine[c]	0.9	1	0.9	1		
Isoleucine					1.6[e]	3
Leucine			2.1	2		
Tyrosine			2.1	2	2.7	3
Phenylalanine	0.9	1	0.8	1	1.1	1
Half-cystine				4[d]		3[d]
Total residues		13		49		45

[a] All values not recorded were less than 0.2 mol of amino acid per mol of peptide, and have been corrected for hydrolysis losses (Rupley and Scheraga, 1963). [b] Calculated from the sequence of bovine pancreatic ribonuclease determined by Smyth et al. (1963). [c] Also represents values for homoserine in cyanogen bromide fragments. [d] The remaining half-cystine is in fragment 14–29. [e] The Ile-107–Val-108 bond is incompletely hydrolyzed under these conditions (24-h hydrolysis).

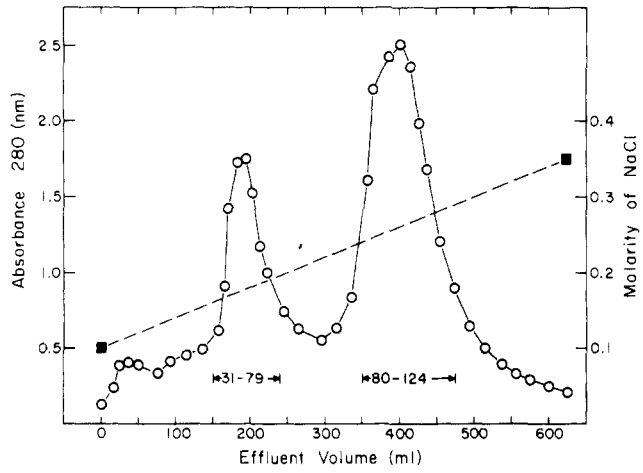

FIGURE 1: Fractionation of a cyanogen bromide digest of ribonuclease. After reduction and carboxymethylation, the desalted digest (0.5 g) was applied to a DEAE-A25 Sephadex column (44 × 2.3 cm) in 0.005 M Tris-HCl (pH 8.0). A gradient of 0.1 to 0.35 M NaCl was used for elution, 5-mL fractions were collected, and the absorbance was measured at 280 nm. Fractions 31–79 and 80–124 were pooled as indicated, and later rechromatographed under the same conditions.

antigenic sites acquire their native conformation during the air oxidation of reduced ribonuclease. A model for the folding pathway, based on the x-ray structure of ribonuclease S (Wyckoff et al., 1970) and on earlier studies of folding, was postulated by Burgess and Scheraga (1975), and the experiments described here were designed to test and improve this model.

Methods

Preparation of Ribonuclease Fragments. Bovine pancreatic ribonuclease A (Sigma Type II-A) was used without further purification. Fragments of ribonuclease were prepared by cyanogen bromide (Eastman Kodak, Rochester, N.Y.) digestion. Fragment 1–13 was prepared from the CNBr digest of ribonuclease with intact disulfide bonds, using the procedure of Brown and Klee (1971). The remaining portion, containing residues 14–124, was reduced in 8.0 M urea, 0.1 M Tris-HCl, and 0.6 M 2-mercaptoethanol (pH 8.0). The sulfhydryl groups were carboxymethylated over a period of 30 min by addition of a 1:1 (w/w) ratio of iodoacetic acid to protein at pH 8.0 (2 M Tris). This solution was desalted on a Sephadex G-25 (coarse) column (160 × 2.5 cm) in 0.2 M acetic acid, and the pooled digest was lyophilized. This material was fractionated by ion exchange chromatography on a DEAE-A25 Sephadex column (44 × 2.3 cm) in 0.005 M Tris-HCl (pH 8.0), using a 0.1–0.35 M NaCl linear gradient (Figure 1) in the same buffer, to obtain fragments 31–79 and 80–124. Fragment 14–29 could not be purified adequately by these procedures, and therefore was not available in these studies.

The three peptide fragments were desalted as above, lyophilized, and hydrolyzed in 6 N HCl for 24 h, and their amino acid compositions were determined with a Technicon TSM automated amino acid analyzer. The results are given in Table I.

Low voltage electrophoresis on thin-layer cellulose plates at pH 5.4 (loaded with ~20 μg of peptide) showed only single ninhydrin-positive spots for each of these three peptides.

Preparation of Rabbit Anti-RNase$_N$ Serum.[1] The procedure of Brown (1962) was used to immunize New Zealand white rabbits (kindly donated by Dr. D. C. Benjamin) with native ribonuclease to produce anti-RNase$_N$. Antibody titers leveled off after the sixth booster injection. Hyperimmune antisera were pooled from two rabbits which had been given at least six booster injections. Bleeding of each rabbit was carried out 1 week after the booster injections. Monthly booster injections and bleedings continued until a substantial antiserum pool was obtained.

