Kekwick, R. A., and Cannan, R. K. (1936), *Biochem. J. 30*, 227.
Murachi, T. (1964), *Biochemistry 3*, 932.
Murachi, T., Inagami, T., and Yasui, M. (1965), *Biochemistry 4*, 2815.
Murachi, T., Yasui, M., and Yasuda, Y. (1964), *Biochemistry 3*, 48.
Neuberger, A. (1938), *Biochem. J. 32*, 1435.
Ota, S., Moore, S., and Stein, W. H. (1964), *Biochemistry 3*, 180.
Sanger, F., and Thompson, E. O. P. (1953), *Biochem. J. 53*, 353.
Svennerholm, L. (1957a), *Arkiv Kemi 10*, 577.
Svennerholm, L. (1957b), *Chem. Abstr. 51*, 9400*h*.
Takahashi, N., Sato, M., and Murachi, T. (1965), Abstracts, 16th Symposium on Protein Structure, Fukuoka, Japan, p 22.
Takahashi, N., Yasuda, Y., Kuzuya, M., Suzuki, A., and Murachi, T. (1967), Abstracts, 7th International Congress of Biochemistry, Tokyo, Japan, p 706.
Winzler, R. L. (1955), *Methods Biochem. Anal. 2*, 279.
Yamashina, I., and Makino, M. (1962), *J. Biochem. (Tokyo) 51*, 359.
Yemm, E. W., and Cocking, E. C. (1955), *Analyst 80*, 209.

Intermediate Stages in the Thermally Induced Transconformation Reactions of Bovine Pancreatic Ribonuclease A*

Werner A. Klee

ABSTRACT: The nature of the thermally induced unfolding reactions of bovine pancreatic ribonuclease has been studied using proteolytic enzymes as conformational probes. The initial rates of proteolysis, as catalyzed by aminopeptidase, trypsin, chymotrypsin, and carboxypeptidase, were studied both with ribonuclease and performic acid oxidized ribonuclease as substrates. The latter protein served as a control substrate so as to take account of the effect of temperature on the proteases.

The temperature range studied was between 30 and 60° at pH 8 in 0.1 M NaCl. The results of these studies indicate that the conformation of ribonuclease changes in a gradual manner with increasing temperature and that some central regions become disordered at lower temperatures than does either end of the molecule. Concomitant optical rotatory dispersion studies also indicate that the thermally induced conformation changes of ribonuclease take place in a gradual rather than a two-step process.

The reversible thermal unfolding of bovine pancreatic ribonuclease (RNase) has been the object of much study since its discovery by Harrington and Schellman (1956). This process has often been treated as the reflection of a simple equilibrium between a native and a denatured state, with no thermodynamically stable intermediates (Harrington and Schellman, 1956; Foss and Schellman, 1959; Kalnitsky and Resnik, 1959; Hermans and Scheraga, 1961; Brandts, 1965; Lumry *et al.*, 1966). However, it has been observed by some that much of the data is not consistent with this simple scheme and that intermediate stages of unfolding probably exist (Foss, 1961; Holcomb and Van Holde, 1962; Scott and Scheraga, 1963; Poland and Scheraga, 1965; Beck *et al.*, 1965; Ginsburg and Carroll, 1965). It has been the object of this investigation to demonstrate in a more clear-cut manner than heretofore that the thermal unfolding of RNase is a gradual or multistate rather than an abrupt or two-state process and to learn something of the pathway which the molecule follows as its conformation is changed from the native or low-temperature form, to that which exists at high temperatures. The approach used has been to study the kinetics of the proteolysis of ribonuclease, with aminopeptidase, chymotrypsin, trypsin, and carboxypeptidase, as a function of temperature throughout the transition region. The specificity of these enzymes provides the means of following changes in RNase conformation at the amino terminus, in a central region or regions and at the carboxyl terminus, respectively. To take account of changes in the properties of these proteases with temperature, all experiments were performed both with RNase and with performic acid oxidized RNase (Ribox)[1] as substrates. The latter

* From the Laboratory of General and Comparative Biochemistry, National Institute of Mental Health, U. S. Department of Health, Education, and Welfare, U. S. Public Health Service, National Institutes of Health, Bethesda, Maryland 20014. *Received July 17, 1967.*

[1] Abbreviation used: Ribox, performic acid oxidized RNase.

protein has been shown to behave as if no appreciable amount of three-dimensional structure remains (Harrington and Schellman, 1956), a result which has been also confirmed and extended somewhat in the present study.

