Laser Raman Spectroscopic Studies of the Thermal Unfolding of Ribonuclease A†

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ABSTRACT: The reversible thermal denaturation of bovine pancreatic ribonuclease A at pH 5 in 0.1 M NaCl over the range 32–70 °C as studied by Raman spectroscopy proceeds in a gradual manner consistent with a stepwise unfolding process rather than as a transition between two states. Conversion of residues from helical or pleated-sheet geometry to some intermediate geometry, as followed by means of the amide I and III lines, reveals that substantial amounts of the helical and pleated-sheet conformations remain at 70 °C. Changes in the strength of hydrogen bonding by the tyrosyl residues are indicated by the intensity ratio of the doublet at 830–850 cm^{-1} and changes in the geometry of the disulfide bridges by the frequency and half-width of the Raman line near 510 cm^{-1} due to the S–S vibration. Vibrations of C–S bonds in the methionines and cystines are used to monitor conformational changes in these residues. While there are small quantitative differences in temperature dependence among these probes, all agree in placing the melting temperature at or near 62 °C. The Raman data are quantitatively consistent with the six-stage scheme of unfolding of A. W. Burgess and H. A. Scheraga [(1975), *J. Theor. Biol. 53, 403*], except that no change in the environment of the tyrosines is seen until 45 °C.

Since the discovery of the reversible thermal denaturation of bovine pancreatic ribonuclease A (RNase A) by Harrington and Schellman (1956), much effort has been devoted to understanding the mechanism of this process. In early studies with optical rotatory dispersion (Harrington and Schellman, 1956; Foss and Schellman, 1959), ultraviolet absorption (Hermans and Scheraga, 1960; Brandts and Hunt, 1967), and enzyme activity (Kalnitsky and Resnick, 1959), the results were interpreted in terms of two states, the native and the denatured molecule, with no stable intermediate, especially in a solution of low pH. However, recent studies by temperature-jump kinetics (Tsong et al., 1972; Tsong and Baldwin, 1972), viscosity and sedimentation (Holcomb and Van Holde, 1962), calorimetric measurements (Beck et al., 1965; Tsong et al., 1970; Winchester et al., 1970; Privalov et al., 1973), proteolytic digestions (Klee, 1967), ultraviolet absorption (Foss, 1961; Scott and Scheraga, 1963), circular dichroism (Simons et al., 1969), and nuclear magnetic resonance spectroscopy (McDonald and Phillips, 1967; Zaborsky and Milliman, 1972; Roberts and Benz, 1973; Benz and Roberts, 1975) have shown that appreciable concentrations of intermediate conformations exist in the process of unfolding. Much of this work and earlier chemical studies have been drawn together by Burgess and Scheraga (1975) in their detailed proposal of a six-stage pathway of reversible unfolding in the temperature range 15–70 °C at neutral pH.

Previous studies of proteins in aqueous solution by Raman spectroscopy (Lord and Yu, 1970a,b; Yu et al., 1972; Chen et al., 1973, 1974) have provided information on peptide backbone conformations, as revealed by the amide I and III frequencies, which lie, respectively, at 1650–1675 and 1220–1300 cm^{-1}, and also by the C–C–N skeletal stretching vibrations in the 900–1150 cm^{-1} range. In addition, the 500–750 cm^{-1} region is useful studying the local conformations of the S–S and C–S bonds. Furthermore, Raman lines due to side chains with aromatic rings, such as those of phenylalanine, tryptophan, and tyrosine, are sharp and well defined, and often are sensitive to the local environment of these residues. Thus, the Raman effect is potentially valuable for studies of denaturation, and has recently been used to examine conformational changes associated with denaturation and renaturation in lysozyme (Chen et al., 1973, 1974) and RNase A (Yu et al., 1972).

The Raman spectrum of RNase A in aqueous solution was first reported and interpreted by Lord and Yu (1970b), but study of thermal denaturation at that time was unsuccessful because of experimental difficulties. With the use of a more powerful laser and a better spectrometer, it has proved possible to obtain satisfactory spectra. In the present work the nature of the conformational changes with temperature of the RNase A molecule in aqueous solution as revealed by the Raman spectrum is examined in detail, and a consideration of the data in the light of the pathway for unfolding proposed by Burgess and Scheraga (1975) is carried out.

Experimental Methods

Chromatographically homogeneous bovine pancreatic RNase A was purchased from Miles Laboratories, Inc., as a "salt-free" dried ethanol precipitate and was used without further treatment. Doubly distilled water was used to prepare 7% solutions of RNase A in 0.1 M NaCl with no buffer added (measured pH was 5).

