Location of the Antigenic Determinants of Bovine Pancreatic Ribonuclease†

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

ABSTRACT: Four antigenic regions of native bovine pancreatic ribonuclease have been located by using antibodies that react specifically with segments 1–13, 31–79, and 80–124. These antibodies were purified by affinity chromatography on columns to which these peptide segments were bound. Analysis of precipitin curves indicates that there are *at least* three antigenic determinants to which antibody molecules can bind simultaneously in the presence of excess antibodies. Analysis of binding data, however, for each purified specific antibody preparation, carried out by the method of Berzofsky et al. [Berzofsky, J. A., Curd, J. G., & Schechter, A. N. (1976) *Biochemistry 15*, 2113], leads to an estimate of four for the number of antigenic determinants in ribonuclease; this estimate had also been made earlier by Stelos et al. [Stelos, P., Fothergill, J. E., & Singer, S. J. (1960) *J. Am. Chem. Soc. 82*, 6034]. We find that one determinant is associated with each of segments 1–13 and 80–124 and two with segment 31–79. No antigenic activity could be detected for segment 14–29 either in native ribonuclease or in the free fragment. These conclusions are based on (1) the use of specific peptides to isolate purified antibodies by affinity chromatography, (2) immunoprecipitation of an antigenic peptide from the peptic digest of ribonuclease, (3) competitive inhibition studies with various peptide and protein fragments [cyanogen bromide fragments 1–13, 31–79, and 80–124, the tryptic peptides 40–61 and 105–124, S-peptide, S-protein, and des(121–124)-RNase], and (4) comparison and evaluation of the published effects on antigenicity of chemical and enzymatic modifications and changes in sequence among homologous ribonucleases. These approaches provide evidence that the four antigenic determinants are localized around the α-helical portion of segment 1–10, somewhere in segment 40–61, at the β bend in segment 63–75, and either at the β bend or β sheet in segment 87–104 of native ribonuclease.

This paper is concerned with the location of the antigenic determinants in the native three-dimensional structure of bovine pancreatic ribonuclease ($RNase_N$).[1,2] The identification of all of the antigenic regions of two proteins (sperm whale myoglobin and lysozyme) has been accomplished only recently (Atassi, 1975, 1978). In general, several approaches have been used to locate antigenic regions. These include (1) determination of the effect of conformational changes on immunochemical behavior, (2) determination of the effect of modification of specific residues on the antigenic activity of intact proteins and their immunologically active peptides, and (3) studies of inhibition of binding of antibodies to the native protein by overlapping peptides which were derived by hydrolysis or by direct synthesis.

The antigenic structure of $RNase_N$ has been the subject of numerous investigations. However, very little direct information about the location of the antigenic determinants in the native protein has been reported. Brown (1962, 1963) and Brown et al. (1967) studied the immunological activities of the tryptic fragments of performic acid oxidized ribonuclease ($RNase_{ox}$) and found that peptides 38–61 and 105–124 possess significant inhibitory activity in the binding of anti-$RNase_{ox}$ to $RNase_{ox}$. These results shed little light on the location of the antigenic determinants in the *native* molecule since only slight cross-reaction between $RNase_N$ and high concentrations of $RNase_{ox}$ was observed by using anti-$RNase_N$ (Brown et al., 1967). Such a small degree of cross-reaction would be expected if $RNase_{ox}$ contained a small amount of native structure, in accordance with the conformational equilibrium theory developed by Sachs et al. (1972), or if anti-$RNase_N$ induced some native conformation in $RNase_{ox}$.

Chemical modification of RNase has been used with moderate success in identifying the role of specific residues and structural features (such as α-helical regions) in antigenic activity. Brown et al. (1959a) proposed that bovine RNase A and B are immunochemically identical. However, using the phage-inactivation technique of Haimovich et al. (1970a,b), Welling & Groen (1976) found a small difference in antigenic behavior between these two proteins which differ only by the presence of a carbohydrate bound to asparagine-34 of RNase B. By examining the cross-reactions among homologous RNase proteins from several species, differing at a limited number of positions, Welling & Groen (1976) implicated residues 34, 35, 50, 99, and 103 in either a direct antigenic or an important conformational role.

Studies of the modifications of particular types of residues have also provided some information about antigenic activity. The antigenic activity of $RNase_N$ is greatly reduced by deamidation (Brown et al., 1959b; van Vunakis et al., 1960), by performic acid oxidation of the disulfide bonds (Brown,

† From the Baker Laboratory of Chemistry, Cornell University, Ithaca, New York 14853. *Received May 29, 1979.* This work was supported by research grants from the National Science Foundation (PCM 75-08691) and from the National Institute of General Medical Sciences, National Institutes of Health, U.S. Public Health Service (GM-14312).

‡ National Institutes of Health Postdoctoral Fellow, 1975–1978.

[1] Abbreviations used: $RNase_N$ or simply RNase, bovine pancreatic ribonuclease A; $RNase_{ox}$, performic acid oxidized RNase; anti-X, antibody against X, where X can be $RNase_N$ or fragments thereof; des(121–124)-RNase, species lacking the C-terminal tetrapeptide; S-peptide and S-protein, the species produced *by limited* subtilisin digestion and which consist of residues 1–20 and 21–124, respectively; IgG, immunoglobulin-G fraction; K_{conf}, the equilibrium constant between the unfolded and native conformations of a protein fragment.

[2] An antigenic determinant is defined as a region of the native protein containing residues which interact *directly* with the antibody plus any intervening residues within a peptide of 6–7 residues. Since Atassi (1975) has found that antigenic determinants "possess sharp boundaries", nonreactive parts of the peptide outside these boundaries are excluded from the antigenic determinant, although the nonreactive parts "are important for appropriate orientation and fit of the reactive region onto the antibody combining site".

0006-2960/79/0418-4386$01.00/0

1962), and by esterification of free carboxyl groups (Acharya et al., 1977). These modifications probably affect the antigenic region not only by modification of particular residues but also by inducing conformational changes elsewhere in the protein molecule. On the other hand, modification of tyrosine-76 and -115 and arginine-39 and -85 has no effect on the reactivity of antibodies toward $RNase_N$ (van der Zee et al., 1977); thus, these residues are not located in an antigenic site, nor are they responsible for maintaining the three-dimensional structure of such an antigenic site.

The effects of enzymatic modification on the antigenic activity of RNase have also been studied. The digestion of RNase by subtilisin at pH 8 produces two fragments, S-peptide and S-protein (Richards & Vithayathil, 1959). Singer & Richards (1959) found that the antigenic activity of S-protein was reduced greatly, while S-peptide did not possess any activity at the relatively low concentrations of S-peptide used in their experiments. Brown et al. (1959b) found that removal of the C-terminal tetrapeptide or the N-terminal dipeptide diminished antigenic activity. In these studies, it was not possible to distinguish between changes within the antigenic region or conformational alterations of other parts of the protein molecule as the source of altered antigenic activity. It is now possible, however, to distinguish between these two effects because of the development of very sensitive radioimmunoassays (Farr, 1958; Teale & Benjamin, 1976; Chavez & Scheraga, 1977; Chavez & Benjamin, 1978) and phage-inactivation assays (Haimovich et al., 1970a,b); i.e., various (specific) regions on the surface of a protein can be probed individually by using several different purified antibodies which can detect whether an antigenic determinant is lost completely or merely destabilized. For example, using radioimmunoassays to study the cross-reactions of homologous myoglobins, Hurrell et al. (1977) and I. East and P. E. E. Todd (private communication) have found that changes in amino acid sequence inside and outside the region of an antigenic determinant greatly affect its reactivity with antibodies.

In this paper, we localize the antigenic determinants of $RNase_N$ by, first of all, determining the number of such determinants by a quantitative precipitin assay and also by an analysis of binding data. Then, by application of the radioimmunoassay (Chavez & Scheraga, 1977), we identify the antigenic peptide and protein fragments by competitive inhibition and, by the immunoprecipitation method of Sanders et al. (1970), isolate an antigenic peptide.

Methods

Preparation of Ribonuclease and Derivatives. Bovine pancreatic ribonuclease A (Sigma type II-a) was purified further by the method of Taborsky (1959) on carboxymethylcellulose (Whatman CM-52).

The production and isolation of the cyanogen bromide fragments of RNase were accomplished according to Brown & Klee (1971) and Chavez & Scheraga (1977) with the following modifications. The S-carboxymethylated fragments 14–29, 31–79, and 80–124 were fractionated by chromatography on a column (2 × 150 cm) of Sephadex G-50 (superfine) (Pharmacia Fine Chemicals Inc., Piscataway, NJ) equilibrated in 5% acetic acid (Figure 1). The amino acid analyses of fragments 1–13, 31–79, and 80–124 were reported previously (Chavez & Scheraga, 1977). The fractions containing fragment 14–29 were pooled and lyophilized; the amino acid analysis of this fragment is presented in Table I.