In conjunction with these experiments a detailed study has been performed of the variation with temperature of the optical rotatory dispersion of RNase and of Ribox. These measurements confirm the complex nature of the unfolding process.

Methods

Ribonuclease A was prepared from crystalline ribonuclease by chromatography by the method of Hirs *et al.* (1953) followed by rechromatography on SE Sephadex as described by Crestfield *et al.* (1962). The material was free of phosphate by adsorption onto carboxymethylcellulose at pH 5 after 1:4 dilution with water. After washing the column with water the RNase was eluted as a concentrated solution with 1 M Tris-HCl (pH 8.0). The protein was freed of buffer by dialysis for 3 days *vs.* many changes of deionized water using 18–32 Visking casing. Solutions for study were made by diluting this salt-free solution with the appropriate solvent. All studies were performed in 0.1 M NaCl–0.02 M $MgCl_2$ (pH 8.0).

Ribox was prepared from ribonuclease A by the procedure of Hirs (1956). The lyophilized product was dialyzed *vs.* deionized water for 3 days with frequent changes. Trypsin and chymotrypsin were products of Worthington. Carboxypeptidase A and leucine aminopeptidase were both diisopropylfluorophosphate-treated products of Worthington.

The rate of trypsin and chymotrypsin digestion of RNase and Ribox was followed in a pH-Stat as described previously (Klee, 1965). Samples (6.7 mg) of protein in 1.1 ml of 0.1 M NaCl–0.02 M $MgCl_2$ were maintained at pH 8.0 by the automatic addition of 0.05 N NaOH using a 50-μl Hamilton syringe as the buret. Trypsin or chymotrypsin (0.35–50 μg depending on the temperature) was added to start the reaction. The amount of protease added was generally sufficient to ensure that approximately 25–75% of the chart reading (5–15 μl of NaOH added) was achieved in 10 min with Ribox as substrate. At the higher temperatures (55° and above) protease inactivation occurred to an appreciable extent during the 10-min assay. In these cases, rates are reported as extrapolated initial slopes. In other cases, actual base uptake after 10 min is recorded (as per cent of the chart) since the curves were approximately straight lines during this time interval.

Exopeptidase digestions were followed by ninhydrin analysis since there is no appreciable pH change near pH 8 due to carboxypeptidase or aminopeptidase hydrolysis of RNase or Ribox. Five time points were taken at each temperature in order to establish a rate. The rate of hydrolysis was measured on 200-μl aliquots of the reaction mixtures which were incubated separately in sealed vials in order to avoid evaporation. At suitable times, the protein was precipitated with 1 ml of either 5% phosphotungstic acid (RNase) or of a mixture of 5% trichloroacetic acid–0.2% sodium tungstate (Ribox). After centrifugation, an 800-μl aliquot of the supernatant fluid was analyzed with ninhydrin for amino acids, according to Moore and Stein (1954). The concentration of protein was that used in the trypsin experiments with 20–40 μg of carboxypeptidase and 250–500 μg of aminopeptidase used for each 1.1 ml of reaction mixture; the actual concentration used depended on temperature. Leucine aminopeptidase was incubated at 37° for 1 hr in 0.02 M $MgCl_2$ at pH 8 immediately prior to each experiment (Smith and Spackman, 1955). Paper chromatographic analysis of the products of the reaction performed at 50° shows that the enzymes are uncontaminated with endopeptidase activity since the expected products are formed.