The sample-handling technique and instrumentation for recording Raman spectra of solutions have been described previously (Chen et al., 1973). All spectra were recorded with a spectral slit width of 7 cm^{-1} in one continuous set of recordings (32–75 °C) without disturbing the Raman cell, except in those experiments described in the next paragraph. Each spectrum was recorded in 1 hr. Temperature was raised in steps

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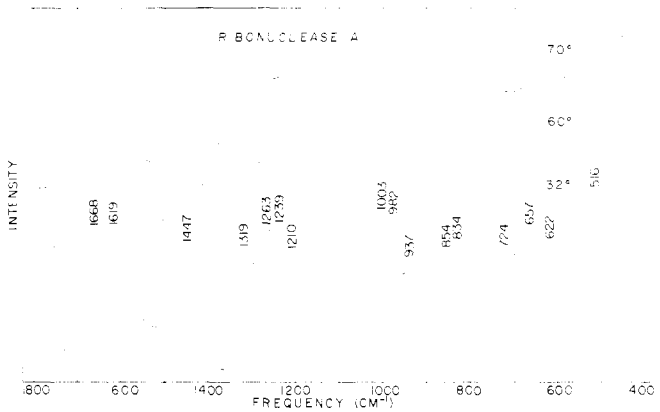

FIGURE 1: Original recordings of Raman spectra of 7% aqueous RNase A at 32, 60, and 70 °C, ionic strength 0.1 M NaCl, pH 5.

of 5 °C, for which about 5 min was required. After the temperature of the thermostat had become constant at a new value, a period of at least 10 min was allowed for the sample to reach equilibrium. Raman peak intensities at each temperature were normalized to the intensity of the methylene deformation frequency at 1447 cm^{-1}, taken as 10.

In order to examine the cumulative effect of continuous heating and laser irradiation in RNase A, a series of fresh, unheated identical samples of 7% solution was incubated for 15 min at a given temperature and their Raman spectra then recorded (about 1 h for each recording) at the same temperature. Temperatures used were 45, 50, 55, 60, 65, and 70 °C. The results were essentially the same as those obtained for the corresponding temperature by continuous recording as described above. In a parallel fashion a series of samples previously incubated at 70 °C for 1 h was each incubated at 65, 60, 55, 50, 45, and 32 °C for 15 min prior to Raman measurement, and the spectra were also similar to those obtained with a single sample at the corresponding temperature. Thus, under the conditions used in the present series of experiments (concentration range 5–15%), the spectroscopic changes observed are characteristic of the molecule at a particular temperature, and are not the consequence of the heating and/or laser irradiation used. Moreover, the spectroscopic changes produced by heating are reversible provided the heating is not protracted, that is, the sample is not held at the highest temperature (75 °C) longer than a few minutes. Protracted heating at a fixed high temperature produces a definite amount of irreversible denaturation that is, of course, related to the time of heating and the temperature.

Results and Discussion

One of the potential advantages of Raman spectroscopy for the study of proteins lies in the correlation of the vibrational frequencies of the peptide backbone with various protein conformations. Previous Raman spectroscopic studies of polypeptides as conformational models (Chen and Lord, 1974; Frushour and Koenig, 1974; Yu et al., 1973) have shown that the frequency of the amide III vibration, composed of a mixture of C–N stretching coupled with N–H in-plane bending, is more sensitive to changes in the conformation of the polypeptide backbone than the amide I frequency, which is due to the amide C=O stretching motion. The characteristic amide III lines of the α-helical geometry (with ϕ,ψ angles in the neighborhood of $-58°,-47°$) are medium in intensity and positioned at 1265–1300 cm^{-1}, the β-sheet conformation (ϕ,ψ angles near $-120°,+135°$) gives a strong sharp line at 1229–1235 cm^{-1}, and peptide units with ϕ,ψ geometry intermediate between the α and β structures ($-75° < \phi < -100°$, $0° < \psi < +90°$) have amide III frequencies of medium strong intensity in the range 1240–1260 cm^{-1}.

In the Raman spectra of 7% RNase A at pH 5 and 32 °C there are two medium strong amide III lines at 1239 and 1263 cm^{-1} with the former slightly stronger (Figure 1). Yu et al. (1972) have assigned these lines to the β-sheet and α-helical components of the protein, respectively. However, in RNase A the line at 1239 cm^{-1} is probably the result of overlap of a strong β-sheet line at about 1232 cm^{-1} with a moderately strong and broad set of lines centered at about 1248 cm^{-1} and due to peptide units of intermediate geometry. This set also overlaps with the α-helical line and results in the maximum at 1263 cm^{-1}.

