Intrinsic Stabilities of Portions of the Ribonuclease Molecule†

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ABSTRACT: The equilibrium constant, K_{conf}, for the formation of the native conformation from the unfolded form is determined for various protein and peptide derivatives of bovine pancreatic ribonuclease. Purified antibodies, specific for native antigenic determinants in segments 1–13, 31–79, and 80–124 are used to obtain the values of K_{conf}. The observed variations in K_{conf} from one type of molecule to another are consistent with the degree of chemical modification introduced. Reduction of the disulfide bonds of ribonuclease decreases K_{conf} to ~0.06 compared to a very large value for native ribonuclease, whereas S-carboxymethylation of the resulting sulfhydryl groups decreases K_{conf} to $\sim 10^{-3}$. These results indicate the degree of importance of the disulfide bonds and the effect of the bulky charged carboxymethyl groups and *also provide evidence that there is significant native structure in the reduced protein.* The loss of residues 1–20 from the N terminus of native ribonuclease decreases K_{conf} to ~0.06. The loss of the C-terminal tetrapeptide by treatment with pepsin decreases K_{conf} to ~0.3, and the loss of the C-terminal hexapeptide lowers K_{conf} to ~0.006. Thus, the stability of the entire molecule depends strongly on the C terminus. The values of K_{conf} for the cyanogen bromide peptides, segments 1–13, 31–79, and 80–124, provide an indication of the extent to which short- and medium-range interactions restrict the conformational space available to these peptides. An approximate value of K_{conf} of 10^{-4}, observed for these three peptides, is similar to the value determined previously for a peptide from staphylococcal nuclease [Sachs, D. H., Schechter, A. N., Eastlake, A., & Anfinsen, C. B. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3790]. The temperature dependence of K_{conf} for three different regions of des(121–124)-RNase yields three different transition curves with the concomitant values of ΔH for the three antigenic regions, indicating that intermediates exist in the unfolding transition.

For a polypeptide chain to fold in a limited amount of time, its available conformational space must be greatly restricted. Theoretical studies have indicated how such restrictions can arise (Ramachandran & Sasisekharan, 1968; Scheraga, 1968, 1971, 1973). The amino acid sequences of proteins presumably have evolved so that short-range interactions lead to structures in which short segments (in the unfolded form of the protein) are fairly stable in the ultimate conformation of the native protein, of which they are a part; also, such short segments can direct the folding of other sections of the protein along a specific pathway (Taniuchi & Anfinsen, 1968, 1969; Lewis et al., 1970, 1971; Scheraga, 1973; Ponnuswamy et al., 1973). However, while most of the residues of the protein are required for proper folding, in some cases several residues can be dispensed with (Haber & Anfinsen, 1961; Chavez & Scheraga, 1980). The number and location of dispensable residues probably varies from one protein to another.

Various attempts have been made to determine the relative importance of individual residues in the folding process. For example, the sequences in which residues become exposed to solvent during denaturation have been determined (Rupley & Scheraga, 1963; Ooi & Scheraga, 1964; Klee, 1967; Nakanishi et al., 1972; Imoto et al., 1974; Burgess et al., 1975; Nakanishi & Tsuboi, 1978), and, in the case of bovine pancreatic ribonuclease ($RNase_N$),[1] this information has been utilized to postulate a pathway for unfolding in which the relative stabilities of various intermediates could be discerned (Burgess & Scheraga, 1975). In general, it has been difficult to determine the stabilities of such intermediates because their concentrations are usually below the limits of detectability of most of the currently available experimental techniques that are used in equilibrium studies.

This situation, however, has been improved recently with the introduction of immunological techniques (Sachs et al., 1972; Furie et al., 1975) that are several orders of magnitude more sensitive than physicochemical methods for making quantitative determinations of the amounts of various conformations present in solutions. The associated theory (Sachs et al., 1972; Furie et al., 1975) has been summarized by Anfinsen & Scheraga (1975). This approach is based on the assumptions that (1) a dynamic equilibrium exists between a native conformation and a number of unfolded forms, (2) the antibody produced against either the native or nonnative forms remains specific for that species, and (3) the antibody binds with the same affinity to its corresponding native determinant no matter whether the determinant is in the intact protein or in a fragment thereof. In this approach, the conformational stability of only the region of an antigenic determinant can be evaluated. Thus, the conformational equilibrium constant, K_{conf}, for the conversion of the unfolded forms to the native one has been determined for the isolated peptide, residues 99–149, from staphylococcal nuclease to be 2×10^{-4} at 25 °C (Sachs et al., 1972) and also for the partially α-helical antigenic region, residues 99–126, in intact staphylococcal nuclease to be 2900 at 25 °C (Furie et al., 1975). Using both the approaches of Sachs et al. (1972) and Furie et al. (1975), Hurrell et al. (1977) have found that the isolated peptide, residues 132–153, of myoglobin, which is largely α helical in

† From the Baker Laboratory of Chemistry, Cornell University, Ithaca, New York 14853. *Received August 13, 1979.* 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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[1] Abbreviations used: $RNase_N$ or simply RNase, bovine pancreatic ribonuclease A; C-peptide and C-protein, the species produced by cyanogen bromide digestion without disruption of disulfide bonds (with the methionine residues converted to homoserine lactone) and consisting of residues 1–13 and 14–124 (with Met-30 missing), respectively; S-peptide and S-protein, the species produced *by limited* subtilisin digestion and consisting of residues 1–20 and 21–124, respectively; SCM-X, where X is a fragment which has been S-carboxymethylated; CNBr-OX-RNase, the cyanogen bromide digest of performic acid oxidized RNase; des-(121–124)-RNase, species lacking the C-terminal tetrapeptide; [^{125}I]-$RNase_N$, iodinated native RNase; anti-X, antibody against X, where X is $RNase_N$ or a fragment thereof; K_{conf}, the equilibrium constant between the unfolded and native conformations of a protein fragment.

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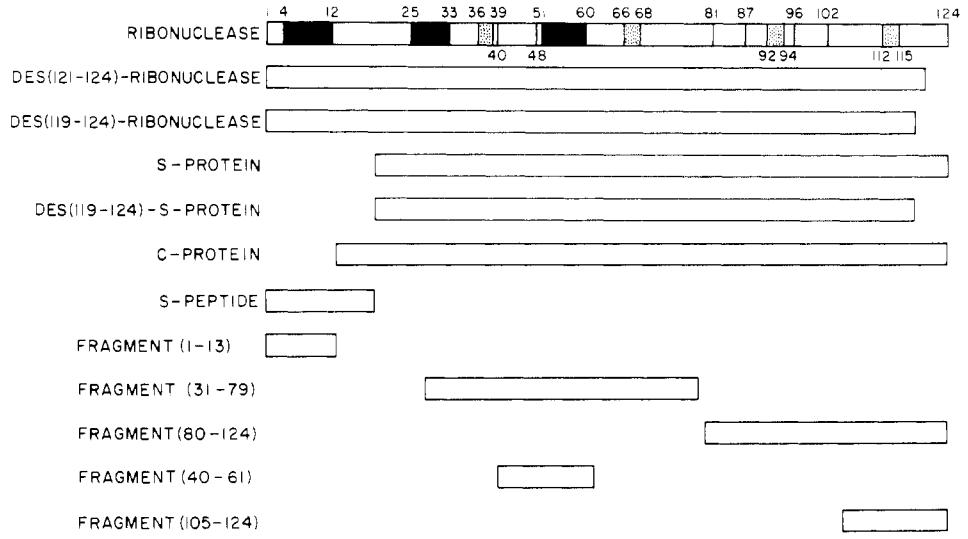