Optical rotatory dispersion experiments were performed with a Cary Model 60 spectropolarimeter using a water-jacketed cell holder for temperature control. Temperatures were measured inside the cell using a Yellow Springs Instrument Co. thermocouple. Samples were equilibrated at each temperature for 30 min and new solutions were used for each run. Solvent spectra were taken before and after each sample and, in general, showed that the drift of the instrument was negligible. Spectra were obtained with RNase solutions of 0.21 mg/ml in a 0.01-dm cell (250 mμ and below) or with a solution of 0.42 mg/ml in a 0.10-dm cell (250–300 mμ). Ribox was studied at a concentration of 0.40 mg/ml in the 0.01-dm cell. All experiments at a given temperature were performed on the same day during which time there was no measurable drift in the temperature of the sample compartment. The solvent was, in all cases, 0.1 M NaCl–0.02 M $MgCl_2$ (pH 8.0). Data are recorded as $[m'] = (3/(n^2 + 2))(MRW/100)[\alpha]$. In making the calculations, the mean residue weights of RNase and Ribox were taken to be 110 and 113, respectively, and the refractive index dispersion of pure water was used.

Results

Proteolysis Studies. RNase is known to be strongly resistant to the action of all of the proteases used in the present study when in its native low-temperature conformation (Dubos and Thompson, 1938; Sela *et al.*, 1957; Brown *et al.*, 1959; Spackman *et al.*, 1960). A number of observations have shown that this resistance is lost at high temperatures (Brown *et al.*, 1959; Ruplay and Scheraga, 1963; Ooi *et al.*, 1963), although no systematic study of the kinetics of proteolysis at higher temperatures has been reported. It seemed reasonable to expect that the progress of the thermally induced structural changes in various parts of the RNase molecule could be followed by a study of the kinetics of proteolysis using proteolytic enzymes of the appropriate specificity as conformational probes. The results of such studies are shown in Table I for aminopeptidase, trypsin, chymotrypsin, and carboxypeptidase. In the table, the rate of hydrolysis of both RNase and Ribox

TABLE I: Rates of Proteolysis of RNase and of Ribox at Various Temperatures.[a]

Temp (°C)	Carboxypeptidase (μg)	Rate		Aminopeptidase (μg)	Rate		Trypsin (μg)	Rate		Chymotrypsin (μg)	Rate	
		RNase	Ribox		RNase	Ribox		RNase	Ribox		RNase	Ribox
25	20	0	46	250	0	12						
35	20	0	135	250	0	25	0.35	0	21	2.1	1.6	26
40	20	0	135	250	0	50	0.7	1	38	2.1	5.5	50
45	20	0	180	250	0	50	0.7	6.5	62	1	5.1	36
50	20	14	200	250	0	65	0.7	16	54	6	16	57
55	20	40	280	250	0	125	0.7	16	22	50	16	21
56	40	210	480									
57.5	40	460	320	500	20	85						
58.5				500	92	140						
60	40	300	210	250	25	18	5	34	44			
63							5	8	5			

[a] Reaction mixtures contained 6 mg/ml of RNase or Ribox in 0.1 M NaCl–0.02 $MgCl_2$ (pH 8) and the amount of proteolytic enzyme shown. Assays are performed as indicated in the Methods section of the text. The rates are expressed as either increase in ninhydrin color ($OD_{570} \times 10^3$) (exopeptidases) or pH-Stat reading (per cent of chart) (endopeptidases) for 10-min reaction time and represent initial rates in all cases.

by the appropriate enzyme is shown as a function of temperature. All four enzymes are able to hydrolyze Ribox throughout the temperature range studied, but RNase is only attacked at the higher temperatures. The temperature at which RNase becomes appreciably susceptible to proteolysis varies significantly with the protease used. Thus, whereas chymotrypsin is able to attack RNase at a significant rate at 40°, trypsin attack begins near 45°, carboxypeptidase near 50°, and aminopeptidase does not attack RNase before 55°. A possibly more meaningful treatment of the data is shown in Figure 1 in which the rate of hydrolysis of RNase relative to that of Ribox under the same conditions is shown for all of the enzymes. This presentation has the advantage of correcting, to some extent, for the effect which temperature changes may have on the catalytic efficiency of the proteases used. If this type of correction were completely valid, the curves shown in Figure 1 could be interpretable as plots of the degree of unfolding of the carboxyl and amino termini and of internal regions of the RNase molecule as a function of temperature. There is, however, some question of whether the degree of inactivation of the proteases at high temperatures is the same in the presence of RNase and of Ribox and so there is danger in an overly detailed interpretation of these curves. Furthermore it is reasonable to expect that thermally unfolded RNase is a better substrate for proteolytic enzymes than is Ribox because of the hindrance which the charged cysteic acid residues are believed to exert (Crestfield *et al.*, 1963). Nevertheless, it seems clear that various parts of the molecule are unfolded to different extents at different temperatures. It may also be reasonable to conclude that regions in the middle of the RNase molecule tend to unfold before the carboxyl terminus does and that the amino terminus is particularly resistant to thermal denaturation.