According to the structure determined by x-ray diffraction (Kartha et al., 1967; Wyckoff et al., 1970; Richards et al., 1973), the α-helical content of the molecule is about 18%, while that of the β conformation is 35–40% and that of units of intermediate geometry about 45%. The substantial discrepancies between these numbers and those inferred from the uncorrected amide III region suggest that there may be other lines contributing to the intensity at 1263 cm^{-1}. Examination of the spectra of the constituent amino acids (Figure 2) shows that histidine and tyrosine have lines at 1272 and 1266 cm^{-1}, respectively, in the region of 1235–1275 cm^{-1} (Lord and Yu, 1970a,b). However, there are only four histidine and six tyrosine residues in RNase A, and these two lines contribute relatively little intensity to the amide III region, as can be seen by noting the tyrosine line at 1210 cm^{-1}, which is known to be three times more intense than the line at 1266 cm^{-1}. From a consideration of the spectrum of a concentrated solution (20%) of RNase in D_2O, in which the D_2O line at 1210 cm^{-1} gives no interference at 1250 cm^{-1} (Lord and Yu, 1970b), the contribution of tyrosine-*O*-*d* and histidine-*N*-*d* at 1260 cm^{-1} is estimated to be no more than 20% of the amide III intensity at 1263 cm^{-1} in the undeuterated sample.

Unfortunately the errors inherent in trying to correct for an intensity of this magnitude (errors in baseline, line shape, peak intensity, and peak frequency) are substantial. Even after correction the uncertainty in the estimate of the amount of helical conformation from the amide III band is of the order of 10%.

The decision about the inclusion of certain residues in an α-helical segment from the x-ray data, moreover, may be based on a more restrictive criterion. For example, Kartha et al. (1967) in their counting of residues 5–12, 28–35, and 51–58 as helical segments add that "there are a couple of other regions where there is a suggestion of helical conformation". If the geometry of the individual residues as defined by the values of the ϕ,ψ angles in such regions is that of the α helix, the resulting amide III frequencies should fall in the α-helical range (R. C. Lord, to be published) and thus indicate a higher α-helical content than the more restricted criterion of one or more "complete turns" of helical conformation.

This way of interpreting the Raman data should then always lead to a higher helical content from Raman studies than by crystallographic evaluation. It should also be expected that a similar "overestimation" would be made by optical rotatory dispersion methods, and that Raman and optical rotatory dispersion methods should tend to agree except when one or the other technique suffers from complications associated with other factors than peptide geometry.

Temperature Dependence of the Spectrum of RNase A. In Figure 1 are presented spectra of 7% solutions, recorded at 32,