[1] The following nomenclature is used to designate each of the antibody preparations: anti-native ribonuclease, anti-RNase$_N$; anti-native (1–13), anti-(1–13)$_N$; anti-native (31–79), anti-(31–79)$_N$; and anti-native (80–124), anti-(80–124)$_N$.

Trace Labeling of Native Ribonuclease. Trace labeling of ribonuclease was accomplished by the procedure of McConahey and Dixon (1966) with carrier-free sodium [^{125}I]iodide (Schwarz/Mann). Double immunodiffusion analysis (Ouchterlony, 1948) of labeled ribonuclease and unlabeled native ribonuclease with unfractionated anti-RNase$_N$ antiserum indicated that labeling did not disrupt the ribonuclease structure detectably in the regions of the antigenic determinants, since the antiserum reacted identically with unlabeled and labeled ribonuclease.

Fractionation of Antibodies. The antibodies were fractionated on columns to which native ribonuclease and peptide fragments 1–13, 31–79, and 80–124 were attached. Each peptide or protein was coupled [through its lysine ϵ-amino group(s)] to Sepharose 4B (Pharmacia Fine Chemicals), using the method of Sachs et al. (1972a). Approximately 1 μmol of peptide or protein was coupled to 1 mL of packed Sepharose as determined by the nitrogen content of 0.1 mL of coupled Sepharose.

Hyperimmune antisera were freed of complement by precipitation at pH 5.4 (carried out by dialysis against 0.02 M sodium acetate (pH 5.4) overnight) and centrifugation. The supernatant was then dialyzed against 0.15 M NaCl and applied and reapplied to a column to which one of the fragments was bound. The final effluent from this column was then applied in a similar manner to a second column (to which a second fragment was bound), and then to a third column. Each column was washed with 0.15 M NaCl and eluted with 0.2 M sodium citrate (pH 2.2) and the eluent was neutralized with 1.0 M NaOH and dialyzed against 0.15 M NaCl, according to Sachs et al. (1972a) to yield purified antibodies against the various fragments. Only the antibody that was bound to each affinity column was assumed to be specific for the particular peptide (or intact ribonuclease) bound to the column. No further protein could be released from these affinity columns by washing with either 2 M acetic acid or 8 M urea, indicating that the elution buffer had removed all of the specifically bound antibody. It should be emphasized, as pointed out by Sachs et al. (1972b), that antibodies isolated in this way bind only to that region of the fragment which is folded in the same conformation as in native ribonuclease.

The specificity of each purified antibody for the peptide with which it was isolated, i.e., the absence of cross-reactivity of the peptides used in these studies, was measured by determining the extent of binding between [^{125}I]RNase and the purified antibodies in the presence of excesses of the nonhomologous peptides in the standard inhibition assay described below. The results showed that there was no inhibition activity with any of the nonhomologous peptides at up to five orders of magnitude molar excess of peptide to [^{125}I]RNase. However, inhibition was evident for the homologous peptides at lower molar excesses. Therefore, within the limits of detectability indicated here, there was no cross-reactivity between peptides.

Reduction of Ribonuclease. Ribonuclease was reduced by the method of Anfinsen and Haber (1961), as modified by Teale and Benjamin (1976a). Ribonuclease was reduced by adding 2-mercaptoethanol (to a final concentration of 0.3 M) to a solution of the protein (20 mg/mL) in 8.0 M urea–0.1 M Tris-HCl (pH 8.0). The solution was allowed to stand for 3 h (under nitrogen), and was then desalted on Sephadex G-25 in 0.1 M acetic acid or 0.1 M HCl and stored frozen at −40 °C. Under these conditions, reduced ribonuclease is stable for at least 6 months (but was used here within 2 weeks of its preparation). To determine the extent of reduction, an aliquot of the desalted solution was removed, and the sulfhydryl groups were carboxymethylated by addition of excess iodoacetic acid in 0.1 M Tris-HCl (pH 8.0). After extensive dialysis against 0.1 M Tris-HCl (pH 8.0) the protein was subjected to acid hydrolysis (6 N HCl for 24 h) and amino acid analysis. The finding of 7.8 mol of *S*-carboxymethylcysteine per 124 amino acids indicated that reduction had been complete.

Inhibition Assay of Refolding. The kinetics of oxidation of reduced ribonuclease (at a concentration of 9.1×10^{-7} M) were studied as follows. The reduced protein was stirred constantly in 0.1 M Tris-HCl (pH 8.0) with free access to air at 23 °C. At various times, aliquots of this solution were tested to determine how well they competed with [^{125}I]RNase for each of the purified antibodies (against segments 1–13, 31–79, and 80–124, respectively). Only that portion of oxidized RNase which had folded to the native conformation in the region of the antigenic site, at the given time, would be expected to compete with [^{125}I]RNase for the corresponding antibody.