Optical Rotatory Dispersion Studies. Although there have been many studies of the variation of the physical properties of RNase with temperature, it was considered important to make such measurements under the conditions used in the proteolysis experiments. Furthermore, the temperature dependence of optical rotation in the far-ultraviolet region has not been examined in detail and shows, as will be seen, that the thermal unfolding of RNase proceeds in a complex manner.

Figure 2 shows the optical rotatory dispersion curves of RNase and Ribox at low temperature. The major features of the spectrum of RNase in this region are a trough with a minimum near 227.5 mμ (with a possible shoulder near 233), a shoulder at 210 mμ, and a peak at 200 mμ. This curve is the resultant of a number of optically active transitions; no attempt will be made at a detailed analysis. It will be seen below, however, that the variation, with temperature, of the optical rotation depends strongly on the particular wavelength which is being followed. At very high temperatures, the ORD curves of RNase and Ribox are almost indistinguishable as may be seen in Figure 3. The small differences between the two curves are probably within experimental error. It will be noted on comparing the

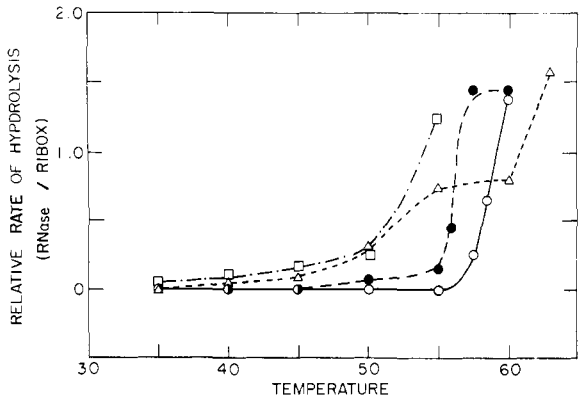

FIGURE 1: The rate of hydrolysis of RNase relative to that of Ribox as a function of temperature. The proteases used were (—○—) aminopeptidase, (—△—) trypsin, (—·—□—·—) chymotrypsin, and (—●—) carboxypeptidase.

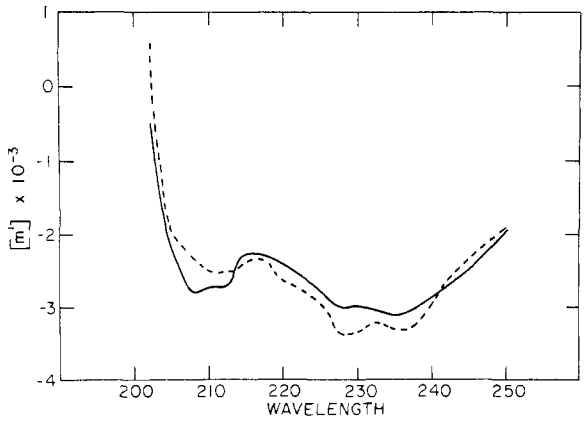

FIGURE 3: The optical rotatory dispersion of RNase (dashed line) and of Ribox (solid line) at 73°.