S-Carboxymethylated RNase at a concentration of 5 mg/mL in 0.1 M sodium phosphate buffer, pH 7.2, was digested with trypsin (3× crystallized, Worthington Biochemicals) at a concentration of 0.025 mg/mL at room temperature for 24 h. The pH was then lowered to 2.2, and the digestion mixture was applied to a column of Dowex 50W-X2 (1.5 × 30 cm) and chromatographed by the method of Hirs et al. (1956). The fractions containing fragments 40–61 and 105–124 were pooled, lyophilized, and desalted on Sephadex G-10 in 0.001 N HCl. The amino acid analysis of these two fragments is given in Table I. Partial aminopeptidase M digestion of fragment 105–124 gave a ratio of histidine to isoleucine of 1:1.5; these results are consistent with the amino-terminal sequence of His-Ile-Ile for fragment 105–124. Aminopeptidase M digestion was not carried out on fragment 40–61 because of the limited amount of this fragment that was available. The amino acid composition of both peptides is

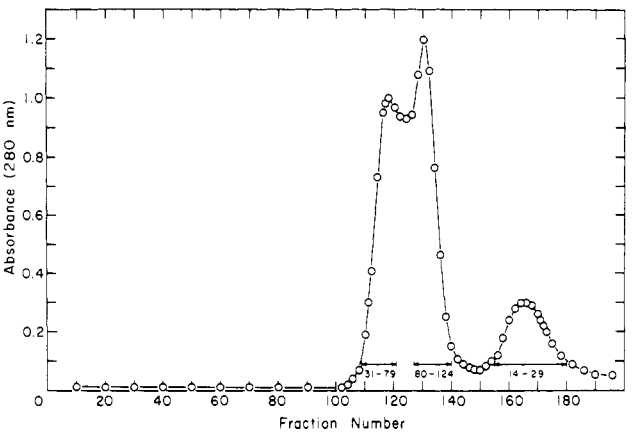

FIGURE 1: Fractionation of the S-carboxymethylated cyanogen bromide fragments after the cyanogen bromide digestion of RNase. Segment 1–13 was separated previously according to the procedure of Brown & Klee (1971). The remaining cyanogen bromide cleaved segment 14–124 was reduced and carboxymethylated; 200 mg of this material was desalted, applied to a column (2 × 50 cm) of Sephadex G-50 (superfine) equilibrated with 5% acetic acid, and eluted with the same solvent. Fractions 14–29, 31–79, and 80–124 were pooled as indicated and rechromatographed on the same column.

Table I: Amino Acid Analyses of the Peptides Used in the Immunochemical Studies[a,b]

amino acid residue	peptide 40–61		peptide 63–75		peptide 105–124		peptide 14–29	
	theory[c]	found	theory[c]	found	theory[c]	found	theory[c]	found
Asp	2	2.8	2	1.8	2	2.1	3	2.8
Thr	1	0.8	1	0.8			1	1.1
Ser	2	1.9	1	1.4	1	0.9	6	5.7
Glu	3	2.6	2	1.7	1	1.0	1	1.6
Pro	1	ND[d]			2	2.1		
Gly			1	1.1	1	0.8		
Ala	2	2.1	1	1.0	2	2.0	2	2.0
Val	4	4.0	1	1.0	4	4.0		
Met							1[e]	
Ile					2	1.7		
Leu	1	0.5						
Tyr			1	0.8	1	0.7	1	1.1
Phe	1	0.7			1	0.7		
His	1	1.3			2	2.3		
Lys	2	1.6	1	1.0				
Arg								
SCM-Cys[f]	2	1.7	2	2.1	1	0.9	1	1.1

[a] Hydrolysis was in 6 N HCl at 105 °C for 48 h under vacuum. [b] 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/mol of peptide. [c] Calculated from the sequence of bovine pancreatic ribonuclease determined by Smyth et al. (1963). [d] No data available. [e] Methionine was converted to homoserine lactone in cyanogen bromide digestion. [f] SCM, *S*-carboxymethyl.

consistent with the expected composition reported by Hirs et al. (1956) (Table I).

The preparation and purification of S-protein and des-(121–124)-RNase were carried out by the procedures of Richards & Vithayathil (1959) and Lin (1970), respectively, as modified by Chavez & Scheraga (1980a).

The production of peptic peptides was accomplished by digestion of purified RNAse A (5 mg/mL) with pepsin (Worthington) (0.03 mg/mL) in 5% formic acid at 37 °C for 24 h. The digest was lyophilized and stored as a powder at 4 °C. This mixture of peptides was not separated from pepsin because it was used in the antibody absorption experiments at pH 7.0, a pH at which pepsin is inactive.

Amino Acid Analysis. The amino acid composition of each fragment used was determined by hydrolysis in 6 N HCl (prepared from constant-boiling HCl) for 48 h at 105 °C, followed by chromatography on a Technicon TSM automated amino acid analyzer. The results are presented in Table I.

Preparation and Fractionation of Antibodies. Anti-$RNase_N$ serum was prepared by the method of Brown (1962). Each of four nonsibling New Zealand white rabbits was immunized initially with 1 mg of native ribonuclease in complete Freund's adjuvant. Subsequent booster injections (1 mg in incomplete Freund's adjuvant) were given at monthly intervals. Antiserum was obtained from the four rabbits 1 week after each booster injection. The antisera obtained from the monthly bleedings following the 7th through the 15th booster injections were pooled. The average association constant of the pooled anti-$RNase_N$ antiserum, determined here in a binding experiment with [^{125}I]$RNase_N$, was 1×10^8 M^{-1}. The antiserum was fractionated on specific immunoabsorbent columns [producing anti-$(1–13)_N$, anti-$(31–79)_N$, and anti-$(80–124)_N$] by the methods of Chavez & Scheraga (1977). Attempts were made to obtain anti-$(14–29)_N$ by fractionation of anti-$RNase_N$ antiserum on an immunoabsorbent column bearing fragment 14–29. No antibody binding, however, could be detected with hyperimmune antiserum.

The immunoglobulin-G fraction of anti-$RNase_N$ serum was prepared by five consecutive precipitations in 40% saturated ammonium sulfate. Each precipitate was dissolved in 0.15 M NaCl and reprecipitated. After the final precipitation, the redissolved material was dialyzed against four changes of 0.15 M NaCl at 4 °C.

Quantitative Precipitin Analysis. A uniform volume (0.2 mL) of whole anti-$RNase_N$ antiserum was added to a series of tubes at 4 °C (final pH $\sim$ 7.4, buffered by the antiserum itself) containing 0.3 mL of $RNase_N$ solutions in 0.15 M NaCl at concentrations between 0.7 and 394 μg of N mL^{-1}. A control tube containing 0.15 M NaCl instead of $RNase_N$ was included. After being mixed, the tubes were incubated at 37 °C for 30 min and then for 6 days at 4 °C. The precipitates were resuspended and mixed once each day. After 6 days, the precipitates were centrifuged at 1000*g* at 0 °C, and the supernatant was decanted. In order to remove protein impurities, we inverted the tubes containing the precipitates and allowed them to drain for 20 min at 4 °C. Cold 0.15 M NaCl (2.0 mL) was added, and the precipitates were resuspended. After 20 min at 4 °C, the precipitates were centrifuged and the washing procedure was repeated. The precipitates were drained and dried overnight in a vacuum desiccator. The entire precipitate was subjected to nitrogen analysis, as described below.

Nitrogen Analysis. Micro-Kjeldahl nitrogen analyses were carried out with the procedures of Lang (1958) and Noel & Hambleton (1976a,b).

Isolation of Antigenic Peptides. The immunoprecipitation of peptides, produced by peptic digestion of native RNase, was carried out by the method of Sanders et al. (1970). A peptic digest of 50 mg of RNase was incubated with 40–50 mg of the immunoglobulin-G fraction of anti-$RNase_N$ serum in 5.0 mL of 0.15 M NaCl at 37 °C (pH 7.0) for 1 h. Then the reaction mixture was cooled to 4 °C, and saturated ammonium sulfate at pH 6.8 and 4 °C was added to 40% saturation. After 24 h at 4 °C, the precipitate was centrifuged in a clinical centrifuge and washed 4 times with 40% saturated ammonium sulfate. This immunoprecipitation step was repeated with six different 50-mg batches of peptic digest in order to obtain sufficient material for further analysis. The combined precipitates (complex of immunoglobulin-G fraction with antigenic peptides) were then dissolved in 3.0 mL of 0.2 M acetic acid and enough solid urea to clarify the solution. The pH was adjusted to 2.0 with 6 N HCl, and the solution was applied to a Sephadex G-75 column (3.0 × 150 cm) equilibrated in 0.2 M acetic acid; elution was carried out with the same solution. The fractions containing low molecular weight peptides were pooled and lyophilized. These peptides were purified further by ion-exchange chromatography on Dowex 50W-X2 according to Hirs et al. (1956) with their sodium formate and sodium acetate buffer systems. The peptides were detected by their ninhydrin color (570 nm), produced by incubating a small aliquot (0.3 mL) of each tube with 0.2 mL of a 1% ninhydrin solution in 1.0 N sodium acetate, pH 5.51, at 95 °C for 10 min.