The inhibition assay used was that of Farr (1958), as modified by Teale and Benjamin (1976a) and Chavez and Benjamin (manuscript in preparation). Standard inhibition assay tubes were prepared in triplicate by adding 100 μL of [^{125}I]RNase (1.25 μg of protein), 100 μL of a 1:4 dilution of normal rabbit serum,[2] and 100 μL of the oxidation solution (1.25 μg of protein as inhibitor) to each tube, the final pH being 7.2. Purified antibody that was specific for a given fragment was then added, and the solution mixed. The amount of antibody added (contained in 100 μL of 1% normal rabbit serum in 0.1 M sodium phosphate buffer, pH 7.2) was sufficient to bind 50% of the [^{125}I]RNase in the absence of any inhibitor.[3] The solution was incubated for 30 min at room temperature and then placed in an ice bath. The 30-min incubation period was chosen since no further change in precipitable [^{125}I]RNase could be measured at and beyond 30 min. Ice-cold saturated ammonium sulfate (500 μL) was then added and mixed to precipitate the antigen–antibody complex. After 30 min, the precipitate was removed by centrifugation at 1500*g* at 0 °C for 30 min. The supernatant was discarded, and the precipitate was washed with ice-cold 50% saturated ammonium sulfate. The tubes were centrifuged, the supernatant was discarded, and the [^{125}I]RNase in the precipitate was counted in a Beckman Biogamma II γ counter.

The control assay used to analyze for nonspecific precipitation of [^{125}I]RNase contained no antibody in the assay tube. The control assay used to check for maximum precipitation of [^{125}I]RNase contained no inhibitor in the assay tube. The control assay used to determine maximum inhibition contained an equivalent amount of native ribonuclease instead of reduced (and partially oxidized) ribonuclease. All samples were corrected for nonspecific precipitation.

As more of the reduced RNase refolds to the native form, it displaces more of the [^{125}I]RNase from the complex with the antibody; i.e., it inhibits the binding of antibody to

[2] The oxidation process was shown here to be slowed in this mixture. It is known that air oxidation of thiol groups is catalyzed by divalent cations in trace amounts (Ahmed et al., 1975); however, the presence of Cu^{2+}-binding proteins in serum could deplete the concentration of free divalent cations. Also, the rate of oxidation of thiol groups decreases when the pH is lowered from 8.0 to 7.2. The addition of normal rabbit serum accomplished three purposes, viz. (i) it slowed the rate of oxidation of reduced ribonuclease, (ii) it served as a carrier for the precipitation of the [^{125}I]RNase–antibody complex, and (iii) it stabilized the purified antibody against denaturation.

[3] To determine the amount of purified specific antibody that binds 50% of the [^{125}I]RNase, dilutions of the antibody were made with 1% normal rabbit serum in 0.1 M sodium phosphate buffer (pH 7.2).

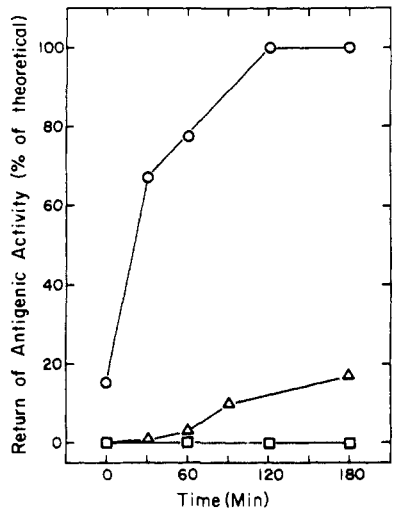

FIGURE 2: Kinetics of return of native antigenic structure in reduced ribonuclease as determined with anti-RNase$_N$. The reaction conditions during air oxidation and in the assay were those described in the text. Oxidation of reduced ribonuclease in: (○) 0.1 M Tris-HCl (pH 8.0); (△) 0.1 M Tris-HCl (pH 8.0), but in the presence of a 1:4 dilution of normal rabbit serum; (□) 0.1 M acetic acid. The concentration of ribonuclease was the same in all three solutions.

[^{125}I]RNase. The percent inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{MP - EXP}{MP} \times 100 \quad (1)$$

where *MP* is the counts per minute in the precipitate of the no-inhibitor control and *EXP* is the counts per minute in the precipitate of the experimental tube. The return of antigenic activity (percent of theoretical) was calculated for each experimental precipitate as follows:

$$\text{return of antigenic act. (\% of theor.)} = \frac{\% \text{ inhibition}}{MI} \times 100 \quad (2)$$

where *MI* is the percent inhibition obtained by an equivalent amount of native ribonuclease.

A similar inhibition assay was used to compare the antigenic activity of various peptides and S-carboxymethylated RNase. Various amounts of these derivatives were added to the standard amount of purified antibody before the addition of [^{125}I]RNase as described above. The percent inhibition was calculated as above (eq 1), and the results are presented as a plot of percent inhibition against amount of inhibitor added.

Spectroscopic and Analytic Methods. Several experimental approaches were used in order to compare the structural characteristics of reduced and native ribonuclease.