data in Figures 2 and 3, that the optical rotatory dispersion of RNase changed markedly in both magnitude and shape on going from 9 to 73°; whereas, that of Ribox changed only to a small degree. This result is not unexpected and is consistent with the well-documented lack of organized structure of Ribox when compared to the highly compact conformation which native RNase is known to exhibit (Scheraga and Rupley, 1962). When the optical rotatory dispersion of RNase and of Ribox is examined at a series of intermediate temperatures it is found that, as expected, the temperature dependence is quite different for the two proteins. Figure 4 shows the temperature dependence of the optical rotation of Ribox at 210 mμ. The data at this wavelength are representative of the results in the low-ultraviolet region and were chosen because the magnitude of the changes seen here is larger than in most other regions. The data are seen to fit a straight line reasonably well. There is no evidence for a conformational transition in the temperature range studied with the possible exception of the region around 30° where two points are somewhat displaced from the line. In Figure 5, on the other hand, are data for RNase at two wavelengths. Both sets of data show a marked transition region near 60° as expected from previous results (Harrington and Schellman, 1956). Note that the two curves of Figure 5 are different both in shape and in the apparent midpoint of the high temperature transition. The data at 210 mμ show almost no temperature dependence below 50° in contrast to the marked negative slope of the data obtained at 227.5 mμ in this temperature region. Thus, it appears that a part of the structure of RNase which is strongly reflected in $[m']_{227.5}$, but not in $[m']_{210}$, changes gradually in this temperature region. Furthermore, although both sets of data show a marked transition at higher tempera-

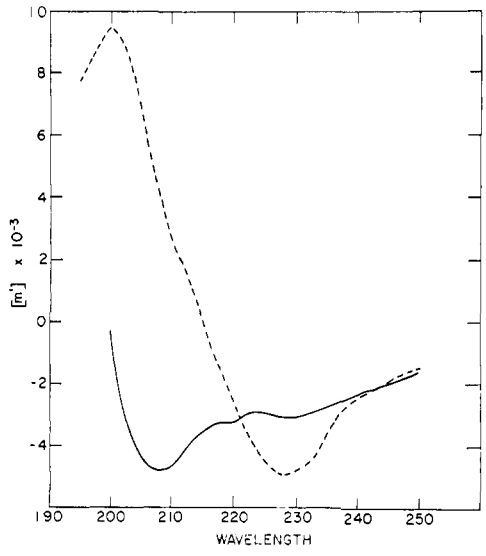

FIGURE 2: The optical rotatory dispersion of RNase (dashed line) and of Ribox (solid line) at 9°.

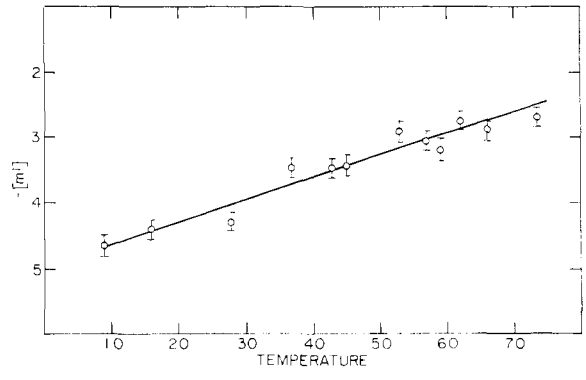

FIGURE 4: The temperature dependence of the optical rotation of Ribox at 210 mμ.

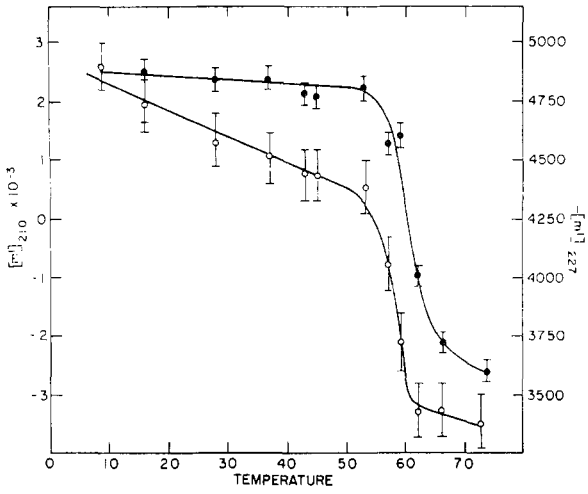

FIGURE 5: The temperature dependence of the optical rotation of RNase at 210 mμ (filled circles and left-hand ordinate) and at 227.5 mμ (open circles and right-hand ordinate).