Radioimmunoassay for Inhibition by Peptide or Protein Fragments and for Determination of K_{conf}. To determine the relative antigenic activities of various peptides, we carried out competitive inhibition of the reaction between purified antibodies and $RNase_N$ by peptide or protein fragments by a radioimmunoassay (Chavez & Scheraga, 1977) using ^{125}I-labeled RNase. This isotopically labeled RNase was also used to determine K_{conf}, according to the procedure of Furie et al. (1975) as modified by Chavez & Scheraga (1980b). For this purpose, purified RNase was trace-iodinated by the chloramine-T method, as described by McConahey & Dixon (1966) and Chavez & Scheraga (1977). Since, as will be shown in Figure 5, an equivalent concentration of $RNase_N$ gave 50% inhibition of precipitation of [^{125}I]$RNase_N$ by anti-$RNase_N$, there was no inactivation of antigenic activity produced by iodination.

Radioimmunoassay for Determining Binding Curves. In order to determine the equilibrium-binding curves for $RNase_N$ to various antibodies, we performed radioimmunoassays on triplicate samples, essentially as described by Steward & Petty (1972a,b) and as modified by Arend & Mannik (1974), with the following additional modifications: 100–250 μL of 10% normal rabbit serum, 50–200 μL of various dilutions of [^{125}I]$RNase_N$ (1–5 μg/mL) with a specific radioactivity of 5300 cpm/μg, and 100 μL of purified antibody ($\sim$1 μg/mL) were added in sequence to each tube. The final volume was 500 μL. Dilutions of all reagents were made with 0.1 M sodium borate, pH 8.3. The samples were mixed and incubated for 60 min at room temperature and then overnight at 4 °C. Ice-cold saturated ammonium sulfate (500 μL) was added to each tube, and the contents were mixed. After 30 min in an ice bath, the samples were centrifuged at 1500*g* for 30 min at 0 °C. The precipitate, containing "bound" [^{125}I]$RNase_N$, was centrifuged into a pellet, and the supernatant, containing "free" [^{125}I]$RNase_N$, was decanted into a separate tube. The precipitate was washed twice with 2 mL of cold 50% saturated ammonium sulfate. An aliquot (100

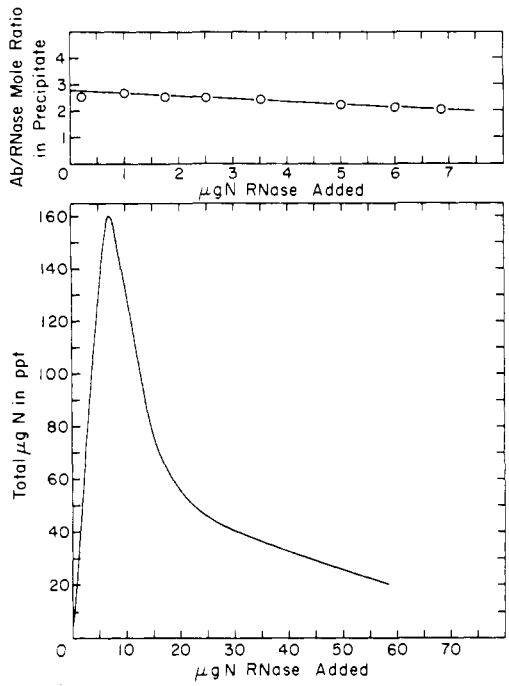

FIGURE 2: (Bottom) Precipitin titration of anti-RNase$_N$ with native RNase. Increasing amounts of RNase were added to incubation mixtures containing a constant volume of whole antiserum as described in the text. (Top) The molar ratio of antibody to RNase in the precipitate, determined from the precipitin titration curve.

μL) of the initial supernatant and the washed precipitate were counted in a Beckman Biogamma II γ counter. Control assays for nonspecific precipitation of [^{125}I]RNase$_N$ substituted 100 μL of 10% normal rabbit serum for antibody. The control assay for maximum precipitable (bound) [^{125}I]RNase$_N$ contained 100 μL of a high-titer antiserum instead of the lower concentration of purified antibody used in the other assay tubes. These controls provided data for calculating values of the ratio of bound to free [^{125}I]RNase for analysis by the procedure of Berzofsky et al. (1976).

Results

Precipitin Analysis. The quantitative precipitin curve is shown at the bottom of Figure 2. The curve at the top of Figure 2 (which pertains to a constant concentration of antibody) was obtained from these data (at low concentrations of added RNase) by using an average molecular weight of 150 000 g/mol for antibody molecules and of 13 683 g/mol for RNase. Extrapolation of this curve to zero concentration of RNase yields a value of 2.7 for the maximum molar ratio of antibody to RNase. This is in good agreement with the value of 2.8 found by Cinader & Pearce (1956), who used a similar procedure. This represents a *minimum* value for the maximum number of antibodies bound per RNase molecule because some antigenic determinants could overlap or lie close to each other; i.e., by competition or steric hindrance (between antibody molecules of different specificities), the binding of one antibody could prevent the binding of another. Thus, there are ≥3 antigenic sites/RNase molecule.

Purified Antibodies for Identification of Antigenic Sites. Three of the cyanogen bromide peptides (1–13, 31–79, and 80–124) of ribonuclease were used by Chavez & Scheraga (1977) to isolate antibodies from antisera which were directed against native RNase. The isolation procedure involved affinity chromatography of antibodies on columns to which each of these fragments was covalently coupled. No antibody binding could be detected on columns which bore fragment

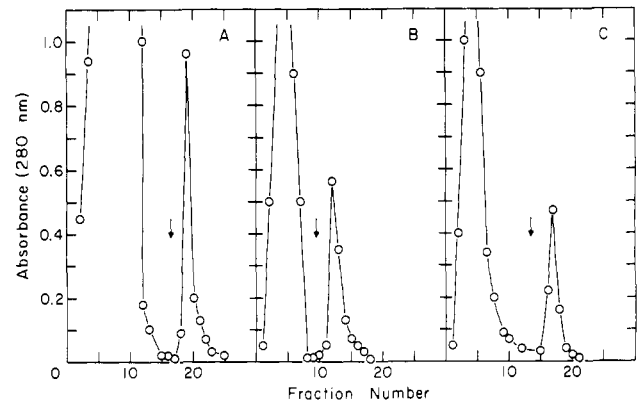

FIGURE 3: Affinity chromatography of anti-RNase$_N$ on Sepharose columns bearing the S-carboxymethylated cyanogen bromide fragments of RNase: (A) 1–13, (B) 31–79, and (C) 80–124. Anti-RNase antiserum (10 mL) which had been dialyzed against 0.15 M NaCl (pH ~7.4) was applied to the columns (1.5 × 5 cm), and the initial elution was performed with 0.15 M NaCl until the absorbance at 280 nm returned to zero. The elution of purified antibody was accomplished in 0.2 M sodium citrate, pH 2.2. The point of buffer change to effect elution of purified antibody is indicated by arrows. The preparation and chromatography of antibody on these columns were carried out according to Chavez & Scheraga (1977).

14–29. Also, this fragment did not inhibit whole anti-RNase$_N$ binding to [^{125}I]RNase$_N$ at a 10^4-fold molar excess. Therefore, it appears that there is no antigenic determinant in the region of residues 14–29 in the native molecule.

Figure 3 shows the elution profiles of antibodies specific for each native antigenic region in the three fragments, 1–13, 31–79, and 80–124. Studies of cross-reactivity between native RNase and the performic acid oxidized or S-carboxymethylated derivatives of RNase demonstrated 50% inhibition with these antibodies only at high molar ratios (>1000-fold) of each of these derivatives to native RNase. Cross-reaction at high molar ratios accounts for the binding of antibodies to the fragment-coupled columns used for the isolation (purification) of specific antibodies; i.e., in the purification procedure, the antibody induces some degree of native conformation in the resin-bound peptide and hence binds to it. On the basis of the known size of the binding site (six to seven amino acids) in the antigenic determinants of myoglobin (Atassi, 1975) and the size of peptide 1–13, it seems that anti-(1–13)$_N$ is probably directed against a single determinant.