All ultraviolet (UV) spectra were obtained with a Cary Model 14 spectrophotometer. UV first-derivative spectra were computed from the absorption curves obtained with the instrument operating at its slowest scan speed. Circular dichroic (CD) spectra were recorded with a CD 6001-equipped Cary Model 60 spectropolarimeter.

Flash photolytic labeling of ribonuclease with *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate (NAP-Taurine) and amino acid analyses were carried out with the apparatus and procedure of Matheson et al. (1977b).

Protein concentrations were determined with a Zeiss Model PMQ II spectrophotometer using the following molar extinction coefficients: 9800 M^{-1} cm^{-1} for native ribonuclease at 277.5 nm (Harrington and Schellman, 1956) and 9390 M^{-1} cm^{-1} for reduced ribonuclease at 276 nm (White, 1961).

Results

Initially, the air oxidation of reduced ribonuclease was followed (at pH 8.0) by determining the rate of return of native antigenic activity with anti-RNase$_N$ (Figure 2). Antigenic activity begins to return immediately as oxidation begins. Although no lag period was observed, the possibility that some oxidation occurred during the 30-min assay period prior to ammonium sulfate precipitation was tested as follows: reduced ribonuclease was incubated at room temperature in 0.1 M acetic acid, and then aliquots were removed at various times and mixed with, and incubated in, the assay solution (pH 7.2), containing normal rabbit serum, [^{125}I]RNase, and anti-RNase$_N$, for 30 min. Figure 2 demonstrates that no inhibition occurred under these conditions; i.e., no refolding of reduced ribonuclease was detectable in 0.1 M acetic acid over a period of 3 h. This experiment was repeated by, initially, omitting the [^{125}I]RNase and anti-RNase$_N$ from the assay solution (which was at pH 8.0), and then adding these two components *after* the 30-min incubation period, and allowing the final mixture to incubate for an additional 5 min. Under these conditions, Figure 2 shows that refolding does occur in the presence of normal rabbit serum, but at a slower rate[2] than in its absence. Since the reaction between [^{125}I]RNase and anti-RNase$_N$ is complete within 1 min, under the conditions used here (unpublished results), the only ribonuclease that can compete with [^{125}I]RNase for the antibody is that which refolded to the native form prior[4] to the addition of [^{125}I]RNase and anti-RNase$_N$. Therefore, even though reduced ribonuclease does oxidize and refold during the 30-min assay period, the material that refolds during this period has no effect on the determination of the amount of protein that refolded prior to the assay.

The return of antigenic activity also was determined with purified anti-(1-13)$_N$, anti-(31-79)$_N$, and anti-(80-124)$_N$, respectively, substituted for anti-RNase$_N$ in the assay mixture. Typical refolding kinetics are shown in Figure 3. The initial rates of folding of the three antigenic regions of ribonuclease are in the order: 80-124 > 1-13 > 31-79. In all cases, the return of antigenic activity reached 100% of the theoretical value expected. Thus, essentially 100% of all of the molecules refolded completely by 240 min of oxidation time.

In order to interpret the results of Figure 3, it is necessary to show that reduced ribonuclease does not have residual amounts of native structure that might react with antibodies against native ribonuclease; i.e., it is necessary to show that there is no antigenic cross-reaction between reduced and native ribonuclease, especially since performic acid oxidized ribonuclease does cross-react very slightly with native ribonuclease (Brown et al., 1967). Because of the difficulty of working with labile reduced ribonuclease, this test was carried out with S-carboxymethylated ribonuclease. The S-carboxymethylated derivative of reduced ribonuclease, therefore, was examined for any cross-reaction by determining its ability to inhibit the reaction of [^{125}I]RNase with anti-RNase$_N$ (see Figure 4). No cross-reaction could be demonstrated, even at a 160-fold molar excess of the S-carboxymethylated derivative. At the molar ratio of reduced ribonuclease to [^{125}I]RNase$_N$ (1:1) used in

[4] As will be shown in the Discussion, the *rate* of dissociation of the [^{125}I]RNase-anti-RNase$_N$ complex is so slow that any reduced ribonuclease that refolds during the 30-min assay period cannot displace [^{125}I]RNase from the complex.

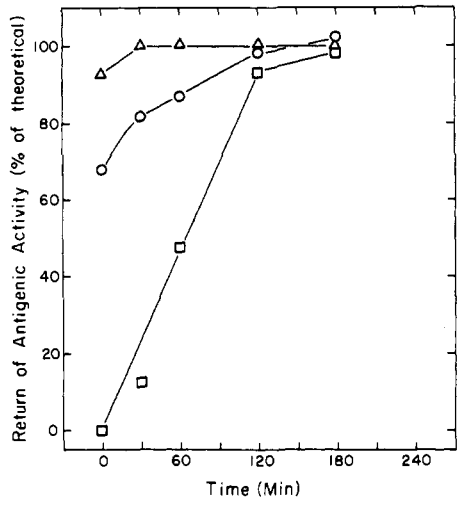

FIGURE 3: Kinetics of return of antigenic activity of reduced ribonuclease as determined with purified antibodies against fragments of ribonuclease. The oxidation conditions were those described in the text. Refolding was measured by: (○) anti-$(1-13)_N$; (□) anti-$(31-79)_N$; (△) anti-$(80-124)_N$.