FIGURE 1: Diagram of the amino acid sequences of ribonuclease and its fragments which were used in these studies. The state of oxidation of the S–S bonds and the presence of S-carboxymethyl groups are not indicated. The X-ray crystallographic studies (Wyckoff et al., 1967, 1970) have shown the presence of α helices at residues 4–12, 25–33, and 51–60, three strands of β pleated sheet at residues 40–48, 81–87, and 96–102, and four segments possessing reverse turns at residues 36–39, 66–68, 92–94, and 112–115. These are indicated on the ribonuclease line by solid bars, open bars, and dotted bars, respectively.

the intact protein, has a value of K_{conf} of 10^{-2} at 36 °C. These results imply that these α-helical segments have relatively high stability. Since the stability of the α helix would increase if it were longer and less exposed to water, such a helical segment could serve to direct a folding process and thereby gain stabilizing interactions with other parts of the chain and a reduction of its contact with the solvent.

The recent identification of four antigenic regions in RNase, with at least three segments of ordered backbone structure (Chavez & Scheraga, 1979), makes it possible to assess the potential of the ordered structure of each segment to direct the folding of RNase. For this purpose, it is important to know the intrinsic stabilities of these ordered structures; thus, in this paper, we use the immunological method to determine the values of K_{conf} of three distinct antigenic regions in several protein and peptide derivatives of RNase in order to determine how they affect the overall stability of the RNase molecule and of derivatives in which several residues are missing.

Methods

Preparation of Ribonuclease and Derivatives. The preparation of purified RNase, des(121–124)-RNase, S-protein, and S-peptide was described previously (Chavez & Scheraga, 1980). Des(119–124)-RNase was prepared from des(121–124)-RNase by the procedure of Lin (1970). Digestion with carboxypeptidase A (Worthington) was allowed to proceed for 20 min at 45 °C and then for 72 h at room temperature.

Reduced RNase was prepared according to the method of Anfinsen & Haber (1961) as modified by Chavez & Scheraga (1977). Complete reduction of RNase was demonstrated by the method of Taniuchi (1970). This same method was used to demonstrate that no oxidation of free sulfhydryl groups occurred during the incubation of reduced RNase with antibody in 0.1 M sodium borate containing 0.01 M of the sodium salt of EDTA at pH 8.3. The initial sulfhydryl titer of 8.1 SH groups per molecule of reduced RNase decreased to 7.8 SH groups per molecule after 24 h. Further criteria that reduction was complete are discussed in the preceding paper (Chavez & Scheraga, 1980).

S-Carboxymethylated RNase, S-carboxymethylated S-protein, C-peptide, C-protein, and the S-carboxymethylated cyanogen bromide peptides of RNase were obtained by the methods of Chavez & Scheraga (1977). SCM-(40–61) and SCM-(105–124) were prepared by the methods of Hirs et al. (1956) and Chavez & Scheraga (1979).

All derivatives were desalted on a 2 × 150 cm column of Sephadex G-10 (Pharmacia Fine Chemicals), equilibrated and eluted with 0.1 M acetic acid. Fractions containing the desired derivatives were pooled, lyophilized, and stored at 4 °C in powder form. Figure 1 summarizes the derivatives used in this study.

Sulfhydryl Titration. Sulfhydryl groups were determined by the method of Taniuchi (1970). Twenty microliters of a solution containing 39.6 mg of 5,5′-dithiobis(2-nitrobenzoic acid) (Aldrich Chemical Co.) in 10 mL of 0.1 M sodium phosphate buffer, pH 7.0, was added to 3 mL of oxidation mixture and allowed to incubate for 10 min at room temperature. Then the absorbance at 412 nm was measured.

Amino Acid Analysis. All derivatives were hydrolyzed for 48 h at 105 °C in 6 N HCl (prepared from constant-boiling HCl) and then chromatographed with a Technicon TSM automated amino acid analyzer. The results are presented in Table I.

Preparation and Fractionation of Antibodies. The preparation of rabbit anti-$RNase_N$ serum and the fractionation of antibodies on columns to which peptide fragments 1–13, 31–79, and 80–124 had been attached have already been described (Chavez & Scheraga, 1977, 1979).

Determination of Peptide and Protein Concentration. The concentrations of all peptide and protein derivatives of RNase and of the antibodies used in the measurements of K_{conf} were determined by micro-Kjeldahl nitrogen analysis carried out with the procedures of Lang (1958) and Noel & Hambleton (1976a,b).

Radioimmunoassay for Determination of K_{conf}. Purified RNase was trace-iodinated (^{125}I) by the Chloramine-T method, as described by McConahey & Dixon (1966) and Chavez & Scheraga (1977). The labeled protein had a specific activity of 22 000 cpm per 9.1×10^{-12} mol.

The radioimmunoassay used to determine how various RNase protein and peptide derivatives competitively inhibited the binding of antibodies to [^{125}I]$RNase_N$ was that of Chavez & Scheraga (1977), as modified below, in order to obtain K_{conf} by the method of Furie et al. (1975). The equilibrium constant

Table I: Amino Acid Compositions of Derivatives of Bovine Pancreatic Ribonuclease[a]