Evidence for Four Antigenic Sites. We can apply the reasoning of Berzofsky et al. (1976) to verify the conclusion that there are *at least* three antigenic determinants in RNase. These authors showed that, for a multideterminant antigen, the slope of the curve of the ratio of bound to free antigen vs. total antigen concentration depends on whether the antibody is a mixture or not of species of differing specificities which can bind simultaneously to the same antigen. If each type of antigenic determinant is independent in its interaction with antibody and appears only once in a given antigen molecule, then the slope of the binding curve increases with an increase in the number of determinants per antigen molecule. Actually, it is not necessary for the antigenic determinants to be independent in order to observe an increase in the slope (as will be discussed below). The reason for this behavior is that an antigen counts as "bound" in the assay procedure used irrespecitve of whether it binds one or more than one type of antibody.

The binding curves for [^{125}I]RNase with anti-(1–13)$_N$, anti-(31–79)$_N$, and anti-(80–124)$_N$, respectively, are shown in Figure 4. Within the limits of error of the data, the slopes (at an RNase concentration of 6×10^{-8} M) are the same (~−1

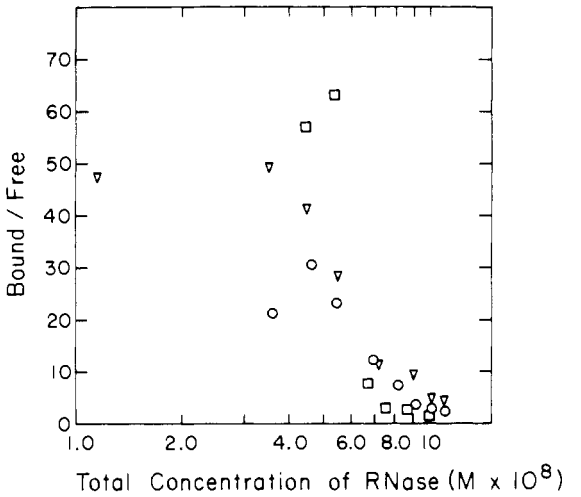

FIGURE 4: Titration of specific antibodies to subregions of native RNase with increasing concentrations of [^{125}I]RNase$_N$, plotted according to the method of Berzofsky et al. (1976). Bound and free refer to the number of moles of RNase. The radioimmunoassays were performed in quadruplicate as described in the text. The antibody preparations used were anti-(1–13)$_N$ (○), anti-(31–79)$_N$ (□), and anti-(80–124)$_N$ (▽).

× 10^9 M^{-1}) for anti-(1–13)$_N$ and anti-(80–124)$_N$ and more than 4 times larger for anti-(31–79)$_N$. Berzofsky et al. (1976) obtained similar results for single-determinant antigens. When they used a mixture of purified antibodies against two separate determinants, they obtained a curve with a slope that was more than 4 times larger than that of the binding curves for each of the two separate antibodies; this is also similar to our results. On the basis of these observations, we conclude that there are at least two antigenic determinants in segment 31–79 and at least one each in segments 1–13 and 80–124. Since the data of Figure 2, however, indicate that only three antibody molecules can bind simultaneously to one RNase molecule, there appears to be some competition for binding sites. Such competition violates the assumption of independence in the model of Berzofsky et al. (1976). Our analysis of this model indicates that competition between two antibody molecules would effectively increase the total concentration of antibody in the assay. As a result, the bound to free antigen ratio at each antigen concentration used would be higher than if binding were independent, i.e., noncompetitive. Since the ratio of the slopes that we obtained for antibody binding to segments 80–124 and 1–13 is consistent with the ratio of the slopes for single determinants found by Berzofsky et al., the increase in the bound to free antigen ratio in segment 31–79 compared to segments 1–13 and 80–124 is consistent with competition between binding sites, arising from at least two different determinants.

Inhibition by Protein Fragments. In order to ascertain or eliminate possible locations of antigenic determinants, we investigated two easily obtainable protein fragments of RNase A. The retention of antigenicity in S-protein and des(121–124)-RNase was examined by determining the ability of these fragments to inhibit the binding of whole anti-RNase$_N$ to [^{125}I]RNase$_N$. If antigenicity were retained, but with lower affinity, then a higher concentration of inhibitor would be required to attain 100% inhibition. If antigenicity were completely destroyed, then no inhibition would be observed. If one antigenic site were destroyed but another one retained, then a degree of inhibition less than 100% would be attained at high concentrations of inhibitor since all antigenic sites must be intact to obtain 100% inhibition. The inhibition curve of Figure 5 for S-protein demonstrates that this fragment has

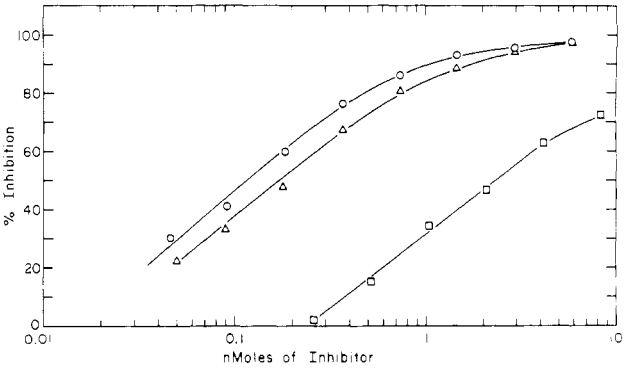

FIGURE 5: Antigenic activity of RNase (○), des(121–124)-RNase (△), and S-protein (□) vs. *whole* anti-RNase$_N$. Increasing amounts of each compound were added as an inhibitor to 0.091 nmol of [^{125}]RNase$_N$ at 4 °C and pH 8.0 in the inhibition assay described in the text.

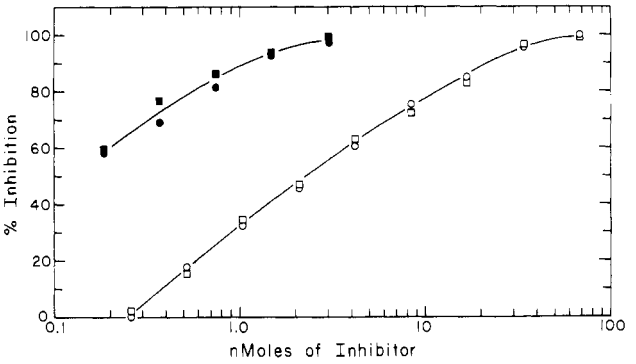

FIGURE 6: Antigenic activity of RNase$_N$ vs. *purified* anti-(31–79)$_N$ (●) or *purified* anti-(80–124)$_N$ (■) and of "native" S-protein vs. the same antibodies (○ and □, respectively). The conditions for the assay were the same as those in Figure 5.

lost one or more antigenic determinants since it leveled off at 75% inhibition even when ~100 nmol of S-protein (not shown in Figure 5) was added. The inhibition curves of Figure 6 for S-protein were obtained with either *purified* anti-(31–79)$_N$ or *purified* anti-(80–124)$_N$; they show that the antigenic determinants in the rest of the protein are not destroyed (since 100% inhibition is obtained) but that the binding affinities of the remaining determinants are lower than those of the native protein. On the other hand, the binding affinities of the antigenic determinants of des(121–124)-RNase are only slightly lower than those of native RNase, with no decrease in the number of antigenic determinants (Figure 5). This result for des(121–124)-RNase accounts for the diminished antigenic activity found by Brown et al. (1959b). These workers were unable to distinguish between changes within the antigenic region or conformational alterations of other parts of the protein (caused by the loss of the C-terminal tetrapeptide) as the reason for the decrease in antigenic activity. Our results (i.e., attainment of 100% inhibition, but at a higher concentration of inhibitor) indicate that it is a conformational alteration that is responsible for the observation of Brown et al. (1959b). Therefore, segment 1–20 does contain an antigenic region, but segment 121–124 does not.

Inhibition by Peptide Fragments. Another approach was taken to study inhibition by the purified small peptides from the cyanogen bromide digestion and from the tryptic hydrolysis respectively of S-carboxymethylated RNase. Using the experimental protocol and theory of Furie et al. (1975) as modified by Chavez & Scheraga (1980b) for the evaluation of K_{conf}, we used the cyanogen bromide peptides 1–13, 31–79, and 80–124 as inhibitors in the inhibition assay with their

corresponding purified antibodies. The values of K_{conf} were found to be 5.0×10^{-4}, 2.1×10^{-4}, and 3.1×10^{-4}, respectively, at 4 °C (Chavez & Scheraga, 1980b). These values clearly demonstrate the antigenic activity retained by the excised fragments and are comparable to the value of 2×10^{-4} found for fragment 99–149 from staphylococcal nuclease (Sachs et al., 1972).