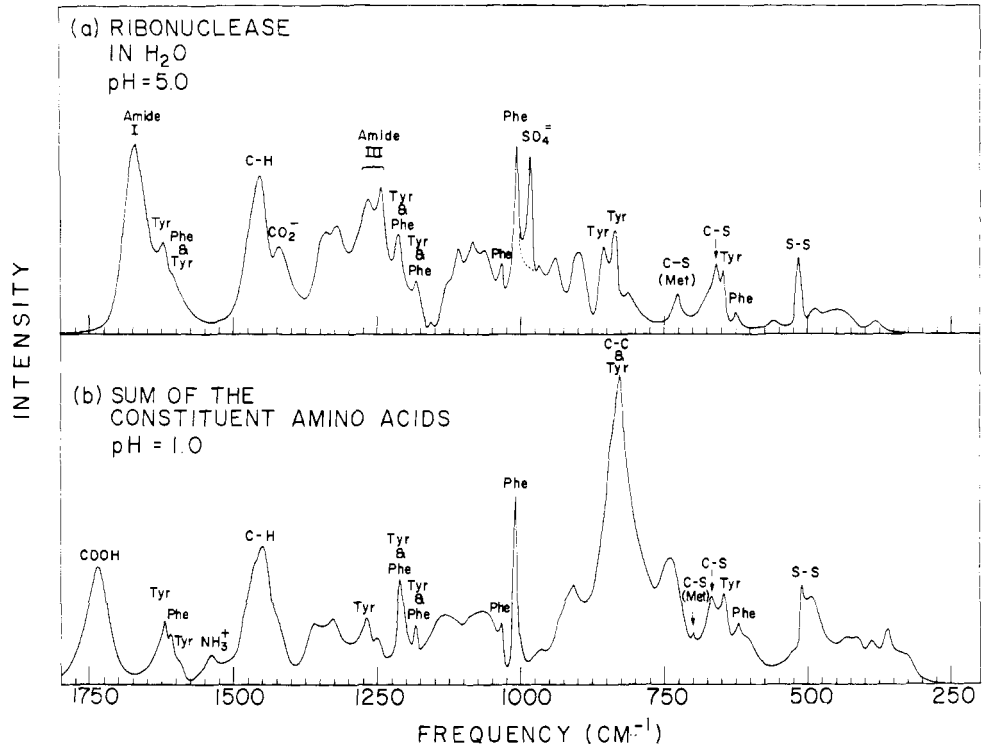

FIGURE 2: Comparison of Raman spectrum of native RNase A with the spectrum of the sum of the constituent amino acids at pH 1 (from Lord and Yu, 1970b). Note that tyrosine has a weak line at 1265 cm^{-1}.

60, and 70 °C. Spectra taken at intermediate temperatures show intermediate intensity changes, while a series determined at 75 °C is very nearly the same as that at 70 °C. For comparison the spectra at 30 and 70 °C, after being redrawn to remove noise and background, and normalized to the 1447-cm^{-1} methylene deformation line, are superimposed in Figure 3 and tabulated in Table I.

Characteristic Frequencies of the Peptide Backbone. We begin with a consideration of the changes in the amide III region with temperature. Qualitatively this region shows a large increase in intensity at 1250 cm^{-1}, with parallel decreases at 1265 and 1235 cm^{-1} due to a decrease in residues with helical and pleated-sheet geometry as a result of denaturation. However, even after correcting for side-chain contributions near 1265 cm^{-1} some intensity near these frequencies is still present at 70 °C, suggesting some residual structure of α-helix and β-pleated sheet geometry as defined by their dihedral angles (IUPAC-IUB, 1970).[1] The minimum at 1250 cm^{-1} in the spectrum of native RNase is almost wiped out at 70 °C, as Figures 1 and 3 show.

Figure 4a gives a temperature plot of the intensity at 1250 cm^{-1}, from which it can be seen that the "melting temperature", T_m, taken as the half-way point between the plateaus of the native and denatured forms, is about 60 °C. This temperature agrees with values for T_m obtained by several other techniques (Harrington and Schellman, 1956; Foss and Schellman, 1959; Kalnitsky and Resnick, 1959; Hermans and Scheraga, 1960; Privalov et al., 1973; Foss, 1961; Klee, 1967; Winchester et al., 1970; Garel and Baldwin, 1973). There is some indication that the transformation is not a smooth one, with signs of "premelting" below 60 °C. These data suggest that the course of melting is not cooperative but corresponds to a multiple process in which the residues of the peptide backbone convert continuously from their native distribution of the ϕ,ψ angles to one with more residues of intermediate geometry.

A parallel but less definite conclusion is indicated by the amide I line. As was found earlier in the irreversible denaturation of lysozyme (Chen et al., 1973), the amide I frequency increases from a value (1668 cm^{-1}) corresponding to a weighted mean of frequencies from the native protein to 1671 cm^{-1}, indicative of a more disordered structure. Denaturation is more clearly indicated (so far as the amide I line alone can do so) by an increase in the half-width of the line from about 70 to 80 cm^{-1}.

The remaining frequency regions sensitive to backbone conformation are those from 870 to 950 cm^{-1} and from 1040 to 1150 cm^{-1}. The former is commonly associated with C_α-C bond-stretching vibrations and the latter with C_α-N stretching. The peak at 937 cm^{-1} decreases markedly with temperature (Figure 4b) and those at 892–902 cm^{-1} become diffuse though probably with little decrease in integrated intensity. Frushour and Koenig (1974) have ascribed a weak line in the Raman spectrum of poly(L-alanine) at 931 cm^{-1} to the α-helical configuration of the backbone and the change seen in Figure 4b may be caused by the decrease in the number of residues with α-helical geometry. In any case this region shows the qualitative changes encountered in other proteins (Chen et al., 1973) upon denaturation and reflects the increased randomness of the peptide backbone at elevated temperatures. In the range 1050–1100 cm^{-1} a parallel diffuseness is observed with increasing temperature.

The Behavior of the Tyrosyl Doublet. It has been shown recently (Siamwiza et al., 1975) that the doublet due to tyrosyl residues at 850 and 830 cm^{-1} arises from Fermi resonance between a fundamental of one phenyl ring vibration and the first overtone of another. These authors give clear evidence that this resonance is a function of the H bonding associated with

[1] Residual structure at high temperature has also been inferred from optical rotatory dispersion (Aune et al., 1967) and from chemical studies (Ooi et al., 1963; Rupley and Scheraga, 1963).