	RNase	des(121-124)-RNase		des(119-124)-RNase		S-protein		S-peptide		C-peptide		C-protein		SCM-(31-79)		SCM-(80-124)		SCM-(40-61)		(SCM-105-124)	
amino acid	T[b]	T[b]	F[c]	T[b]	F	T[b]	F	T[b]	F	T[b]	F	T[b]	F	T[b]	F	T[b]	F	T[b]	F	T[b]	F
aspartic acid	15	14	14.0	14	14.4	14	13.9	1	1.1			15	14.7	7	7.0	5	5.8	2	2.8	2	2.0
threonine	10	10	10.0	10	9.9	8	8.3	2	1.9	1	1.2	9	8.9	4	4.1	4	4.2	1	0.8		
serine	15	14	12.5	14	13.7	12	11.6	3	2.7			15	14.8	5	5.2	4	4.3	2	1.9	1	0.7
glutamic acid	12	12	12.3	12	12.0	9	9.8	3	2.9	3	3.6	9	8.8	5	4.7	3	3.5	3	2.6	1	0.9
proline	4	4	4.3	4	4.3	4	4.3					4	3.9	1	1.0	3	2.9	1	0.9	2	2.3
glycine	3	3	3.6	3	4.1	3	3.2					3	3.2	1	1.8	2	2.0			1	1.0
alanine	12	11	10.7	11	10.7	7	8.4	5	4.7	3	3.0	9	8.9	3	3.3	4	4.0	2	2.1	2	1.8
valine	9	8	7.8	8	8.2	9	9.2					9	8.6	5	4.7	4	4.1	4	4.0	4	3.8
$^1/_2$-cystine[d]	8	8	8.5	8	8.0	8	6.9					8	7.5	4	4.1	3	3.0	2	1.7	1	1.0
methionine[e]	4	4	3.6	4	2.9	3	3.0	1	1.0	1	1.0	3	2.6								
isoleucine	3	3	2.7	3	2.7	3	2.6					3	2.6			3	2.5			2	1.6
leucine	2	2	2.0	2	1.9	2	1.8					2	1.8	2	2.1			1	0.5		
tyrosine	6	6	6.3	6	5.8	6	6.0					6	5.5	2	1.9	3	3.3			1	0.8
phenylalanine	3	3	3.1	2	1.6	2	2.2	1	1.0	1	1.0	2	2.0	1	0.8	1	1.2	1	0.7	1	1.0
histidine	4	4	3.9	3	3.4	3	2.9	1	1.0	1	1.2	3	3.4	1	0.8	2	2.0	1	1.3	2	2.5?
lysine	10	10	10.7	10	9.7	8	9.0	2	2.0	2	2.1	8	7.7	5	4.7	3	3.7	2	1.6		
arginine	4	4	4.0	4	4.2	3	3.2	1	1.0	1	1.0	3	3.0	2	1.8	1	1.3				

[a] Hydrolysis was in 6 N HCl at 105 °C for 48 h under vacuum. Corrected for hydrolysis losses (Rupley & Scheraga, 1963) and given as moles of amino acid per mole of peptide. All values not recorded were less than 0.2 mol of amino acid per mol of peptide. [b] T = theory. Calculated from the sequence of bovine pancreatic ribonuclease determined by Smyth et al. (1963). [c] F = found. [d] Also represents values for S-carboxymethylated cysteine in the SCM fragments. [e] Also represents values for homoserine in the cyanogen bromide fragments.

for the formation of a complex between a specific antibody [either anti-$(1-13)_N$ or anti-$(31-79)_N$ or anti-$(80-124)_N$] and native RNase (or one of its derivatives) was measured by using [^{125}I]$RNase_N$ of known specific radioactivity to determine the amount of the antibody–$RNase_N$ complex formed. Bound [^{125}I]$RNase_N$ was separated from free [^{125}I]$RNase_N$ by the ammonium sulfate precipitation method of Farr (1958).

Assay tubes were prepared in quadruplicate by adding to each one 100 μL of [^{125}I]$RNase_N$ (0.125 μg of protein), 200 μL of a 1:4 dilution of normal rabbit serum, and 100 μL of a solution containing 0.1–200 μg of a peptide or protein to be tested for inhibition. The amount of antibody added (contained in 100 μL of a 1:4 dilution of normal rabbit serum) was sufficient to bind 50% of the [^{125}I]$RNase_N$ in the absence of any inhibition (at ~50% inhibition, the degree of inhibition is quite linear in the concentration of inhibitor). All solutions and dilutions were made in 0.1 M sodium borate, pH 8.3, unless stated otherwise. The solution was incubated for 3 h at 4 °C, unless stated otherwise, and then placed in an ice bath. At this stage, the antigen–antibody complexes are soluble because the antigen is in slight excess. Ice-cold saturated ammonium sulfate (500 μL) was added and mixed to precipitate the antigen–antibody complex. An incubation period of 3 h was chosen since trial experiments showed that equilibrium (for formation of a complex between antibody and [^{125}I]$RNase_N$) was reached before this time. After the precipitate was incubated for 30 min, it was removed by centrifugation at 1500g at 0 °C for 30 min. The supernatant was discarded, and the precipitate was washed twice with 2 mL of ice-cold half-saturated ammonium sulfate. The radioactivity in the precipitate was then counted in a Beckman Biogamma II γ counter.

The control assay used to analyze for nonspecific precipitation of [^{125}I]$RNase_N$ contained no antibody in the assay tube.[2] The control assay used to determine the maximum amount of precipitable [^{125}I]$RNase_N$ (i.e., antibody-bound [^{125}I]$RNase_N$) contained no inhibitor in the assay tube. The data were analyzed by the procedure of Furie et al. (1975). With this assay, we have studied the interaction of protein and peptide derivatives of RNase with antibodies to the native antigenic determinants in segments 1–13, 31–79, and 80–124.

When reduced RNase (with free sulfhydryl groups) was used as the inhibitor in the radioimmunoassay, incubation of the assay mixture was carried out for 3, 20, and 24 h, instead of the 3 h mentioned above, to determine the effect of time of incubation with antibody on the value of K_{conf} of reduced RNase [since protein folding was found to proceed during incubation in similar experiments with reduced bovine serum albumin (Chavez & Benjamin, 1978)]. Since air oxidation of sulfhydryl groups of proteins is catalyzed by metal ions (Ahmed et al., 1975), the assay solution was made 0.01 M in EDTA to prevent oxidation.

The effectiveness with which a polypeptide derivative of RNase inhibits the binding of one of these purified antibodies, Ab_N, to [^{125}I]$RNase_N$ depends on the magnitude of the equilibrium constant, K_{conf}, for the equilibrium

$$P_R \overset{K_{conf}}{\rightleftharpoons} P_N \qquad (1)$$

where P_R and P_N designate the (randomly) unfolded and (native) folded forms, respectively, of the polypeptide being tested for inhibition. The two competing reactions are

$$Ab_N + P_N \overset{K_1}{\rightleftharpoons} Ab_N{\cdot}P_N \qquad (2)$$

$$Ab_N + RNase_N \overset{K_2}{\rightleftharpoons} Ab_N{\cdot}RNase_N \qquad (3)$$

The forward and reverse rate constants of eq 2 and 3 are such that these reactions proceed in both directions with measurable rates under our experimental conditions (Anfinsen & Scheraga, 1975). Equations 1–3 represent simultaneous equilibria which can be analyzed by the procedure described below. If, as postulated by Furie et al. (1975), $K_1 = K_2$, then eq 2 and 3 may be combined to give

$$[P_N] = \frac{[Ab_N{\cdot}P_N][RNase_N]}{[Ab_N{\cdot}RNase_N]} \qquad (4)$$

where $[RNase_N] = [RNase_{tot}] - [Ab_N{\cdot}RNase_N]$, the con-

[2] In half-saturated ammonium sulfate, [^{125}I]$RNase_N$ is soluble whereas rabbit Ab and rabbit Ab-antigen complex are insoluble.