Tryptic fragment 105–124 exhibited no inhibition at a 3400-fold molar excess over [^{125}I]RNase$_N$ in the presence of anti-(80–124)$_N$, while tryptic fragment 40–61 in the presence of anti-(31–79)$_N$ exhibited a value of $K_{conf} = 1.2 \times 10^{-5}$ at 4 °C (determined at a 2000-fold molar excess). The inability to measure K_{conf} for fragment 105–124 is due to the absence of an antigenic determinant in this segment rather than to the existence of an antigenic determinant that is not in its native conformation. The second alternative is eliminated for the following reason. Anfinsen & Scheraga (1975) showed that the value of K_{conf} for a peptide with a random structure would be $\sim 10^{-6}$. Under the conditions of our experiments, we could have measured a value of K_{conf} as small as 1×10^{-6}; i.e., had there been an antigenic determinant in 105–124 in the random conformation, we could have detected it. Therefore, the inability to measure a value of K_{conf} indicates that fragment 105–124 does not contain the antigenic determinant(s) that is present in segment 80–124.

Fragment 40–61 does possess antigenic activity, although with K_{conf} of an order of magnitude less than that found for the cyanogen bromide peptides of RNase. At least four possible explanations can be suggested for this low value of K_{conf}. (1) Since an extensive test of the purity of this peptide could not be made (because of the limited amount of the material available; see Methods section), the observed value of K_{conf} could have been obtained if another antigenic peptide impurity were present to the extent of $\sim$10%. However, we can eliminate this possibility by the following argument. The only peptide produced by the tryptic digestion of S-carboxymethylated RNase, which is large enough and has the appropriate sequence to react with anti-(31–79)$_N$, is the peptide 67–85 (Hirs et al., 1956). This peptide, however, contains one arginine and two tyrosine residues, whereas the amino acid analysis of 3.3 nmol of peptide 40–61 indicated that there was less than 0.05 nmol of each of these residues per mol of peptide. Thus, from the amino acid analysis data, the contamination of peptide 40–61 by peptide 67–85 was less than 1.5%. At this level of contamination, the value of K_{conf} of fragment 67–85 would have to be of the order of 10^{-3}, which is unlikely in view of the value of 2.1×10^{-4} observed for fragment 31–79 (Chavez & Scheraga, 1980b). Thus, the presence of such an impurity cannot be responsible for the observed low value of K_{conf}. (2) The peptide could be essentially in the statistically random, unstructured state. (3) The whole antigenic determinant may not be present in the linear sequence of peptide 40–61, but may involve additional residues, removed in the sequence from this segment. (4) If anti-(31–79)$_N$ is a mixture of antibodies specific for segment 40–61 and for another determinant(s) not in the segment, then only anti-(40–61)$_N$ could be involved in competition between fragment 40–61 and [^{125}I]RNase$_N$, while the antibody for the other determinant(s) would bind freely to [^{125}I]RNase$_N$. This would result in a deceptively low value of K_{conf}. The last explanation certainly is involved in accounting for the low value of K_{conf} for segment 40–61 since the available data indicate that anti-(31–79)$_N$ is a mixture of antibodies with at least two different specificities (see Evidence for Four Antigenic Sites). The extent to which the second and third possible reasons account for the low value of K_{conf} cannot be determined, but segment 40–61 appears to contain an antigenic region, at least in part, because a measurable value of K_{conf} is obtained.

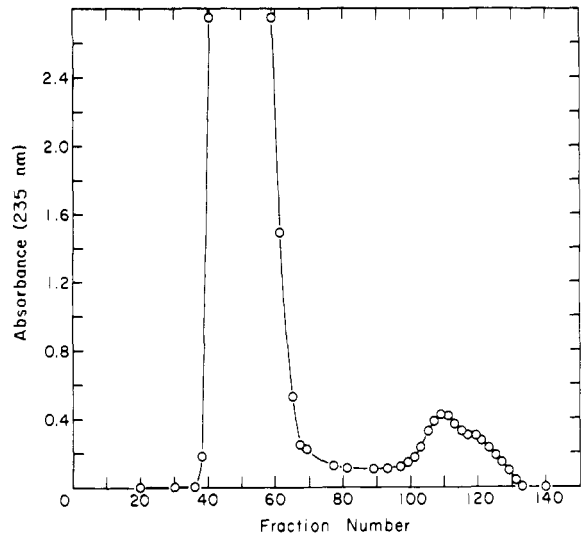

FIGURE 7: Separation of the immunologically active peptic peptides from the antibodies precipitated by ammonium sulfate. The precipitate was dissolved in 3.0 mL of 0.2 M acetic acid (the solution being clarified by addition of solid urea), and the pH was adjusted to 2.0. This solution was applied to a column (3.0 × 150 cm) of Sephadex G-75 equilibrated in 0.2 M acetic acid, and 5-mL fractions were collected. The absorbance at 235 nm was measured, and the low molecular weight peptides (fractions 100–130) were pooled.

Isolation of Antigenic Peptides. Sanders et al. (1970) introduced a procedure for the isolation of antigenic peptides from a digest of the original protein by using the immunoglobulin fraction of antiserum. The method will work only if it is possible to produce small enough peptides that still retain their antigenic activity. The retention of antigenic activity after digestion could be surveyed by examining the ability of the whole protein digest to inhibit (competitively) the binding of anti-RNase$_N$ to [^{125}I]RNase$_N$. If the peptide binds only weakly, then the amount of peptide required would be too large for this procedure to work practically. From their work on the antigenic peptides from a chymotryptic digest of sperm whale myoglobin, Crumpton & Wilkerson (1965) indicated that molar excesses of more than 300 (of small antigenic peptides to native protein) might be required in order to obtain 50% inhibition. Also, if the protein is digested to too great an extent, then the resulting peptides will be too small for retention of antigenic activity. The only digestion technique that yielded any inhibition was the peptic digestion of native ribonuclease in 5% formic acid for 20 h. No inhibition could be detected, even at a 10^3-fold molar excess of digested material, after digestion by trypsin. However, as shown earlier, an antigenic peptide does arise from the tryptic digestion of S-carboxymethylated RNase which was not detected by this preliminary screening technique because the concentration of this antigenically active peptide was not high enough in the total digest for detection of any inhibitory activity.

The immunoprecipitation of antigenic peptides from the peptic digest of native RNase was accomplished with the immunoglobulin-G fraction (IgG) of anti-RNase$_N$ antiserum. Extensive dialysis of the IgG fraction against 0.15 M NaCl was first necessary to remove all low molecular weight protein components which could coelute with the bound antigenic peptides in the subsequent separation procedure. After complexation (at pH 7.0), precipitation (at pH 6.8), and washing, the precipitate was dissolved in 0.2 M acetic acid since immune complexes are dissociated at low pH (and

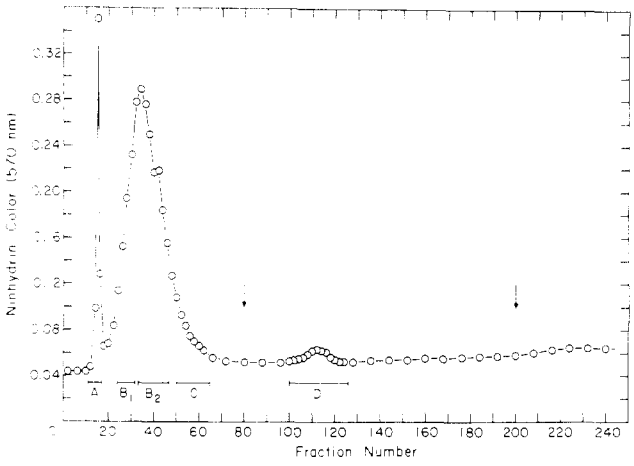

FIGURE 8: Chromatography of the pooled peptic peptide fractions obtained from the chromatogram shown in Figure 7 on a 1.8 × 30 cm column of Dowex 50W-X2. Initial elution was accomplished at 35 °C by washing with 500 mL of 0.2 N sodium formate, pH 3.08. The arrows indicate a change in the elution buffer. A gradient at 35 °C consisting of 500 mL of 0.2 N sodium formate, pH 3.08, as the first component and 500 mL of 2.0 N sodium acetate, pH 5.09, as the second component was begun at the first arrow. This was followed by 500 mL of 2.0 N sodium acetate, pH 6.6, at 50 °C at the second arrow. The effluent was collected in 3.0-mL fractions, and 0.3-mL aliquots were removed for analysis by the ninhydrin method. The fractions were pooled as indicated.

enough urea had been added to clarify the solution). Figure 7 shows a typical elution profile of the high molecular weight components and the low molecular weight peptides from the precipitate on Sephadex G-75 in 0.2 M acetic acid.