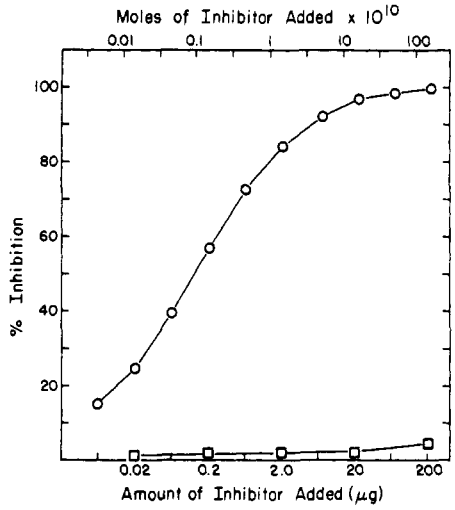

FIGURE 4: Antigenic activity of native ribonuclease and of S-carboxymethylated ribonuclease. Increasing concentrations of either compound were added as an inhibitor in the inhibition assay described in the text: (○) native protein; (□) S-carboxymethylated protein.

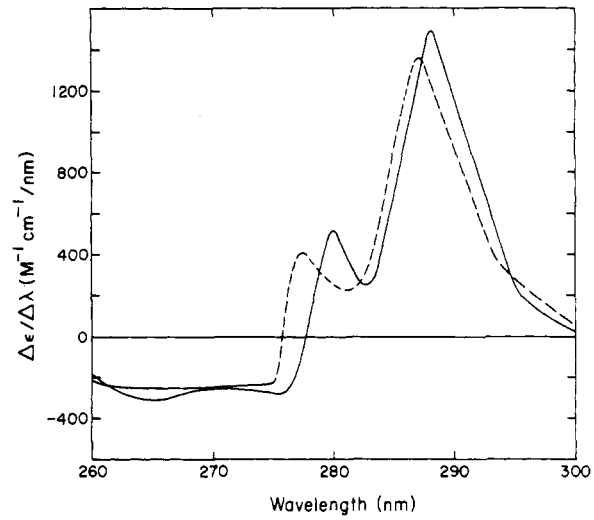

FIGURE 5: Ultraviolet derivative spectra of native ribonuclease in 0.1 M sodium phosphate buffer (pH 7.2) (solid curve) and reduced ribonuclease in 0.001 N HCl (broken curve).

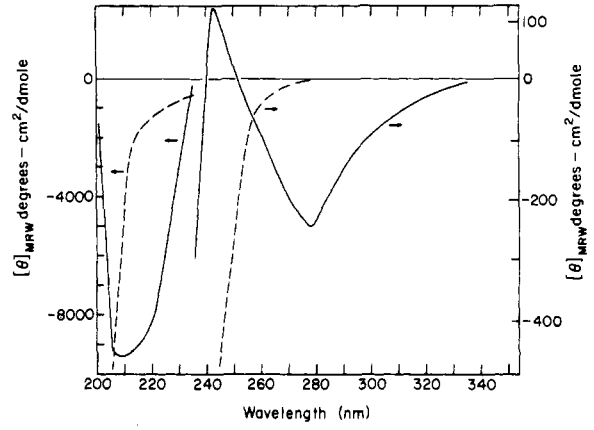

FIGURE 6: Circular dichroism spectra of native ribonuclease in 0.1 M sodium phosphate buffer (pH 7.2), 24.0 °C (solid curve); reduced ribonuclease in 0.001 N HCl at 24.0 °C (broken curve).

the refolding assay, cross-reaction therefore could not account for the results of Figure 3.

The extent of denaturation of reduced ribonuclease was also examined by several other methods. Since the reduced material was stored at low pH (0.001 N HCl), a comparison was made of the physical properties of reduced RNase at low pH with those of native RNase at neutral pH. First, the UV derivative spectrum of reduced ribonuclease in 0.001 N HCl was distinctly different from the derivative spectrum of the native protein at pH 7.2 (Figure 5). Second, the circular dichroism spectrum of reduced ribonuclease in 0.001 N HCl differs from that of the native protein at pH 7.2 (Figure 6). Lastly, using a flash photolytic labeling technique developed by Matheson et al. (1977b), a comparison of the accessibilities of each amino acid to the photolytic label in reduced ribonuclease, thermally denatured ribonuclease (pH 3.0), and native ribonuclease (pH 6) demonstrates that the residues in reduced ribonuclease are exposed significantly more than in the native protein and slightly more than in the thermally denatured protein at pH 3 (Table II). These data are consistent with the evidence that reduced ribonuclease assumes a disordered conformation in solution (Harrington and Sela, 1959). However, complete assurance that no residual native structure is left in reduced ribonuclease cannot be given. Nevertheless, if we can assume that the conformation of reduced ribonuclease resembles that of S-carboxymethylated ribonuclease, and the latter does not cross-react with native ribonuclease, it may be concluded that reduced ribonuclease is devoid of residual structure at the antigenic sites of native ribonuclease.