Table II: Values of K_{conf} for Protein Derivatives of Bovine Pancreatic Ribonuclease at 4 °C and pH 8.3

inhibitor protein	concn of inhibitor (M)	antibody used[a]	K_{conf}
native RNase	1.6×10^{-5} to 4.5×10^{-7}	anti-$(1-13)_N$	∞[g]
		anti-$(31-79)_N$	∞[g]
		anti-$(80-124)_N$	∞[g]
SCM-RNase[b]	6.3×10^{-4} to 9.9×10^{-6}	anti-$(1-13)_N$	1.21×10^{-3}
		anti-$(31-79)_N$	1.53×10^{-3}
		anti-$(80-124)_N$	1.18×10^{-3}
CNBr-OX-RNase[c]	1.5×10^{-4} to 2.3×10^{-6}	anti-$(1-13)_N$	1.47×10^{-4}
		anti-$(31-79)_N$	3.23×10^{-5}
		anti-$(80-124)_N$	9.66×10^{-5}
des(121-124)-RNase[d]	1.8×10^{-5} to 2.4×10^{-7}	anti-$(1-13)_N$	0.27
		anti-$(31-79)_N$	0.33
		anti-$(80-124)_N$	0.29
des(119-124)-RNase[d]	1.3×10^{-5} to 2.0×10^{-7}	anti-$(1-13)_N$	6.89×10^{-3}
		anti-$(31-79)_N$	6.68×10^{-3}
		anti-$(80-124)_N$	4.88×10^{-3}
S-protein (residues 21-124)[d]	1.7×10^{-5} to 1.7×10^{-8}	anti-$(31-79)_N$	0.086
		anti-$(80-124)_N$	0.036
SCM-S-protein[e]	6.2×10^{-5} to 9.7×10^{-7}	anti-$(31-79)_N$	1.34×10^{-4}
		anti-$(80-124)_N$	3.39×10^{-4}
C-protein (residues 14-124)[d]	2.1×10^{-5} to 3.3×10^{-7}	anti-$(31-79)_N$	2.25×10^{-3}
		anti-$(80-124)_N$	3.16×10^{-3}
reduced RNase[f]	7.3×10^{-6} to 1.1×10^{-7}	anti-$(1-13)_N$	0.073
		anti-$(31-79)_N$	0.029
		anti-$(80-124)_N$	0.078

[a] The antibody concentration and [^{125}I]$RNase_N$ concentration were 8.9×10^{-9} and 1.8×10^{-8} M throughout. The mixture of all species was incubated for 3 h at 4 °C. [b] S-Carboxymethylated RNase. [c] The mixture of peptides produced by cyanogen bromide treatment of RNase, followed by performic acid oxidation (before removal of peptide 1-13). [d] With *intact* disulfide bonds. [e] S-Carboxymethylated S-protein. [f] With free sulfhydryl groups. [g] Equation 6 leads to infinite theoretical values because of the neglect of $RNase_R$. If this term were included, it would make a very small contribution in both the numerator and denominator of eq 6. Thus, the actual values are really finite and very large, but inaccurate because of the inaccuracy in small differences between two large numbers in the denominator of eq 6.

Table III: K_{conf} for Peptide Derivatives of Bovine Pancreatic Ribonuclease at 4 °C and pH 8.3

inhibitor peptide	concn of inhibitor (M)	antibody used[a]	K_{conf}
S-peptide	1.3×10^{-4} to 2.0×10^{-6}	anti-$(1-13)_N$	5.23×10^{-3}
C-peptide[b]	2.8×10^{-4} to 4.3×10^{-6}	anti-$(1-13)_N$	5.01×10^{-4}
SCM-(31-79)[c]	1.1×10^{-5} to 1.8×10^{-7}	anti-$(31-79)_N$	2.13×10^{-4}
SCM-(80-124)[d]	3.7×10^{-4} to 5.8×10^{-6}	anti-$(80-124)_N$	3.13×10^{-4}
SCM-(40-61)[e]	1.5×10^{-4} to 2.4×10^{-6}	anti-$(31-79)_N$	1.19×10^{-5}
SCM-(105-124)[f]	6.3×10^{-5} to 9.7×10^{-7}	anti-$(80-124)_N$	[g]

[a] The concentrations of antibody and [^{125}I]$RNase_N$ were 8.9×10^{-9} and 1.84×10^{-8} M, respectively, throughout. The mixture was incubated for 3 h at 4 °C. [b] Cyanogen bromide peptide 1-13. [c] S-Carboxymethylated cyanogen bromide peptide 31-79. [d] S-Carboxymethylated cyanogen bromide peptide 80-124. [e] S-Carboxymethylated tryptic peptide 40-61. [f] S-Carboxymethylated tryptic peptide 105-124. [g] Not measurable.

centration of $RNase_R$ being assumed to be negligible. Therefore

$$K_{conf} = \frac{[P_N]}{[P_R]} = \frac{[Ab_N{\cdot}P_N]([RNase_{tot}] - [Ab_N{\cdot}RNase_N])}{[Ab_N{\cdot}RNase_N][P_R]} \quad (5)$$

where $[P_R] = [P_{tot}] - [Ab_N{\cdot}P_N] - [P_N]$. By making the substitutions and combining terms, we obtain

$$K_{conf} = \frac{[Ab_N{\cdot}P_N]([RNase_{tot}] - [Ab_N{\cdot}RNase_N])}{[P_{tot}][Ab_N{\cdot}RNase_N] - [Ab_N{\cdot}P_N][RNase_{tot}]} \quad (6)$$

The mole ratio of Ab_N to [^{125}I]RNase in the systems was adjusted to be ~0.5. The quantities $[RNase_{tot}]$ and $[P_{tot}]$ were total concentrations of [^{125}I]RNase and inhibitor added, respectively, as determined by nitrogen analysis. By use of [^{125}I]$RNase_N$, the concentration of $Ab_N{\cdot}RNase_N$ at equilibrium is the quantity determined from the radioactivity present in the precipitate in the inhibition assay. The concentration of $Ab_N{\cdot}P_N$ is calculated by subtracting the amount of [^{125}I]-RNase in the precipitate (in the presence of competing inhibitor) from the amount of [^{125}I]RNase in the precipitate (in the absence of competing inhibitor).

Results

The values of K_{conf} for various derivatives of RNase are shown in Tables II and III. Each value represents the average of at least four determinations (each being carried out in quadruplicate) at inhibitor concentrations of 6.3×10^{-4} to 1.7×10^{-8} M and a concentration of [^{125}I]$RNase_N$ of 1.8×10^{-8} M.

When unlabeled $RNase_N$ is used as the inhibitor (i.e., if $Ab_N{\cdot}RNase_N$ is substituted for $Ab_N{\cdot}P_N$ and ($[RNase_N] + [Ab_N{\cdot}RNase_N]$) is substituted for $[P_{tot}]$ in eq 6), then the value of K_{conf} should become infinite since $RNase_R$ is taken as zero. Of course, K_{conf} for $RNase_N$ is not infinite but is a very large finite number. The fact that the *experimental* values are very large for all three antibodies (see Table II) assures us that the trace-labeled [^{125}I]$RNase_N$ has the native conformation and is not detectably denatured in the regions of the antigenic determinants. Consequently, the values of K_{conf} for those derivatives of RNase which possess lower stabilities than $RNase_N$, in the region of the antigenic determinant, should be less than the large values observed for $RNase_N$.