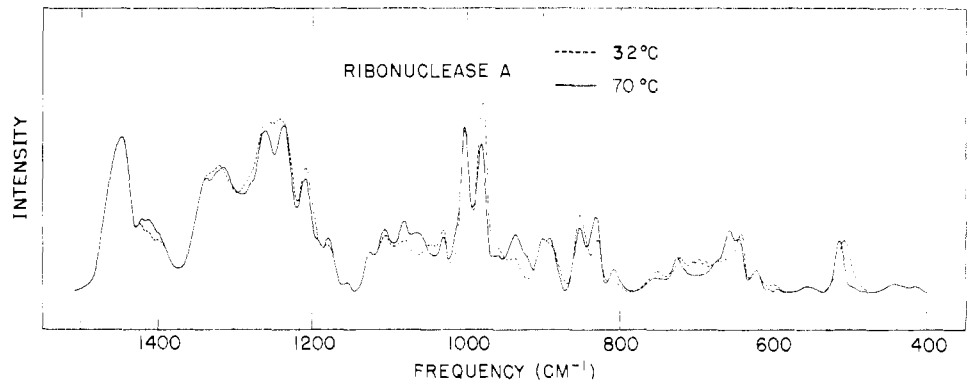

FIGURE 3: Raman spectra (400–1500 cm^{-1}) of native and denatured RNase A, at 32 and 70 °C, respectively, after correction of the water background and being normalized to the intensity of the methylene deformation mode at 1447 cm^{-1}.

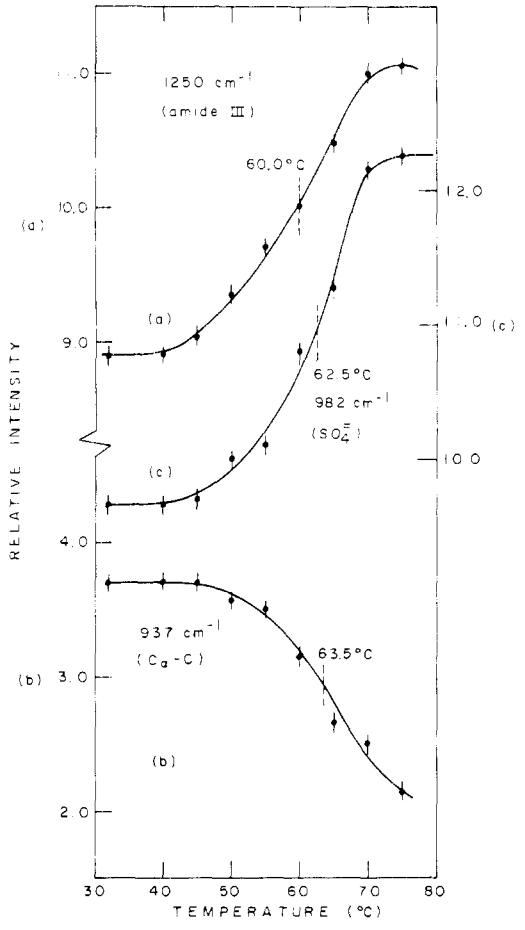

FIGURE 4: Thermal transition curves of 7% RNase A as monitored by (a) the amide III vibration at 1250 cm^{-1}; (b) C_α–C stretching at 937 cm^{-1}; (c) SO_4^{2-} stretching at 982 cm^{-1}.

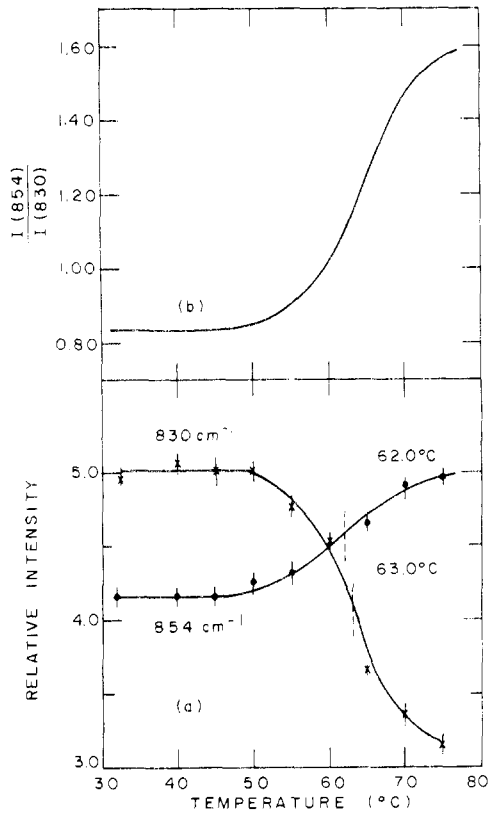

FIGURE 5: Thermal transition curves of 7% RNase A as monitored by the tyrosine doublet: (a) intensities of the separate components as measured at the intensity maxima; (b) ratio of the intensity of the higher to that of the lower component.