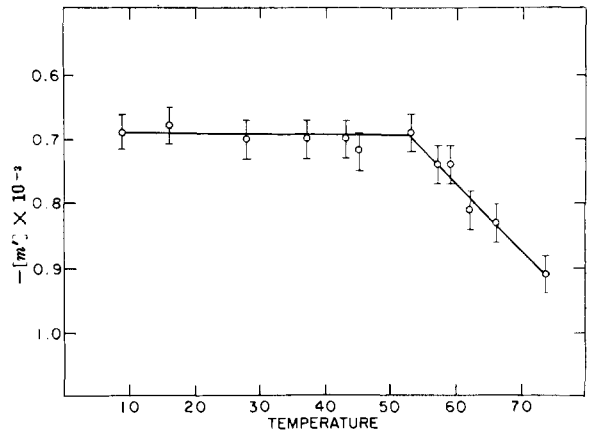

FIGURE 7: The temperature dependence of the optical rotation of RNase at 275 mμ.

ture, the midpoint of the transition is close to 58° when measured at 277.5 mμ, but is closer to 61° when measured at 210 mμ. Since both sets of data are taken from identical optical rotatory dispersion curves it is likely that this difference, though small, is significant.

Cotton effects associated with side-chain chromophores have been observed for RNase (Glazer and Simmons, 1965) and many other proteins. Figure 6 shows the region of the optical rotatory dispersion curve of RNase which encompasses at least some of the tyrosine, phenylalanine, and cystine absorption bands and their resultant Cotton effects. The marked flattening of the low-temperature curve around 275 mμ is a reflection of a small negative Cotton effect associated with tyrosine absorption. Cotton effects due to other chromophores are masked by the strong rotation in the 230-mμ region but are presumably contributing significantly to the observed rotation in the region between 240 and 270 mμ. It is seen in the figure that the tyrosine Cotton effect is diminished at high temperatures, but as shown more clearly in Figure 7, there appears to be no leveling off of rotation at this wavelength (275 mμ) even at the highest temperature studied. Clearly, the data in Figure 6 do not suggest a transition with a midpoint near 60°. In Figure 8 are data obtained at 252.5 mμ which is also in a region of side-chain absorption (in this case cystine and perhaps phenylalanine). The figure shows the existence of transitions taking place at temperatures well above the generally accepted transition temperature for RNase.

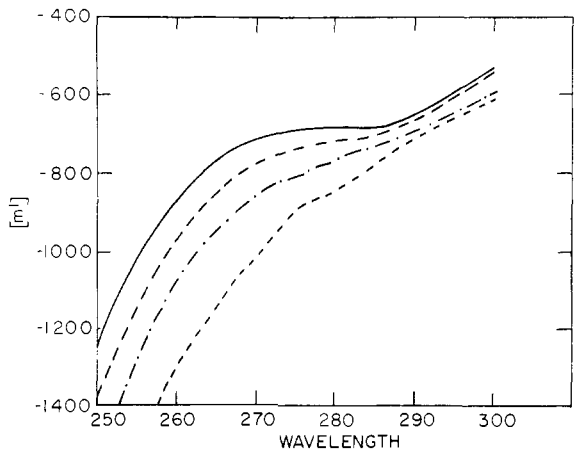

FIGURE 6: The optical rotatory dispersion of RNase in the wavelength region of side-chain absorption at (from top to bottom) 9, 59, 62, and 73°.

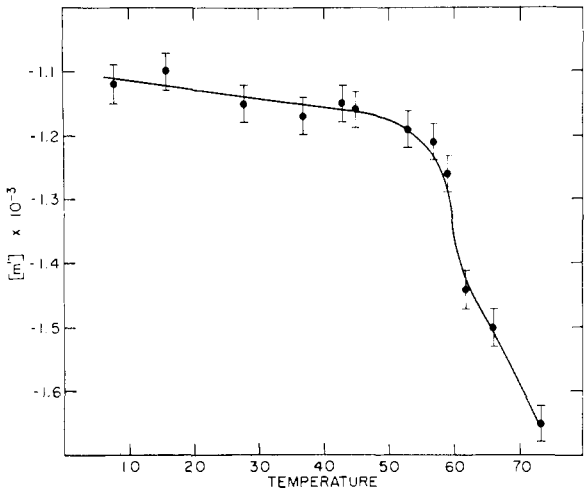

FIGURE 8: The temperature dependence of the optical rotation of RNase at 252.5 mμ.