The values of K_{conf} correlate well with the degree and location of chemical or enzymatic modification of the RNase molecule. It should be noted that probably none of these modifications affects the covalent structures of the individual antigenic determinants, but more likely the modifications alter their conformations (Chavez & Scheraga, 1979). Removal of four amino acids from the C terminus of $RNase_N$ affects the value of K_{conf} for each segment to approximately the same extent. This reduction probably arises from a general distortion of the hydrophobic pocket (that includes Ile-106 and -107, Val-108 and -118, and Phe-120) due to its increased exposure in the absence of peptide 121-124. As can be seen from the X-ray structure of Wyckoff et al. (1967, 1970), Phe-8 has intimate contact with this region, and distortion of this interaction would appear to be responsible for the destabilization of segment 1-13. When two additional residues (Phe-120 and His-119) are removed to produce des(119-124)-RNase, the value of K_{conf} of each of the three determinant regions decreases markedly (see Table II). The conformation of the entire RNase molecule appears to depend on the proper burial of the small number of residues in the region 106-120. Residues 106-120, containing the alleged nucleation site 106-118 (Matheson & Scheraga, 1978), interact mostly with each other. The role of the C terminus was pointed out previously by Burgess & Scheraga (1975) and Burgess et al. (1975).

The N terminus of native RNase also plays a role in stabilizing the rest of the molecule, as also pointed out by Burgess

& Scheraga (1975). Deletion of S-peptide, residues 1–20, reduces K_{conf} for S-protein to about 0.06. Further distortion of the structure of S-protein accompanies the S-carboxymethylation of the sulfhydryl groups of reduced S-protein by decreasing K_{conf} 2 orders of magnitude compared to that of S-protein. Comparison of the values of K_{conf} for S-carboxymethylated RNase and S-carboxymethylated S-protein shows the stabilizing effect of S-peptide in the structure of the whole molecule. These results demonstrate the importance of the disulfide bonds and of segments 1–20 and 119–124 in stabilizing the structure of native RNase.

Cyanogen bromide cleavage of native RNase, producing C-protein (residues 14–124 with disulfide bonds intact, but missing Met-30, and with the other methionine residues converted to homoserine lactone), decreases K_{conf} to a level comparable to that of S-carboxymethylated RNase. Comparison of the value of K_{conf} of C-protein with that of S-protein demonstrates that the cleavage of peptide bonds following Met-29, -30 and -79, and the conversion of methionine to homoserine lactone, in C-protein increases the overall flexibility of the peptide chain significantly, even though C-protein contains more residues (residues 14–124, minus Met-30) than S-protein (residues 20–124). Performic acid oxidation of cyanogen bromide treated RNase (before removal of peptide 1–13) gives a mixture of peptides devoid of disulfide bonds (CNBr-OX-RNase) with increased conformational distortion compared to native RNase; i.e., the values of K_{conf} for CNBr-OX-RNase are lower than those of any other derivative of Table II and are comparable in magnitude to the values for the purified S-carboxymethylated cyanogen bromide fragments (see Table III). By comparing K_{conf} for C-protein and CNBr-OX-RNase, we see that stabilizing interactions are present in C-protein, probably because of its intact disulfide bonds.

There is no indication from the values of K_{conf} of any significant differences in stability among the purified cyanogen bromide fragments 1–13 (C-peptide), 31–79, or 80–124 (see Table III). However, increasing the size of the C-peptide to that of the S-peptide increases the value of K_{conf} of the antigenic determinant 10-fold. This is consistent with the observations of Klee (1967) and Silverman et al. (1972) that the α-helical portion of the isolated S-peptide has some stability. [The values of K_{conf} for SCM-(40–61) and SCM-(105–124) were discussed earlier (Chavez & Scheraga, 1979)]. From these results, we can see that the antigenicity of the determinant regions depends not only on their amino acid sequences but also on their proper conformation. The ability of small or highly modified peptides to interact with antibodies against the native structure implies the existence of a nonnegligible concentration of P_N in the equilibrium of eq 1 (Furie et al., 1975).

When reduced RNase was used as the inhibitor in the radioimmunoassay, various incubation times were used to test for the absence of oxidation of disulfide bonds during the assay period for K_{conf}. In control experiments, no oxidation of the sulfhydryl groups of reduced RNase could be demonstrated during a 24-h incubation in the presence of 0.01 M EDTA, pH 8.0, by sulfhydryl titration. When reduced RNase was used as the inhibitor, in the presence of 0.1 M Tris-HCl containing 0.01 M EDTA, pH 8.3, the values of K_{conf}, given in Table II, were found to be independent of time over a period of 24 h, within the experimental error (see Figure 2). This demonstrated that no appreciable oxidation of sulfhydryl groups had occurred during the assay. These values of K_{conf} indicate that the conformation of reduced RNase is somewhat similar to that of the native protein. This result is in agreement

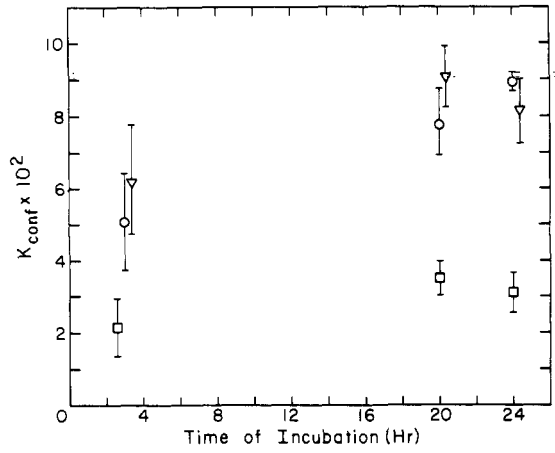

FIGURE 2: Time dependence of K_{conf} at 4 °C for reduced RNase incubated at pH 8.3 with [^{125}I]RNase$_N$ and the purified antibodies against fragments of RNase. K_{conf} was measured with anti-(1–13) (○), anti-(31–79) (□), and anti-(80–124)$_N$ (▽).

with computations (carried out with an Ising model) that show that the conformations of the residues of a reduced protein are similar to those of the native protein (L. G. Dunfield and H. A. Scheraga, unpublished calculations).