the phenolic hydroxyl and is not the result of other environmental or structural changes. In native RNase A (pH 5) the intensity ratio of the high-frequency to the low-frequency component of the doublet (Figure 5b) is about 8:10, which shows that a substantial portion of the phenolic hydroxyls is strongly H bonded, most probably in the ratio of three strongly bonded to three weakly or moderately bonded. The three strongly bonded residues are probably to be identified with the three "buried" residues (Tyr-25, -92, and -97) that were presumed by Scheraga and coworkers (Li et al., 1966; Scheraga, 1967) to be strongly paired with the carboxylate ions of aspartic acid residues 14, 38, and 83. When these aspartic acid residues are protonated (pH 1.7), the intensity ratio of the doublet changes to 10:9, as was shown by Yu et al. (1972). This change indicates that two of these three "buried" tyrosines are no longer strongly H bonded.

An analogous but more extensive set of changes occurs in the doublet when RNase A is thermally denatured. The native intensity ratio of 8:10 (Figures 1 and 3) is converted to 10:6 at 70 °C. The latter ratio indicates that all of the phenolic hydroxyls in the six tyrosyl residues are weakly hydrogen bonded, for example to the oxygen of water or to other uncharged acceptor atoms. To the extent that this change in H bonding results from disruption of the above-mentioned bonding to carboxylate ions of aspartic residues, the tyrosyl residues concerned may be regarded as changing from "buried" status to "exposed".

Table I: Raman Frequencies and Intensities of Native and Thermally Denatured RNase A in Aqueous Solution.[a]

Frequency (cm^{-1})		Tentative Assignment
32 °C	70 °C	
412 (0)	412 (0)	
440 (0)	440 (0)	
496 (0 s)		
516 (3)	507 (3)	ν(S-S)
555 (0)	555 (0)	
594 (0)	598 (0)	
605 (0)		
622 (1)	622 (1)	Phe
644 (3)	642 (4)	Tyr
657 (4)	662 (2)	
675 (2 s)	675 (2 s)	
	687 (2)	
	695 (2)	
	703 (2)	ν(C-S)
714 (0 s)		
724 (2)	720 (2)	
	735 (1 s)	
755 (1)	750 (1)	
808 (1)	806 (1)	
834 (5)	829 (3)	Tyr
854 (4)	854 (5)	
892 (3)	886 (3)	
902 (3)	902 (3)	ν(C_α-C)
918 (0 s)		
937 (4)	937 (2)	
960 (0)		
982 (9)	982 (12)	SO_4^{2-}
1003 (10)	1003 (10)	Phe
1015 (0 s)		
1030 (3)	1030 (4)	Phe
	1047 (3)	
1062 (4)		ν(C_α-N)
1082 (4)	1080 (3)	
1106 (4)	1104 (4)	
1125 (3)	1125 (3)	
1154 (1)	1154 (1)	
1180 (2)	1180 (2)	Tyr and Phe
1191 (0 s)		
1210 (3)	1210 (4)	Tyr and Phe
1239 (11)	1242 (11)	
1263 (10)	1260 (11)	Amide III
1284 (0 s)	1290 (0 s)	
1315 (8)	1320 (8)	
1324 (1 s)		γ(CH_2)(?)
1337 (3)	1337 (3 s)	
1399 (4)	1399 (3)	
1412 (5)	1412 (4)	ν(CO_2^-)
1420 (5)	1423 (4)	
1447 (10)	1447 (10)	δ(CH_2)
1585 (0 s)	1585 (0 s)	
1603 (1 s)	1603 (1 s)	
1619 (3)	1619 (3)	Tyr and Phe
1668 (24)	1671 (21)	Amide I and H_2O

[a] Solution 7% by weight, pH 5, 0.1 M NaCl. s denotes a shoulder. ν means stretching vibration, δ deformation, γ twisting. Numerical figures in parentheses are relative peak intensities with that at 1447 cm^{-1} taken as 10.