Discussion

The results of the present work indicate that RNase assumes a *minimum* of four conformational states during the course of thermal unfolding. It is likely that this number is a gross underestimate and that the transition from the low-temperature form(s) to those present at higher temperatures is the result of a gradual disordering process involving a very large number of intermediate states (Poland and Scheraga, 1965). Evidence for the existence of multiple states in the course of the thermal unfolding of RNase has been presented by Holcomb and Van Holde (1962), Scott and Scheraga (1963), and Beck *et al.* (1965) on the bases of many types of physical measurements. Phillips and McDonald[2] have recently found evidence for different environmental states for several of the aromatic amino acid residues of RNase at different temperatures by means of nuclear magnetic resonance studies. Bigelow (1963) and Barnard (1964) have shown that a number of conformational states of RNase are found during the course of solvent-induced denaturation reactions. There thus seems little doubt that the two-state hypothesis for the denaturation of ribonuclease is no longer tenable.

At temperatures below 35° RNase is very compactly folded and is consequently resistant to the action of many proteases. Even under these conditions, however, the region of the molecule between amino and residues 19 and 22 is sufficiently unstructured so as to be readily susceptible to the action of subtilisin and elastase (Richards, 1958; Klee, 1965; Gross and Witkop, 1966; Doscher and Hirs, 1967). At temperatures near 40–45° the enzyme has unfolded to the extent of being susceptible to the action of chymotrypsin and trypsin, apparently in that order.

Rupley and Scheraga (1963) and Ooi *et al.* (1963) have shown that, at 60°, the peptide bonds which appear to be most susceptible to attack by trypsin and chymotrypsin are located in the region of the RNase molecule between residues 25 and 34. Although it is not necessarily true that the most susceptible bonds at 40–45° are the same as at the higher temperature this appears to be a reasonable assumption.[3] The proximity of this region of the molecule to that which is susceptible to subtilisin and elastase at low temperatures is perhaps significant. It may be that the molecule starts to unravel about the disordered region 19–22 on heating.

At the next stage of unfolding, near 50°, the carboxyl terminus of the RNase molecule becomes accessible to attack by carboxypeptidase. Finally, the amino terminus of the molecule is unfolded at the very highest temperatures accessible to the proteolytic enzymes. Clearly then, the unfolding of RNase is not a simple unraveling of the molecule starting at one or the other end. The extreme resistance of the amino terminus to proteolysis is not unexpected in view of the tightness with which S peptide (residues 1–20) has been shown to bind to the rest of the RNase molecule (Richards and Vithayathil, 1959), and of the profound influence which removal of the first two amino and residues of a shortened S peptide has on this binding (Finn and Hofmann, 1965). The latter authors have shown that removal of the amino-terminal lysine from an S-peptide derivative results in a 12-fold decrease in apparent affinity and subsequent removal of the penultimate glutamic and residue reduced the affinity by another factor of 40. The next residue appears to have a much less critical influence on binding. The importance of the amino-terminal lysine residue to the structural integrity of the molecule may also be seen from the fact that an RNase missing only this amino acid is significantly more susceptible to tryptic hydrolysis than is the normal enzyme (Eaker *et al.*, 1965).

Proteolysis can detect the presence of even minute amounts of unfolded RNase which are in rapid equilibrium with the native forms. Thus, the intermediate stages of unfolding which have been detected do not necessarily account for a large fraction of the total RNase present. It is nonetheless true, however, that intermediate states of unfolding exist.

The thermally induced conformational changes of RNase can be completely reversible (Harrington and Schellman, 1956; Foss and Schellman, 1959). These studies, therefore, may have relevance to the problem of how the information contained in the amino acid sequence of a protein is used to determine its three-dimensional structure (Anfinsen, 1962). The number of conformations possible for even as small a protein as RNase is extraordinarily large. It has been postulated that in order for the molecule to assume its thermodynamically stable conformation in a finite time it must follow a restricted number of kinetic pathways (Levinthal, 1967), which are most likely to be determined by the existence of particularly probable local interactions (Levinthal, 1967; Matthews *et al.*, 1967). The results of the present study are not inconsistent with such an hypothesis but more direct evidence is clearly needed.

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References

Allende, J. E., and Richards, R. M. (1962), *Biochemistry 1*, 295.