The effect of temperature on the values of K_{conf} of des-(121–124)-RNase was evaluated between 5 and 50 °C. This derivative was selected for studies of the temperature dependence of K_{conf} since [^{125}I]RNase, used in the assay, is stable in the temperature range where des(121–124)-RNase unfolds. The transition temperature, T_m, of des(121–124)-RNase is lower than that of native RNase [Lin (1970) reported a T_m of 44.5 °C in 0.02 M Tris-HCl, pH 7.5, on the basis of changes in ultraviolet absorption at 287 nm, and we observed a T_m of 46.1 °C in 0.1 M Tris-HCl, pH 8.0, using the same technique]. Also, the thermal transition of des(121–124)-RNase is essentially over before that of native RNase begins at approximately 51 °C. Lin (1970) indicated that the thermal transition of des(121–124)-RNase is reversible. However, the thermal transition of native RNase at pH 8.0 becomes progressively more irreversible with increasing temperature and with increasing time at elevated temperatures (Hermans & Scheraga, 1961; Chavez & Scheraga, 1977). Therefore, the transition of des(121–124)-RNase presumably would exhibit similar irreversibility at elevated temperatures. In order to determine the minimum length of time necessary to reach equilibrium in the assay for the determination of K_{conf}, we incubated des(121–124)-RNase at 50 °C for various lengths of time with [^{125}I]RNase$_N$ and antibody (Figure 3). It can be seen that, within experimental error, equilibrium in the assay is reached within 5–15 min. Hence, the measurements of K_{conf} were made as rapidly as possible, i.e., within 15 min, in order to minimize irreversibility in any transition occurring during the measurements. Figure 4 shows the temperature dependence of K_{conf} for each antigenic region of des(121–124)-RNase. Three factors contribute to the temperature dependence of K_{conf}. (1) Thermal effects on the conformation of des(121–124)-RNase will affect its binding to antibody. (2) Thermal effects on the antibody will affect its intrinsic association constant. (3) Although RNase is generally stable to the effects of elevated temperatures below 50 °C, Chavez & Scheraga (1977) and Matheson & Scheraga (1979b) have demonstrated that a conformational transition occurs in the N-terminal region of RNase around 30–40 °C. Thus, the thermal effects on the conformation of [^{125}I]RNase$_N$ in the assay will affect the binding of antibody and, consequently, the value of K_{conf}. The last two contributions were corrected

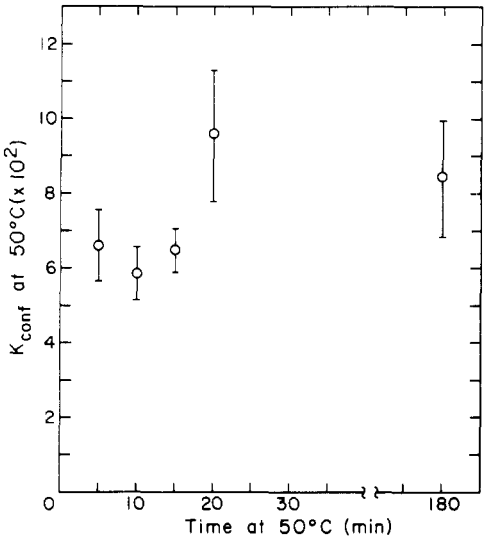

FIGURE 3: Time dependence of K_{conf} at 50 °C. Des(121–124)-RNase (with intact disulfide bonds) was used as inhibitor at concentrations between 2.0×10^{-5} and 2.0×10^{-8} M at pH 8.3. The concentrations of $[^{125}I]RNase_N$ and anti-$(31–79)_N$ were 1.4×10^{-8} and 7.9×10^{-9} M, respectively. The error symbols represent the standard deviation of the mean of 12 separate determinations for each point.

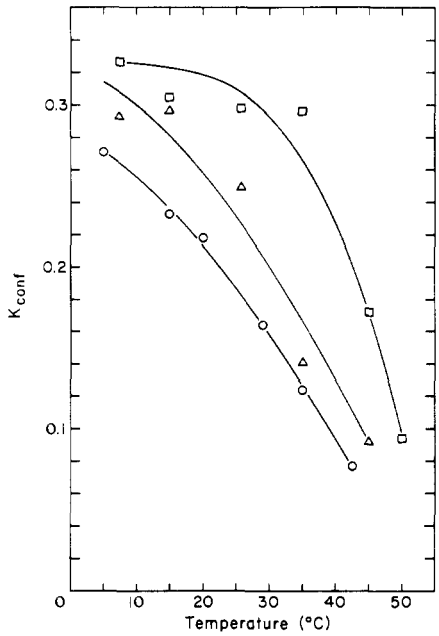

FIGURE 4: K_{conf} values for des(121–124)-RNase as a function of temperature. K_{conf} was determined by incubating the inhibition assay mixture at each temperature for less than 15 min before precipitation of the antigen–antibody complexes with an equal volume of ice-cold saturated ammonium sulfate. Des(121–124)-RNase was incubated at several concentrations between 3.7×10^{-7} and 5.8×10^{-9} M, and the value of K_{conf} represents the average of at least three separate determinations. K_{conf} was measured with anti-$(1–13)_N$ (○), anti-$(31–79)_N$ (□), and anti-$(80–124)_N$ (△). The concentrations of $[^{125}I]RNase_N$ and each antibody were 7.8×10^{-9} and 4×10^{-9} M, respectively.

for by data from control experiments in which antibody and $[^{125}I]RNase_N$ were incubated (in the absence of inhibitor) in the assay mixture. These data represent the maximum amount of $[^{125}I]RNase_N$ precipitable at each assay temperature which is used in the determination of $[Ab_N{\cdot}P_N]$. These results indicate that, with increasing temperature, an increasing fraction of des(121–124)-RNase exists in a denatured form not recognized by the antibodies raised against the native conformation. The sequence of unfolding of the antigenic regions

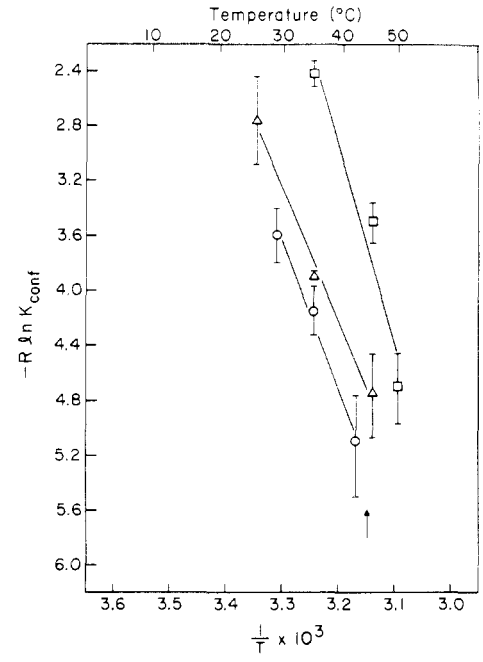

FIGURE 5: van't Hoff plot of the conformational equilibrium constants determined in Figure 4 for each of the antigenic regions of des-(121–124)-RNase in segments 1–13 (○), 31–79 (□), and 80–124 (△). The lines represent the linear least-squares best fit through the experimental points. The arrow indicates the position of the transition temperature, T_m, determined by absorbance measurements.

is in the order of segment 1–13 and then segment 80–124, followed by segment 31–79. Segment 1–13 is significantly less stable since conformational distortions are apparent at 15 °C while the other segments remain stable up to 25 °C. Segment 31–79 does not unfold until after 35 °C.