After lyophilization, the low molecular weight peptides were dissolved in 0.2 M sodium formate, pH 3.08. Total solubilization of these peptides could be accomplished only by the addition of urea to a final concentration of 4.0 M. Fractionation of the peptides was carried out by chromatography on Dowex 50W-X2 (Figure 8). The appropriate fractions were pooled, lyophilized, and desalted on Sephadex G-10 in 0.001 M HCl. Amino acid analyses were performed on all desalted pools, but only one, pool A, gave an amino acid composition that could be identified uniquely with a sequence (residues 63–75) from RNase (see Table I). Presumably, the peptides in the other pools were too degraded by pepsin to retain antigenicity. Segment 63–75 in native ribonuclease contains a small disulfide loop between residues 65 and 72, with a double β bend at residues 66–68 (Wyckoff et al., 1967, 1970). The importance of this loop for the antigenic activity of segment 63–75 can be seen when the loop is destroyed by tryptic digestion [peptide 67–85 is generated by the digestion of S-carboxymethylated RNase A with trypsin (Hirs et al., 1956); i.e., the peptide bond between residues 66 and 67 is split by trypsin. In similar immunoprecipitation experiments using the tryptic digestion products of RNase, no precipitation of peptide 67–85 with anti-$RNase_N$ could be observed, presumably because trypsin splits the antigenic site in this region].

Location of Antigenic Sites. To summarize the results of the preceding experiments we find the following. (1) There are ≥3 antigenic determinants per RNase molecule, based on the analysis of the precipitin curve. (2) The cyanogen bromide fragments, 1–13, 31–79, and 80–124, possess antigenic activities, based on their abilities to bind antibodies on affinity columns and to inhibit the binding of their corresponding antibodies to [^{125}I]$RNase_N$. We conclude that there is at least one determinant per cyanogen bromide fragment (except for fragment 14–29 which possesses no antigenic activity). Binding data for each specific antibody further indicate that segment 31–79 possesses at least two determinants. (3) The peptide 63–75 also possesses antigenic activity on the basis of its ability to precipitate specifically with anti-$RNase_N$. (4) Fragment 40–61 competes with [^{125}I]$RNase_N$ for anti-$(31–79)_N$, while fragment 105–124 displays no such competition for anti-$(80–124)_N$ with [^{125}I]$RNase_N$ [also segment 121–124 is not required for the binding of des(121–124)-RNase to whole anti-$RNase_N$]. These data support the conclusions that region 31–79 contains at least two antigenic determinants, 40–61 and 63–75 [i.e., anti-$(31–79)_N$ is a mixture of two antibodies]. The antigenic determinant(s) in region 80–124 must be limited to residues 80–104.

Trypsin splits peptide bonds in these antigenic determinants as follows: after Lys-7 and Arg-10 in segment 1–13, after Lys-66 in segment 63–75, and after Arg-85, Lys-91, and Lys-98 in segment 80–104 (Hirs et al., 1956). Therefore, we were not able to isolate antigenic peptides in segments 1–13, 63–75, and 80–104 by tryptic digestion in the procedure of Sanders et al. (1970).

Discussion

Kabat (1964) estimated that a globular protein molecule having the molecular weight of RNase could possess three antigenic determinants. This is a lower limit based on estimates of the relative sizes of RNase and antibody molecules, of the size of a determinant from peptide–antibody binding studies, and of the available surface area of RNase. This value of 3 for RNase is supported by our results in Figure 2 and by the similar results of Cinader & Pearce (1956), who also used a precipitin titration to determine the number of antibodies that can bind simultaneously to $RNase_N$. Cinader & Pearce (1956), however, analyzed their data from precipitin experiments further by using the model of Goldberg (1952) and found that a value of 4 fit better for the total number of antibodies that could bind, thus indicating that there is competition between the antigenic sites. Stelos et al. (1960) obtained best agreement between the calculated and experimental equilibrium constant for the antibody–$RNase_N$ reaction when a valency of 4 was used in the computations. When our antigen-binding data for each purified antibody preparation (Figure 4) are compared with the experimental results of Berzofsky et al. (1976), which are based on the analysis of the shape of the antigen-binding curve in relation to the valency of the antigen, we conclude that RNase contains *at least* four antigenic determinants.

The results reported here implicate the following segments as the approximate locations of the antigenic determinants that we have identified in native RNase: lysine-1 to methionine-13, half-cystine-40 to lysine-61, valine-63 to serine-75, and serine-80 to lysine-104. No other regions are implicated as antigenic determinants according to our results. No measurable binding occurs between segment 14–29 and anti-$RNase_N$; hence, we eliminate this region as a possible antigenic site. A major region from which we could not obtain any fragments for analysis was segment 30–39. This region cannot be eliminated as a possible binding site for antibody on the basis of our data. In fact, residues 34 and 35 have been implicated in the antigenic activity of RNase (Welling & Groen, 1976). We will return to this point later.

It is possible that other antigenic determinants exist and were not detected because the coresponding antibodies did not bind to the peptide fragments used here for antibody fractionation. Also, each peptide generated by different digestion methods would have to be subjected individually to inhibition assays in order to determine conclusively whether it is antigenic.

Chemical modification experiments provide further information on the location of the antigenic regions of $RNase_N$. The antigenic activity of native RNase is not affected by treatment of arginine-39 and -85 with 1,2-cyclohexadiene (van der Zee et al., 1977), nitration of tyrosine-76 and -115 (van der Zee et al., 1977), or dinitrophenylation of lysine-41 (Brown et al., 1967). These results support our conclusions since each of these modifications lies outside of the specified antigenic regions, except lysine-41 which is buried in the enzymatic active site of RNase (Richards & Wyckoff, 1971) and would not be accessible to antibody in any case and arginine-85 which is discussed below.

In contrast, increasing degrees of esterifcation of the carboxyl groups lead to a gradual reduction in antigenicity (Acharya et al., 1977). To interpret the data of Acharya et al., who used a precipitin assay to measure the antigenicity of RNase, we make use of the sequential pattern of esterification found by Riehm & Scheraga (1966). (1) The first carboxyl group which is modified is aspartic acid-53, and this decreases the antigenicity and enzymatic activity by only 2% [this residue is involved in an α helix, and its β-carboxyl group is freely exposed to the solvent (Richards & Wyckoff, 1971)]. Thus, Asp-53 is not part of an antigenic determinant. (2) The next residues to be modified are glutamic acid -49 and -111, and the antigenicity decreases to 88% and the enzymatic activity to 58%. Riehm & Scheraga (1966) could not determine the order of esterification of residues 49 and 111; however, Acharya et al. (1977) found that, with only two residues (53 and 49 or 111) esterified, the antigenic activity decreased only to 95%. The γ-carboxyl group of residue 49 is hydrogen bonded to the backbone amide group of serine-80, and that of residue 111 is exposed to the solvent (Richards & Wyckoff, 1971). Modification of glutamic acid-49 would probably distort the N-terminal portion of the α helix beginning at serine-50 and affect the disposition of the N-terminal α helix containing histidine-12 (since residues 49 and 12 are near each other). Thus, residue 49 is probably involved in some way, either as part of an antigenic determinant or in influencing the conformation of an antigenic determinant. Esterification of residue 49 would have a relatively large effect on enzymatic activity because of its close association with histidine-12 (which is involved in the active site of RNase) and on antigenic activity since it would have an effect on the antigenic determinants in segment 1–13 and also in segment 40–61. The motion of the peptide backbone of residue 111 is probably restricted by its proximity to the disulfide bond at half-cystine-110 and, since residue 111 is freely accessible to solvent, esterification of its side chain would not be expected to modify the conformation in the surrounding region significantly. This interpretation is consistent with our finding that segment 105–124 does not contain an antigenic determinant, and therefore glutamic acid-111 would not play a significant role in the antigenic activity. This agrees with the 3% decrease to 95% antigenic activity found by Acharya et al. (1977) after modification of two residues. (3) The next residues to be modified are glutamic acid-9 and -86, and the antigenicity is lowered to 68% but the enzymatic activity is only 16% that of the native protein. Again, while Riehm & Scheraga (1966) were not able to determine the order of modification, Acharya et al. (1977) demonstrated that the modification of four residues (53, 111, 49, and 9 or 86) decreased the antigenic activity from 88 to 83% and the modification of five residues resulted in 68% of the native antigenic activity. The location of glutamic acid-86 on the edge of the active-site cleft is probably responsible for the dramatic decrease in the enzymatic activity. The choice between residue 9 and 86 as the one responsible for the decrease from 83 to 68% antigenic activity is made on the basis of the location of antigenic sites according to our results. It is most reasonable to expect that modification of glutamic acid-9 would produce a large decrease in the antigenic activity in the limited region of 1–13. Hence, modification of residue 86 would be responsible for only slight conformational alterations but would not be involved in an antigenic determinant. Some support for the latter conclusion can be found from the fact that modification of arginine-85 had no effect on antigenic activity (van der Zee et al., 1977). (4) The *last* three free carboxyl groups (14, 38, and 83) which are modified are esterified very slowly (Riehm & Scheraga, 1966). Prior to their modification, three other residues (glutamic acid-2, aspartic acid-121, and C-terminal valine-124) are esterified as a group (Acharya et al., 1977). The modification of these residues can be correlated with the decrease of antigenic activity from 68 to 43%. In order to estimate the contributions that these residues make to the decrease in antigenic activity after esterification, we should consider the following. We have shown that the removal of residues 121–124 decreases the antigenic activity of RNase but does not destroy any antigenic sites (Figure 5). Puett (1972) has shown that des(121–124)-RNase is ~30% less stable than native RNase. If we use these data to approximate the decrease in antigenic activity after modification of carboxyl groups 121 and 124, we find a value of 48% antigenic activity left. Therefore, residues 121–124 are not involved in antigenic determinants but contribute to the stability (hence antigenicity) of the whole $RNase_N$ molecule. Esterification of residue 2 (which is near or in the antigenic site in segment 1–13) may contribute to this value of 48%, which agrees very well with the experimental value of 43%. It is important to realize that interactions involving residues 121–124 greatly affect the conformational stability of each antigenic region, as indicated by significant decreases in the values of K_{conf} for des(121–124)-RNase compared to $RNase_N$ (Chavez & Scheraga, 1980b). (5) The modification of aspartic acid-38 precedes that of residues 14 and 83 (Riehm & Scheraga, 1966) with a concomitant decrease in antigenic activity from 43 to 30% (Acharya et al., 1977). We are unable to associate any antigenic changes with particular residues at this stage of esterification. A combination of changes in conformation and in specific antigenic sites could be responsible for these results. Therefore, the effects of esterification appear consistent with the location of antigenic determinants in the regions around residues 2, 9, and 49.