The temperature dependence of the antigenic activity of each peptide segment was investigated in order to determine whether the order of folding of the different portions of the ribonuclease molecule (Figure 3) bears any relationship to the *thermal* stabilities of the conformations of the chain in the regions of the antigenic sites in the native molecule. [^{125}I]$RNase_N$ was incubated for 1 h at various temperatures in 0.1 M Tris-HCl (pH 8.0) and then added immediately to an ice-cold standard inhibition assay mixture (containing, in turn, each of the purified specific antibodies) in which 0.1 M Tris-HCl (pH 8.0) was substituted for the inhibitor (see Methods for constituents of the standard inhibition assay). Any [^{125}I]$RNase_N$ that was irreversibly denatured at any given

TABLE II: Comparison of Photolytic Labeling of Reduced, Thermally Denatured, and Native Ribonuclease.[a]

Amino Acid Residue[b]	No. of Unlabeled Residues: Native RNase[c] 25 °C, pH 6	Reduced RNase[d] 25 °C, pH 3	Denatured RNase[c,e] 70 °C, pH 3
Lysine (10)	3.5	1.6	2.3
Histidine (4)	0.9	0.4	0.7
Arginine (4)	0.0	0.4	0.2
Aspartic acid (15)	5.4	2.1	2.6
Threonine (10)	3.0	3.0	3.1
Serine (15)	5.3	3.5	3.8
Glutamic acid (12)	6.3	2.5[f]	5.5
Proline (4)	1.1	0.3	0.8
Glycine (3)	2.1	1.3	2.0
Alanine (12)	4.2	2.0	2.4
Half-cystine (8)	2.0	0.4	0.7
Valine (9)	2.6	1.2	1.3
Methionine (4)	1.0	0.4	0.4
Isoleucine (3)	0.6	0.3	0.4
Leucine (2)	0.7	0.4	0.4
Tyrosine (6)	1.1	0.4	0.5
Phenylalanine (3)	1.0	0.3	0.4

[a] Recorded as moles of amino acid/mole of protein and corrected for hydrolysis losses (Rupley and Scheraga, 1963). [b] Numbers in parentheses are numbers of residues in the native molecule. [c] Data of Matheson et al. (1977b) for a label/protein ratio of 212/1. The data of Matheson et al. (1977b) for native RNase were checked here. [d] For a label/protein ratio of 212/1. [e] With disulfide bonds intact. [f] It should be noted that, by reducing the disulfide bonds, it is possible to label more glutamic acid residues.

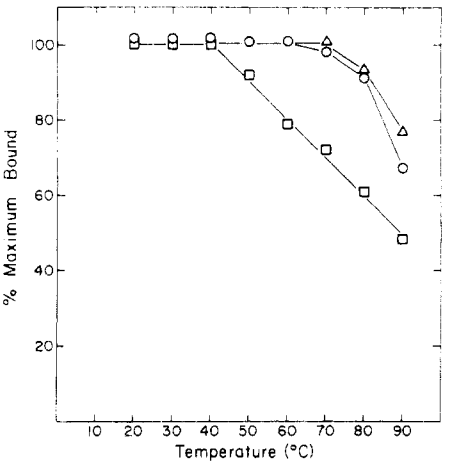

FIGURE 7: Effect of thermal denaturation on the antibody binding capacity of [^{125}I]RNase$_N$. Conditions were those described in the text. Samples of [^{125}I]RNase$_N$, which had been incubated at the indicated temperatures at pH 8.0, were added to an inhibition assay containing each purified specific antibody preparation and 0.1 M Tris-HCl instead of inhibitor. Binding of denatured [^{125}I]RNase$_N$ to: (□) anti-(1–13)$_N$; (○) anti-(31–79)$_N$; (△) anti-(80–124)$_N$.

antigenic site in this heat treatment would not bind the corresponding antibody. [It had been shown previously (Hermans and Scheraga, 1961) that, at pH ≤7, ribonuclease is reversibly denatured at all temperatures below the transition temperature but irreversibly denatured to increasing extents above the transition temperature, depending on the length of time at the elevated temperature. In fact, ribonuclease is completely, irreversibly denatured when incubated at 95 °C for longer than 20 min]. We have found that incubation of ribonuclease for 1 h at pH 8.0 produces 0, 1, and 12% irreversible denaturation at 40, 50, and 60 °C, respectively, as determined by the method of Hermans and Scheraga (1961); similar incubation at pH 7.2 demonstrated no irreversible effects. The extent of irreversible thermal denaturation, as measured by the binding activity between each of the purified specific antibodies and [^{125}I]RNase that had been incubated previously between 20 and 90 °C, is shown in Figure 7. The antigenic regions of ribonuclease show the following thermal stabilities: (1–13) < (31–79) ≤ (80–124). The discrepancy between the amount of irreversible denaturation, as measured by the spectroscopic and immunological techniques, is within the limits of error of these experimental methods. These experiments indicate that region (1–13) is more susceptible to structural alterations than the protein as a whole.