The van't Hoff plots of the data of Figure 4, as $R \ln K_{conf}$ vs. $1/T$, are shown in Figure 5. The apparent enthalpy changes for folding (eq 1), calculated from the slopes of the lines in Figure 5, are -10.7 ± 4.0, -14.3 ± 2.0, and -9.5 ± 2.0 kcal/mol for the antigenic determinants in segments 1–13, 31–79, and 80–124, respectively. Since these values of ΔH for the three antigenic regions are quite similar, a calorimetric measurement of ΔH on the whole des(121–124)-RNase molecule would indicate that the transition is apparently a two-state one. The curves of Figure 4, however, show that intermediates exist, and this conclusion is supported by the three different values of ΔH, even though the errors in ΔH are quite large.

Discussion

The introduction of immunological techniques (Sachs et al., 1972; Furie et al., 1975) in the study of protein folding has made it possible to measure values of K_{conf} for specific antigenic regions. Our approach [similar to that of Hurrell et al. (1977)] is also similar to the one used by Furie et al. (1975), except that we precipitated the immune complexes with ammonium sulfate (Farr, 1958) instead of using the double-antibody precipitation procedure of Furie et al. (1975).

Trace labeling of RNase with ^{125}I provided high specific activities. Under the conditions used in the iodination procedure, the tyrosines (presumably residues 73, 76, and 115; Woody et al., 1966) were iodinated to a level of 0.1 mol of ^{125}I per mol of RNase with a specific activity of 22 000 cpm per 9.1×10^{-12} mol. The positions of these tyrosine residues, with respect to the antigenic determinants in segments 1–13, 40–61, 63–75, and 87–104 (Chavez & Scheraga, 1979), and the assumption that the tyrosines were labeled randomly

suggest that the antigenic determinants in [^{125}I]RNase$_N$ would not be affected by iodination. This expectation was borne out by the very large value of K_{conf} obtained when unlabeled RNase was used as an inhibitor (Table II).

We have located the antigenic determinants and shown that segments 1–13 and 80–124 are probably univalent, segment 31–79 is most likely divalent, and segment 14–29 possesses no antigenic activity (Chavez & Scheraga, 1979). No preliminary absorption of anti-RNase$_N$ antiserum by native RNase was performed in order to remove possible antibodies to denatured RNase. However, Sachs et al. (1972) have shown that the antibody population against the native conformation and the one against the denatured conformation of the same sequence in staphylococcal nuclease cross-react to the extent of only ~0.02%, thus indicating that a native immunogen would not be expected to yield significant levels of antibody to the unfolded protein species, especially for proteins containing disulfide bonds (Anfinsen et al., 1971). It also seems unlikely that significant levels of antibody to the denatured forms of RNase were present since no cross-reaction could be detected between native RNase and S-carboxymethylated RNase, by using the same purified antibodies, except at very high concentrations of SCM-RNase (Chavez & Scheraga, 1977). Because anti-(31–79)$_N$ is divalent, the value of K_{conf} for segment 31–79 is an average one.

The unfolding transition of des(121–124)-RNase has been studied by Puett (1972), using guanidine hydrochloride as the denaturant in 10 mM sodium phosphate and 0.1 M KCl. His results indicated that the transition could be treated adequately by using a two-state model. However, our results indicate that intermediate states of folding occur between the denatured and native macroscopic states. The thermodynamic data of Privalov & Khechinashvili (1974) on RNase also indicated that the two-state model was not adequate to describe protein denaturation but that intermediates were highly unstable. In the case of RNase, we find significant concentrations of intermediate states. Of course, the intermediates must differ significantly in enthalpy in order to be detected by their thermodynamic properties (Privalov & Khechinashvili, 1974; Lumry et al., 1966); as seen in Figure 5, the enthalpies for unfolding of segments 1–13 and 80–124 do not differ much. The broad transition found for segment 1–13 in Figure 4 could indicate the presence of different "native" states prior to unfolding.

The early onset of the unfolding of segment 1–13 in des-(121–124)-RNase (Figure 4) is consistent with similar observations made with intact RNase (Chavez & Scheraga, 1977; Matheson & Scheraga, 1979b). The antigenic determinant in segment 80–124 is destabilized to a greater degree than those in segment 31–79. At least one reason can be proposed for this result. The antigenic determinants in these segments most likely reside in residues 40–61, 63–75, and 98–104. The determinant in residues 63–75 probably involves the β bend at residues 66–68 which is joined on both ends by a disulfide bond between half-cystines-65 and -72. One would expect this determinant to be more stable at elevated temperatures than the determinant in residues 98–104 which most likely depends on the C-terminal residues for stability.

The reduction of the enzymatic activity of des(121–124)-RNase [0.5% of the activity of native RNase (Lin, 1970)] is greater than the aforementioned reduction in the values of K_{conf} and has been attributed by Lin (1970) to a change in the pK_a of His-119. This explanation seems reasonable because the hydrodynamic data of Anfinsen (1956), the spectral data of Puett (1972), and the values of K_{conf} reported here indicate that des(121–124)-RNase possesses a stable folded conformation similar to that of native RNase, but the values of K_{conf} suggest a loosening of the structure near the C terminus which could change the exposure of His-119 to the solvent and thus alter its pK_a.

Comparison of the values of K_{conf} shows a stability scale of RNase > des(121–124)-RNase > S-protein > des(119–124)-RNase. This agrees with the stabilities indicated by the transition temperatures of 61.0 °C (Hermans & Scheraga, 1961; Lin, 1970), 44.5 °C (Lin, 1970), 37.5 °C (Tsong et al., 1970), and 32.5 °C (Lin, 1970) at neutral pH for the same proteins. The removal of Phe-120 and His-119 decreases K_{conf} markedly in all antigenic regions. However, as indicated here and by Puett (1972), some stable structure still persists. Removal of the 20-residue N terminus also decreases K_{conf}, but to a lesser extent. The relative importance of these modifications can be assessed by comparing the abilities of these derivatives to fold to the native conformation after the reduction of the disulfide bonds. The folding of des(119–124)-RNase has not been investigated, but that of des(121–124)-RNase has. The air-oxidized product forms randomly paired disulfide bonds (Taniuchi, 1970). However, the glutathione-oxidized product does regain some native structure, approximately 35% (Chavez & Scheraga, 1980). This indicates that the nucleation site cannot reside in residues 121–124 in RNase A, although these residues are necessary for complete folding. Furthermore, the decrease in the conformational stability of des(119–124)-RNase implicates Phe-120 and His-119 in important stabilizing interactions which would be necessary during the folding of RNase A. On the other hand, when reduced S-protein folds, 100% of the antigenic activity of "native" S-protein is regained, indicating that the nucleation site cannot lie in residues 1–20 (Chavez & Scheraga, 1980). It does appear that S-peptide stabilizes the rest of the molecule significantly, but residues 1–20 do not restrict the conformational space probed by reduced S-protein during folding since the rate of folding is not accelerated but slightly inhibited (Chavez & Scheraga, 1980).