Using the static accessibility data of Lee & Richards (1971) and the X-ray structure of Wyckoff et al. (1967, 1970), we can suggest various residues that are not involved in the binding of antibody to the antigenic sites. The backbones or side chains of residues 8, 11–13, 26, 30, 35, 36, 40, 44, 46–48, 54, 57, 58, 65, 72, 75, 79, 81, 82, 84, 90, 95, 97, 106, 108–110, 117, 118 and 120 have little or no contact with the solvent [see Figure 5 of Richards & Wyckoff (1971)] and therefore probably cannot interact with antibodies. This is not to say that these residues are not important for providing a specific conformational framework for the antigenic sites. However, according to the definition of an antigenic determinant,[2] these data narrow the possible locations for antigenic sites within the particular regions that we have identified. These data can be used only to eliminate residues which occur at the ends of the antigenic peptides used here (i.e., 1–13, 31–79, 40–61, 63–75, and 80–124) because inaccessible (nonreactive) residues

are outside of the boundary of reactive residues. Internal residues may affect antigenicity even if they are buried since they could provide the proper structural orientation for the adjacent residues which do bind directly to antibody.

In region 1–13, residues 11–13 can be eliminated on the basis of lack of exposure. Because of our inability to separate the conformational contributions from the direct participation of internal residues in the antigenic site, we cannot eliminate more than 11–13 (in segment 1–13) at present. In region 40–61, after eliminating the inaccessible residue at the end of the peptide (residue 40) and those whose chemical modification does not affect antigenicity (residues 41 and 53), we are left with residues 42–52 and 54–61 as possible candidates. Localization of the antigenic determinant, however, must be around residue 49 on the basis of our interpretation of the data of Acharya et al. (1977). Therefore, the most likely location of the antigenic determinant must be in residues 42–52. In region 63–75, the most predominant structural feature is the double β bend which occurs in residues 66–68 and which is anchored on each end by a disulfide bond joining residues 65–72. Residues 66–70 are among the most exposed residues on the surface of RNase (Lee & Richards, 1971). This information implicates the β-bend region as the most likely candidate for the antigenic site. In region 80–104, residues 81, 82, and 84–86 can be eliminated on the basis of either the lack of exposure or the ineffectiveness of modification on antigenic activity. Within the remaining residues, residues 83, 87–89, and 96 are only marginally exposed (<0.7% average exposure for each of these residues compared to the average exposure of 0.88% for all the residues of RNase), and two regions which are partially exposed (Lee & Richards, 1971) include the double β bend at residues 92–94 and a portion (residues 98–104) of the extended β-sheet structure at residues 96–110. These latter segments are likely candidates for the antigenic site(s).

Welling & Groen (1976) have compared the antigenic activities of several homologous pancreatic ribonucleases which have limited amino acid substitutions. Their analysis implicates residues 34, 35, 50, 99, and 103 in antigenic determinants. If these substitutions do not affect the three-dimensional structure of the protein, then residues 50, 99, and 103 are consistent with the antigenic determinants that we have located. Hurrell et al. (1977) and I. East and P. E. E. Todd (private communication) have shown that residues that are not part of the antigenic determinant, but nevertheless play a role in determining the conformation of the antigenic determinant, can influence antigenicity. This result should be kept in mind in interpreting the results of experiments designed to test the effect of chemical modification and changes in composition on antigenicity. Therefore, the results of Welling & Groen (1976) could be accounted for if residues 34 and 35 are not part of the antigenic site but instead help to maintain the conformation. This is especially the case for residue 35, which is only slightly exposed to the solvent and therefore cannot be part of an antigenic site.

As already mentioned, our data do not provide any information about the possible antigenicity of region 30–39. It appears that residues 30 and 39 can be eliminated because of the lack of exposure and the ineffectiveness of modification, respectively. The role that residue 34 plays in antigenic activity is not clear. Residue 34 (which is asparagine in bovine RNase A and B) has a carbohydrate chain attached in RNase B. Since the experiments of Welling & Groen (1976) compared ribonuclease proteins from different species using the antibody against bovine RNase A, the differences in the antigenic activities of this position would depend on the presence of a carbohydrate chain as well as on the particular residue at position 34. Residues 34 and 35 may be located in an antigenic site; however, another explanation can adequately account for their results. Inspection of the X-ray structure of bovine RNase A reveals that a carbohydrate moiety could overlap the antigenic region in segment 1–13. This could influence the specificity of the antibody made and the binding affinity of the antibody to segment 1–13. This might account for the difference in the antigenic activity when an amino acid (which can accommodate the carbohydrate chain) is substituted. Amino acid substitutions at position 35 could then influence the conformational disposition of the carbohydrate attached at position 34. This problem could be resolved by the analysis of the antigenic activity of a peptide containing segment 31–38.

Our location of an antigenic determinant in segment 1–13 is in disagreement with the results of Singer & Richards (1959) and Welling & Groen (1976). Singer & Richards found no inhibition of the reaction of anti-$RNase_N$ with $RNase_N$ by a 90-fold molar excess of S-peptide. However, in determining the value of K_{conf} for S-peptide (Chavez & Scheraga, 1980b), an excess of at least 200-fold was necessary to demonstrate 50% inhibition. From the work of Crumpton & Wilkerson (1965) and the corroborating studies of Sachs et al. (1972), it is clear that high molar excesses of peptides over native proteins may be needed in inhibition assays in order to compensate for the decrease in conformational stability. Using a phage-inactivation assay, Welling & Groen (1976) detected no inhibition of the inactivation of phage (to which bovine RNase A was coupled) by a synthetic peptide segment, 1–14. However, their antiserum was only a second course bleeding from one rabbit and was not fractionated. It is well-known that pools of antiserum from different rabbits, especially from early bleedings, can exhibit large differences in the spectrum of antibody specificities and in binding affinities (Brown et al., 1960). In contrast, we used relatively late hyperimmune, high-affinity antisera accumulated over several bleedings, pooled from four nonsibling rabbits and fractionated according to specificity on a column to which fragment 1–13 was coupled (see Preparation and Fractionation of Antibodies). It is likely that antibodies taken later during immunization have a more restricted specificity, not in the number of determinants recognized but in the number of residues recognized within a single determinant (Atassi, 1975). Greater specificity and high affinity may be the reasons that we were able to detect binding to region 1–13 whereas others have not. We believe that these explanations account for the observed discrepancies.