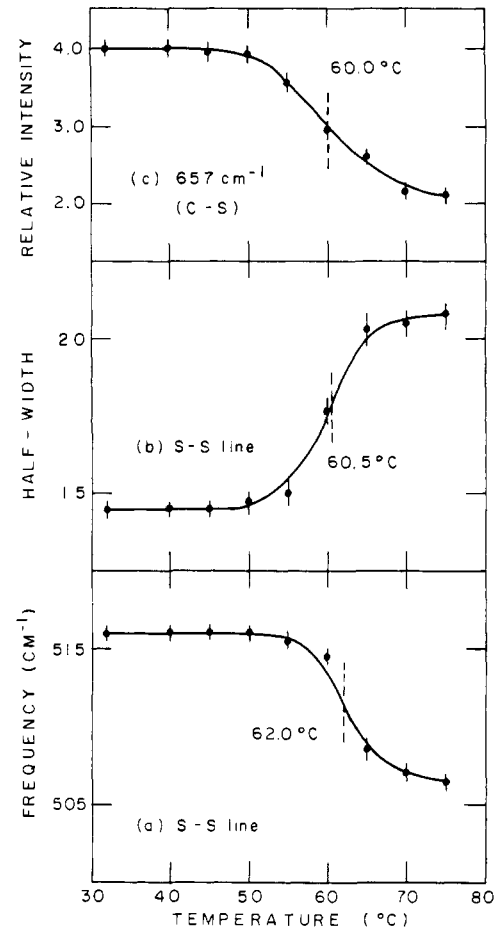

FIGURE 6: Thermal transition curves of 7% RNase A as monitored by (a) the frequency of the S-S stretching line; (b) the half-width of the S-S stretching line; (c) the relative intensity (peak height) of the C-S stretching line at 657 cm^{-1}.

The temperature dependences of the peak intensities of the doublet components are shown in Figure 5a and their ratio is shown in Figure 5b. At pH 5 the ratio is stable up to about 45 °C and the participation of the tyrosines in the denaturation does not begin to show up until almost 50 °C. From 50 to 70 °C there is a steady and rapid change in the ratio which shows all the tyrosyl residues to become weakly hydrogen bonded. The mean melting temperature T_m appears to be about 63 °C.

The Disulfide Frequency. The disulfide frequency in native RNase A at room temperature and pH 5 is higher than usual (516 cm^{-1} as compared to 509 cm^{-1} for a number of other proteins and model compounds). There is no evidence of high-frequency components near 525–540 cm^{-1} and the sharpness of the S-S peak (half-width 14 cm^{-1}) indicates that the geometries of the four disulfide groups are similar. It has been proposed (Miyazawa and Sugeta, 1974) that this is to be expected, since all the eight half-cystine groups have the C^α and C^β atoms in the gauche,gauche,gauche positions. The stability of the peptide backbone in the neighborhood of the disulfide bridges is well indicated by the temperature dependence of the frequency and half-width of the S-S line, plotted in Figure 6. While the former changes only slightly with temperature and the latter is difficult to measure, it may still be said from Figure 6 that these do not indicate any change until about 55 °C, although the T_m they yield is still 61–62 °C. The S-S frequency of the denatured structure (507 cm^{-1}) is near the normal one of 509 cm^{-1}, thus suggesting that the tertiary structure of the native protein puts the disulfide bonds under some strain.

The Region 600–750 cm^{-1}. This region includes the phenylalanine ring-distortion vibration at 622 cm^{-1}, the analogous mode in tyrosine at 642 cm^{-1}, and various C-S stretching modes. These latter are conformation sensitive and have been

interpreted by Miyazawa and Sugeta (1974) as 657 cm^{-1} (methionine gauche,gauche form) and 725 cm^{-1} (cystine "P_c" form plus methionine trans,trans form). The line at 622 cm^{-1} is not sensitive to conformation and has the same intensity and shape at 70 as at 32 °C. The tyrosine line at 642 cm^{-1} shows differences in frequency and intensity between these two temperatures but we cannot decide how much these differences result from changes in tyrosine and how much from a decrease in the C–S line at 657 cm^{-1}. That both are involved is indicated by the remarkable difference in these lines between the spectra of native RNase in the crystal and as a lyophilized powder (Yu et al., 1972; Yu and Jo, 1973). It appears that the distortions produced by lyophilization are sufficient to disrupt two of the three strongly H-bonded tyrosines (shown by the change in the 850:830 doublet intensity ratio from 8:10 to 10:9), which makes the contributions of five of six tyrosines the same at 642 cm^{-1} and thereby sharpens and strengthens this line in the spectrum of the lyophilized powder.