The folding pathway implied in these immunological studies is consistent with the predicted nucleation site at residues 106–118 (Matheson & Scheraga, 1978). The initial folding of the interior hydrophobic residues in the region of 106–118 is followed by folding of the surface (antigenic determinant) region in segment 80–124, which is the first antigenic region to form (Chavez & Scheraga, 1977).

An estimate of the significance of the magnitudes of the values of K_{conf} in Tables II and III can be made, following the similar illustrative calculation of Anfinsen & Scheraga (1975). The expected value of K_{conf} for a single unstructured determinant would be 10^{-5}–10^{-6}. Thus, most of the derivatives examined here, having much higher values of K_{conf}, must acquire their conformations through interactions that greatly restrict their available conformational space. Two of the more striking examples are reduced RNase ($K_{conf} \sim 0.06$) and S-carboxymethylated RNase ($K_{conf} \sim 0.001$). Thus, these so-called "denatured" proteins *have a high degree of native conformation.* In support of this conclusion, Takahashi & Ooi (1976) found significant α-helix and β-structure content in reduced RNase. Slightly lower amounts of native structure were found in reduced RNase by Garel (1978); on the basis of a measurement of enzymatic activity of reduced RNase at pH 7, about 0.04–1.5% of the reduced molecules possess native structure. It thus appears that RNase can fold to a structure resembling that of the native protein without the formation of disulfide bonds.

In summary, our results indicate that intermediates are detectable in the folding of RNase. The process may be initially under control of a nucleating hydrophobic core in residues 106–118. Taken together with results of Matheson & Scheraga (1979a) on the photolabeling of RNase during folding, there appears to be a concerted condensation involving these intermediates. Finally, the rather high value of ~0.06 for K_{conf} of reduced RNase provides strong support for the concept (Scheraga, 1973) that short-range interactions dominate in determining the three-dimensional structure of a globular protein.

Acknowledgments

We are indebted to T. W. Thannhauser for performing the nitrogen analyses and to Drs. Y. Konishi, R. R. Matheson, Jr., and R. Rudolph for helpful discussions.

References

Ahmed, A. K., Schaffer, S. W., & Wetlaufer, D. B. (1975) *J. Biol. Chem. 250*, 8477.
Anfinsen, C. B. (1956) *J. Biol. Chem. 221*, 405.
Anfinsen, C. B., & Haber, E. (1961) *J. Biol. Chem. 236*, 1361.
Anfinsen, C. B., & Scheraga, H. A. (1975) *Adv. Protein Chem. 29*, 205.
Anfinsen, C. B., Cuatrecasas, P., & Taniuchi, H. (1971) *Enzymes, 3rd Ed. 4*, 177.
Burgess, A. W., & Scheraga, H. A. (1975) *J. Theor. Biol. 53*, 403.
Burgess, A. W., Weinstein, L. I., Gabel, D., & Scheraga, H. A. (1975) *Biochemistry 14*, 197.
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. (1979) *Biochemistry 18*, 4386.
Chavez, L. G., Jr., & Scheraga, H. A. (1980) *Biochemistry* (preceding paper in this issue).
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.
Garel, J. R. (1978) *J. Mol. Biol. 118*, 331.
Haber, E., & Anfinsen, C. B. (1961) *J. Biol. Chem. 236*, 422.
Hermans, J., Jr., & Scheraga, H. A. (1961) *J. Am. Chem. Soc. 83*, 3283.
Hirs, C. H. W., Moore, S., & Stein, W. H. (1956) *J. Biol. Chem. 219*, 623.
Hurrell, J. G. R., Smith, J. A., & Leach, S. J. (1977) *Biochemistry 16*, 175.
Imoto, T., Fukuda, K. T., & Yagishita, K. (1974) *Biochim. Biophys. Acta 336*, 264.
Klee, W. A. (1967) *Biochemistry 6*, 3736.
Lang, C. A. (1958) *Anal. Chem. 30*, 1692.
Lewis, P. N., Gō, N., Gō, M., Kotelchuck, D., & Scheraga, H. A. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 810.
Lewis, P. N., Momany, F. A., & Scheraga, H. A. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2293.
Lin, M. C. (1970) *J. Biol. Chem. 245*, 6726.
Lumry, R., Biltonen, R., & Brandts, J. F. (1966) *Biopolymers 4*, 917.
Matheson, R. R., Jr., & Scheraga, H. A. (1978) *Macromolecules 11*, 819.
Matheson, R. R., Jr., & Scheraga, H. A. (1979a) *Biochemistry 18*, 2437.
Matheson, R. R., Jr., & Scheraga, H. A. (1979b) *Biochemistry 18*, 2446.
McConahey, P. J., & Dixon, F. J. (1966) *Int. Arch. Allergy Appl. Immunol. 29*, 185.
Nakanishi, M., & Tsuboi, M. (1978) *J. Am. Chem. Soc. 100*, 1273.
Nakanishi, M., Tsuboi, M., & Ikegami, A. (1972) *J. Mol. Biol. 70*, 351.
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.
Ooi, T., & Scheraga, H. A. (1964) *Biochemistry 3*, 648.
Ponnuswamy, P. K., Warme, P. K., & Scheraga, H. A. (1973) *Proc. Natl. Acad Sci. U.S.A. 70*, 830.
Privalov, P. L., & Khechinashvili, N. N. (1974) *J. Mol. Biol. 86*, 665.
Puett, D. (1972) *Biochemistry 11*, 1980.
Ramachandran, G. N., & Sasisekharan, V. (1968) *Adv. Protein Chem. 23*, 283.
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.
Scheraga, H. A. (1968) *Adv. Phys. Org. Chem. 6*, 103.
Scheraga, H. A. (1971) *Chem. Rev. 71*, 195.
Scheraga, H. A. (1973) *Pure Appl. Chem. 36*, 1.
Silverman, D. N., Kotelchuck, D., Taylor, G. T., & Scheraga, H. A. (1972) *Arch. Biochem. Biophys. 150*, 757.
Smyth, D. G., Stein, W. H., & Moore, S. (1963) *J. Biol. Chem. 238*, 227.
Takahashi, S., & Ooi, T. (1976) *Bull. Inst. Chem. Res., Kyoto Univ. 54*, 141.
Taniuchi, H. (1970) *J. Biol. Chem. 245*, 5459.
Taniuchi, H., & Anfinsen, C. B. (1968) *J. Biol. Chem. 243*, 4778.
Taniuchi, H., & Anfinsen, C. B. (1969) *J. Biol. Chem. 244*, 3864.
Tsong, T. Y., Hearn, R. P., Wrathall, D. P., & Sturtevant, J. M. (1970) *Biochemistry 9*, 2666.
Woody, R. W., Friedman, M. E., & Scheraga, H. A. (1966) *Biochemistry 5*, 2034.
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.