In summary, we propose the following locations for the antigenic determinants in $RNase_N$ (Figure 9): segment 1–10, which includes an α helix at residues 4–10; segment 63–75, which contains a double β bend at residues 66–68; and segment 87–104, which contains portions of a β-pleated sheet in residues 98–104 and a double β bend at residues 92–94. On the basis of the data of Welling & Groen (1976), who implicate residues 99 and 103 in an antigenic determinant, residues 98–104 appear to be the best candidate for the antigenic determinant in segment 87–104. On the basis of the limited amount of information available, the determinant within segment 40–61 appears to be centered around residue 49. It is still possible that segment 31–38 may be part of an antigenic determinant.

Finally, it should be noted that the order in which antigenic determinants appear during folding of the protein to the native structure depends on nucleation and folding of residues at other (interior) sites where antibodies cannot bind. Thus, the

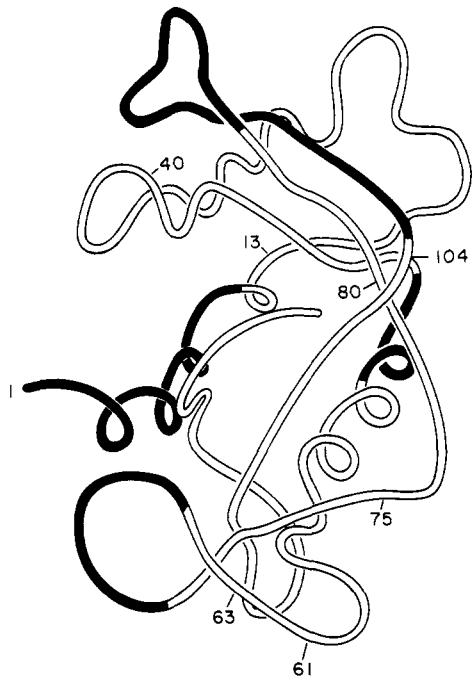

FIGURE 9: An artist's representation of RNase with the N-terminal and C-terminal amino acids of the peptides within which the antigenic site lies indicated by the residue numbers and the proposed locations of the antigenic determinants shaded.

observed formation of the *first* antigenic determinant at (the surface of) segment 80–124 (Chavez & Scheraga, 1977) *follows* the presumed nucleation at residues 106–118 (Matheson & Scheraga, 1978).

Acknowledgments

We are indebted to T. W. Thannhauser for the amino acid and nitrogen analyses and to Drs. Y. Konishi, S. J. Leach, R. R. Matheson, Jr., and R. Rudolph for helpful discussions.

References

Acharya, A. S., Manjula, B. N., Murthy, G. S., & Vithayathil, P. J. (1977) *Int. J. Pept. Protein Res. 9*, 213.
Anfinsen, C. B., & Scheraga, H. A. (1975) *Adv. Protein Chem. 29*, 205.
Arend, W. P., & Mannik, M. (1974) *J. Immunol. 112*, *451*.
Atassi, M. Z. (1975) *Immunochemistry 12*, 423.
Atassi, M. Z. (1978) *Immunochemistry 15*, 909.
Berzofsky, J. A., Curd, J. G., & Schechter, A. N. (1976) *Biochemistry 15*, 2113.
Brown, J. E., & Klee, W. A. (1971) *Biochemistry 10*, 470.
Brown, R. K. (1962) *J. Biol. Chem. 237*, 1162.
Brown, R. K. (1963) *Ann. N.Y. Acad. Sci. 103*, 754.
Brown, R. K. Delaney, R., Levine, L., & van Vunakis, H. (1959a) *J. Biol. Chem. 234*, 2043.
Brown, R. K., Durieux, J., Delaney, R., Leikhim, E., & Clark, B. J. (1959b) *Ann. N.Y. Acad. Sci. 81*, 524.
Brown, R. K., Tacey, B. C., & Anfinsen, C. B. (1960) *Biochim. Biophys. Acta 39*, 528.
Brown, R. K., McEwan, M., Mikoryak, C. A., & Polkowski, J. (1967) *J. Biol. Chem. 242*, 3007.
Cinader, B., & Pearce, J. H. (1956) *Br. J. Exp. Pathol. 37*, 541.
Chavez, L. G., Jr., & Scheraga, H. A. (1977) *Biochemistry 16*, 1849.
Chavez, L. G., Jr., & Benjamin, D. C. (1978) *J. Biol. Chem. 253*, 8081.
Chavez, L. G., Jr., & Scheraga, H. A. (1980a) *Biochemistry*, in press.
Chavez, L. G., Jr., & Scheraga, H. A. (1980b) *Biochemistry*, in press.
Crumpton, M. J., & Wilkerson, J. M. (1965) *Biochem. J. 94*, 545.
Farr, R. S. (1958) *J. Infect. Dis. 103*, 239.
Furie, B., Schechter, A. N., Sachs, D. H., & Anfinsen, C. B. (1975) *J. Mol. Biol. 92*, 497.
Goldberg, R. J. (1952) *J. Am. Chem. Soc. 74*, 5715.
Haimovich, J., Hurwitz, E., Novik, N., & Sela, M. (1970a) *Biochim. Biophys. Acta 207*, 115.
Haimovich, J., Hurwitz, E., Novik, N., & Sela, M. (1970b) *Biochim. Biophys. Acta 207*, 125.
Hirs, C. H. W., Moore, S., & Stein, W. H. (1956) *J. Biol. Chem. 219*, 623.
Hurrell, J. G. R., Smith, J. A., Todd, P. E., & Leach, S. J. (1977) *Immunochemistry 14*, 283.
Kabat, E. A. (1964) *Kabat and Meyer's Experimental Immunochemistry*, 2nd ed., Charles C. Thomas, Springfield, IL.
Lang, C. A. (1958) *Anal. Chem. 30*, 1692.
Lee, B., & Richards, F. M. (1971) *J. Mol. Biol. 55*, 379.
Lin, M. C. (1970) *J. Biol. Chem. 245*, 6726.
Matheson, R. R., Jr., & Scheraga, H. A. (1978) *Macromolecules 11*, 819.
McConahey, P. J., & Dixon, F. J. (1966) *Int. Arch. Allergy Appl. Immunol. 29*, 185.
Noel, R. J., & Hambleton, L. G. (1976a) *J. Assoc. Off. Anal. Chem. 59*, 134.
Noel, R. J., & Hambleton, L. G. (1976b) *Chem. Abstr. 84*, 149347k.
Puett, D. (1972) *Biochemistry 11*, 1980.
Richards, F. M., & Vithayathil, P. J. (1959) *J. Biol. Chem. 234*, 1459
Richards, F. M., & Wyckoff, H. W. (1971) *Enzymes, 3rd Ed. 4*, 647.
Riehm, J. P., & Scheraga, H. A. (1966) *Biochemistry 5*, 99.
Rupley, J. A., & Scheraga, H. A. (1963) *Biochemistry 2*, 421.
Sachs, D. H., Schechter, A. N., Eastlake, A., & Anfinsen, C. B. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3790.
Sanders, M. M., Walsh, K. A., & Arnon, R. (1970) *Biochemistry 9*, 2356.
Singer, S. J., & Richards, F. M. (1959) *J. Biol. Chem. 234*, 2911.
Smyth, D. G., Stein, W. H., & Moore, S. (1963) *J. Biol. Chem. 238*, 227.
Stelos, P., Fothergill, J. E., & Singer, S. J. (1960) *J. Am. Chem. Soc. 82*, 6034.
Steward, M. W., & Petty, R. E. (1972a) *Immunology 22*, 747.
Steward, M. W., & Petty, R. E. (1972b) *Immunology 23*, 881.
Taborsky, G. (1959) *J. Biol. Chem. 234*, 2652.
Teale, J. M., & Benjamin, D. C. (1976) *J. Biol. Chem. 251*, 4603.
van der Zee, R., Duisterwinkel, F. J., & Welling, G. W. (1977) *Eur. J. Biochem. 77*, 125.
van Vunakis, H., Leikhim, E., Delaney, R., Levine, L., & Brown, R. K. (1960) *J. Biol. Chem. 235*, 3430.
Welling, G. W., & Groen, G. (1976) *Biochim. Biophys. Acta 446*, 331.
Wyckoff, H. W., Hardman, K. D., Allewell, N. M., Inagami, T., Johnson, L. N., & Richards, F. M. (1967) *J. Biol. Chem. 242*, 3984.
Wyckoff, H. W., Tsernoglou, D., Hanson, A. W., Knox, J. R., Lee, B., & Richards, F. M. (1970) *J. Biol. Chem. 245*, 305.