Anfinsen, C. B. (1962), *Brookhaven Symp. Biol. 15*, 184.

Barnard, E. A. (1964), *J. Mol. Biol. 10*, 263.

Beck, K., Gill, S. J., and Downing, M. (1965), *J. Am. Chem. Soc. 87*, 901.

Bigelow, C. C. (1963), *J. Mol. Biol. 8*, 696.

Brandts, J. F. (1965), *J. Am. Chem. Soc. 87*, 2759.

[2] W. D. Phillips, personal communication.

[3] Allende and Richards (1962) have shown that the lysyl bond at residue 31 and the arginyl bond at residue 33 of RNase S protein are particularly susceptible to trypsin digestion at 25°.

Brown, R. K., Durieux, J., Delaney, R., Leikhim, E., and Clark, B. J. (1959), *Ann. N. Y. Acad. Sci. 81*, 524.
Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 622.
Crestfield, A. M., Stein, W. H., and Moore, S. (1962), *Arch. Biochem. Biophys., Suppl. 1*, 217.
Doscher, M. S., and Hirs, C. H. W. (1967), *Biochemistry 6*, 304.
Dubos, R. J., and Thompson, R. H. S. (1938), *J. Biol. Chem. 124*, 501.
Eaker, D. L., King, T. P., and Craig, L. C. (1965), *Biochemistry 4*, 1486.
Finn, F. M., and Hofmann, K. (1965), *J. Am. Chem. Soc. 87*, 645.
Foss, J. G. (1961), *Biochim. Biophys. Acta 47*, 569.
Foss, J. G., and Schellman, J. A. (1959), *J. Phys. Chem. 63*, 2007.
Ginsburg, A., and Carroll, W. R. (1965), *Biochemistry 4*, 2159.
Glazer, A. N., and Simmons, N. S. (1965), *J. Am. Chem. Soc. 87*, 3991.
Gross, E., and Witkop, B. (1966), *Biochem. Biophys. Res. Commun. 23*, 720.
Harrington, W. F., and Schellman, J. A. (1956), *Compt. Rend. Trav. Lab. Carlsberg 30*, 21.
Hermans, J., Jr., and Scheraga, H. A. (1961), *J. Am. Chem. Soc. 83*, 3283.
Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.
Hirs, C. H. W., Stein, W. H., and Moore, S. (1953), *J. Biol. Chem. 200*, 493.
Holcomb, D. N., and Van Holde, K. E. (1962), *J. Phys. Chem. 66*, 1999.
Kalnitsky, G., and Resnik, H. (1959), *J. Biol. Chem. 234*, 1714.
Klee, W. A. (1965), *J. Biol. Chem. 240*, 2900.
Levinthal, C. (1967), 17th Meeting of the Societe de Chimie Physique, Paris, May.
Lumry, R., Biltonen, R., and Brandts, J. F. (1966), *Biopolymers 4*, 917.
Matthews, B. W., Sigler, P. B., Henderson, R., and Blow, D. M. (1967), *Nature 214*, 652.
Moore, S., and Stein, W. H. (1954), *J. Biol. Chem. 211*, 907.
Ooi, T., Rupley, J. A., and Scheraga, H. A. (1963), *Biochemistry 2*, 432.
Poland, D. C., and Scheraga, H. A. (1965), *Biopolymers 3*, 401.
Richards, F. M. (1958), *Proc. Natl. Acad. Sci. U. S. 44*, 162.
Richards, F. M., and Vithayathil, P. J. (1959), *J. Biol. Chem. 234*, 1459.
Rupley, J. A., and Scheraga, H. A. (1963), *Biochemistry 2*, 421.
Scheraga, H. A., and Rupley, J. A. (1962), *Advan. Enzymol. 24*, 161.
Scott, R. A., and Scheraga, H. A. (1963), *J. Am. Chem. Soc. 85*, 3866.
Sela, M., Anfinsen, C. B., and Harrington, W. F. (1957), *Biochim. Biophys. Acta 26*, 502.
Smith, E. L., and Spackman, D. H. (1955), *J. Biol. Chem. 212*, 271.
Spackman, D. H., Stein, W. H., and Moore, S. (1960), *J. Biol. Chem. 235*, 648.