Discussion

The immunological approach for studying the pathway of folding of proteins has advantages and disadvantages. By using the great specificity of the antibody probe, one can monitor the folding of a specific segment of the polypeptide chain. However, such kinetic measurements of folding are restricted to relatively slow processes. Other limitations are the paucity of information on (1) the location of the antigenic determinants and (2) the relationship between the antigenic determinants and the conformational nucleation sites which promote folding. The progress made in resolving these questions will be the subject of subsequent reports.

Antibodies against native ribonuclease were produced in rabbits and fractionated on columns to which fragments of ribonuclease were bound. Presumably, this fractionation succeeds because some percentage of the antigenic site of each peptide fragment can adopt the native conformation and bind to the appropriate antibody fraction, in spite of the fact that their cysteines were S-carboxymethylated; it appears that some (even though small) fraction of these carboxymethylated peptides can fold to the native conformation, at least in the region of the antigenic site. This observation, that a very small percentage of a peptide fragment can adopt the native conformation, was made earlier by Sachs et al. (1972a). S-Carboxymethylated ribonuclease showed little cross-reaction at the 160-fold molar excess used here (Figure 4). There is, however, evidence of cross-reaction at 1000-fold molar excess (unpublished results), which no doubt explains why the S-carboxymethylated CNBr fragments could be used to fractionate the antibodies against native ribonuclease.

The use of antibodies to detect stages of folding and the interpretation of the data depend on whether the antibody only recognizes a folded antigenic determinant or whether it induces the folding. If the antibody were to *induce* the folding of reduced ribonuclease, the degree of folding would depend on the length of time of incubation of reduced ribonuclease with the antibody. In these experiments, however, where a constant incubation time (30 min) was used prior to precipitation, the observed degree of folding depended not on the incubation time but on the time of oxidation at pH 8.0 in 0.1 M Tris-HCl (Figure 2). The possibility exists that incompletely folded antigenic determinants could inhibit the binding of the antibody to [^{125}I]RNase. However, the degree of inhibition would be limited by the lower affinity of the antibody for the slightly folded determinant. It is the *association* rate constants for antibody–protein reactions that should be affected most by partially folded determinants (Sachs et al., 1972b). Since the competition between refolded RNase and [^{125}I]RNase for

antibody is not allowed to reach equilibrium, only those determinants that are folded sufficiently compete effectively. Also, under the conditions of these experiments, no significant dissociation of antibody–ribonuclease complexes would occur since their association rates are several orders of magnitude greater than their dissociation rates (Grey, 1963).

There are some correlations between these results and those reported previously for the air oxidation of reduced ribonuclease (Anfinsen et al., 1961). The reported rates of decrease in the sulfhydryl titer and of increase in optical rotation are comparable with the rate of return of antigenic activity for anti-$RNase_N$ (compare Figure 2 here with Figure 1 in Anfinsen et al., 1961). However, while enzymatic activity returns after a lag period of 30 min, antigenic activity begins to return immediately. Two factors can account for the lag in enzymatic activity. First, air oxidation of free sulfhydryl groups is a rather inefficient process (Hantgan et al., 1974), and the rate of oxidation depends on trace amounts of divalent metal ions (Ahmed et al., 1975). Also, the interchange of initially mispaired disulfide bonds can delay proper structural reorientation (Hantgan et al., 1974). Second, the slow return of antigenic determinants in segment 31–79 is consistent with the slow return of enzymatic activity. While the return of antigenic activity depends upon the stepwise reappearance of each conformational determinant in the form recognizable by its appropriate antibodies, the return of enzymatic activity, on the other hand, may depend on the folding of the *entire* molecule. Our kinetic results suggest that the (slower) folding of segment 31–79 may account for the slow return of enzymatic activity.

The kinetic data provide strong evidence for the presence of intermediate conformational states during folding and also suggest a folding pathway for ribonuclease during air oxidation. Burgess and Scheraga (1975) have presented a detailed model for the pathway of folding of thermally denatured disulfide cross-linked ribonuclease, and provided some arguments to support the hypothesis that the folding during oxidation of sulfhydryl groups might follow a similar pathway. The experimental results described here are consistent with this model. However, this does not prove that the pathways of folding are the same for thermally denatured and for reduced ribonuclease, since the results may depend on the methods of measurement used in each case. Nevertheless, Burgess and Scheraga (1975) postulated that segment 81–102 is a nucleation region because of its high thermal stability, as measured by susceptibility to proteolytic enzymes (Burgess et al., 1975), and the kinetics of folding of segment 80–124, reported here, supports this postulate.

The thermal denaturation data of Figure 7 suggest that the segment 1–13 is distorted at lower temperatures than is suggested by the action of either trypsin (Ooi et al., 1963) or aminopeptidase (Klee, 1967) on thermally unfolded ribonuclease,[5] and that the stability of antigenic determinants in regions 31–79 and 80–124 reflects greater stabilizing interactions in these segments than in segment 1–13.

The kinetics of return of native antigenic activity (Figure 2) and structural data provide several interesting comparisons. First, isolated peptide 1–13 and S-peptide have been shown to possess some degree of stabilization of the native α-helical structure, independent of the rest of the protein molecule and, therefore, attributable to short-range interactions (Brown and Klee, 1971; Silverman et al., 1972). The helical state in the peptide is favored by low temperature and high ionic strength. Under the conditions used in these experiments (23 °C and $\mu < 0.1$), the segment 1–13 (during the refolding of the intact protein) would possess only a small degree of α-helical structure according to the data of Brown and Klee (1971). Second, in the intact protein, the helical region of this segment includes residues 4 to 12 (Kartha et al., 1967; Wyckoff et al., 1967, 1970). The antigenic determinant in this segment probably contains elements of the α-helix since the antigenic determinants of myoglobin are of the order of 6–7 amino acids in length (Atassi, 1975). Hence, the folding of at least a portion of this α helix is measured during folding. Third, the intact reduced protein (in 0.001 N HCl) possesses no α-helical structure (Figure 6) and the kinetics of return of antigenic activity (Figure 3) demonstrate that segment 1–13 folds after 80–124. It appears then that the N-terminal α helix gains stability from favorable long-range interactions with other portions of the protein molecule.

The interactions that are provided by the S-peptide are necessary for quantitative recovery of enzymatic activity during the folding of RNase S (Kato and Anfinsen, 1969). However, recovery of approximately 20% enzymatic activity was found in the absence of S-peptide, when S-peptide was added *after* folding the S-protein (Kato and Anfinsen, 1969). Furthermore, since contacts do not exist between segments 1–13 and 80–124 but rather between segments 1–13 and 31–79 (Wyckoff et al., 1967, 1970), a portion of 31–79 (which is *not* antigenic) should fold prior to region 1–13 in order to provide the necessary stabilizing interactions. The nonantigenic portion of region 31–79 responsible for these long-range interactions could be residues 35–50 which form part of an antiparallel β-pleated sheet in the native molecule (Burgess and Scheraga, 1975). These results exclude the possibility that the N-terminal helical region serves as the *primary* nucleation site for folding the rest of the protein.

The early folding of region 80–124 indicates the importance of the C-terminal region in the folding of the ribonuclease molecule. The implications of various modifications in amino acid residues 105–124 have been reviewed by Burgess and Scheraga (1975). Several points are worth repeating. (1) The refolding of reduced des-(121–124) ribonuclease produces randomly paired disulfide bonds (Taniuchi, 1970). (2) The replacement of Phe-120 by other nonpolar residues has very significant effects on the structural stability of ribonuclease (Lin et al., 1972). (3) Iodination of Tyr-115 prevents refolding of reduced ribonuclease (Friedman et al., 1966). (4) The interactions of the charged α-carboxyl group of Val-124 and the ϵ-amino group of Lys-104 are important for the stabilization of the C-terminal loop. It is interesting that the pH dependence of the fast kinetic step in the unfolding and refolding of ribonuclease (with disulfide bonds intact) indicates the involvement of a group with the pK of a carboxyl group (Garel and Baldwin, 1975). All of the above data, in addition to the folding kinetics of the antigenic determinants reported here, provide evidence for the location of a nucleation site in the sequence 80–124.

The particular native structures responsible for our results have not yet been determined. Clearly, long-range contacts are necessary to stabilize organized backbone structures (Epand and Scheraga, 1968), while structures with intrinsic stabilities could serve to direct the folding process toward productive channels (Lewis et al., 1970). Three important questions must be answered. (1) Does the nucleation process depend on the formation of only one nucleation site? (2) Is a nucleation site

[5] It should be noted that the experiments of Ooi et al. (1963) were carried out under conditions where ribonuclease is denatured *reversibly*. However, the data of Figure 7 and those of Klee (1967) pertain to *irreversible* denaturation.

the most stable structure in the protein molecule? (3) Do structures with intrinsic thermal stabilities serve as nucleation sites? Further details of the folding process can come only when distinct intermediate states can be examined and identified.

Acknowledgment

We should like to thank Professor S. J. Leach for helpful comments on this manuscript.

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