Unfortunately it is difficult to separate the effects produced by temperature on the C–S frequencies of cystine from those of the methionine side chains. If we *assume* that the former make no contribution to the intensity at 657 cm^{-1} and the latter none to the 725-cm^{-1} line in the native spectrum, one could conclude from the temperature dependence of the 657-cm^{-1} line that the methionines (Met-13 and -30) begin to change structure in the 45–50 °C range (Figure 6c), whereas the 725-cm^{-1} line indicates that the geometry around the cystine C–S bonds does not begin to alter until about 55 °C. The disappearance of an intensity maximum at 657 cm^{-1} above 65 °C suggests a randomization of the methionine conformations, with a resultant increase in intensity in the range 675–710 cm^{-1}. It appears that the integrated area in this range makes up for the loss of intensity at 657 cm^{-1} in the high-temperature spectrum (see Figure 3).

Finally we comment that the overlap of C–S intensity with that of the tyrosine peak at 642 cm^{-1} makes the use of this peak rather uncertain for the determination of the average tyrosyl environment. It appears that the intensity ratio of the 850:830 doublet is somewhat more reliable for this purpose.

The Sulfate Ion as an Indicator of Conformation. It has been recognized for many years (Sela and Anfinsen, 1957; Sela et al., 1957) that phosphate ion binds so tightly to residues in the active site of RNase A that it acts as an inhibitor of enzymatic action. The locus of binding of a single phosphate or sulfate ion has been established by various methods, in particular x-ray diffraction (Kartha et al., 1967, 1968; Wyckoff et al., 1970), to be immediately adjacent to His-12 and -119. It is well known (Ginsburg and Carroll, 1965; von Hippel and Wong, 1965; Winchester et al., 1970) that sulfate ion stabilizes the native structure of RNase, particularly at low pH. However, at the pH range of the present study and the concentration of sulfate ion present (~0.03 M, see below), its effect on thermal denaturation is very small, as is shown by the close similarity of the values of T_m determined from the various Raman data and those found in the absence of sulfate ion.

The Raman spectrum of sulfate ion bound to RNase A shows an interesting temperature dependence that is of potential value for conformational study.[2] Sulfate ion in aqueous solution at pH >2 produces an intense Raman line at 982 cm^{-1}, due to the symmetric stretching vibration of the ion's tetrahedral structure. When the symmetry is destroyed by protonation, this line disappears and the HSO_4^- ion shows two weaker frequencies (Daly et al., 1972) at 895 and 1053 cm^{-1}. If in the course of binding to one of the histidines (say 119) in RNase the sulfate ion is converted in effect to HSO_4^-, the result would be the elimination of the line at 982 cm^{-1} from the bound species. In Figure 1, the strong sulfate line at this frequency shows considerable free SO_4^{2-} (in concentration approximately 0.03 M in the spectrum at 32 °C as determined by comparison with a dilute solution of Na_2SO_4). This corresponds to five or six sulfate ions per RNase A molecule.

When the enzyme is heated, some of the bound sulfate is released and the 982-cm^{-1} line increases in intensity. The temperature dependence of this line, plotted in Figure 4c, shows that the release of sulfate begins rather early (~40–45 °C) but continues steadily up to 70 °C. The amount released corresponds to an increase of about 25% in free ion or to roughly 1.3–1.5 ions bound per molecule of native RNase. This is in reasonable agreement with earlier studies on the binding of sulfate ion to the protein (Saroff and Carroll, 1962). It is rather remarkable that the "melting temperature" of the protein as indicated by Figure 4c is the same as that found by the other Raman indicators (Figures 4a,b, 5, and 6). It might have been supposed that the sulfate "desorption" would have a different T_m, depending mainly on the history of the specific groups to which it is attached.

Additional information is obtained from the cooling cycle of the study. If the denatured material is kept for a long period (>1 h) at elevated temperature (>70 °C) and is then cooled to room temperature, the intensity of the sulfate line remains essentially constant. If, however, the cooling process is begun shortly after the system reaches 70 °C, the sulfate-line intensity follows the curve of Figure 4c back down to room temperature. Thus, irreversible denaturation by heat impairs the ability of the protein to bind sulfate ion strongly.

Relation of the Raman Data to Models of the Unfolding of Ribonuclease. Several models of the unfolding of the tertiary structure of RNase A have been put forward on the basis of various kinds of data: kinetic studies (Tsong et al., 1972), proton magnetic resonance spectroscopy (^1H NMR) (Benz and Roberts, 1975; Roberts and Benz, 1973), and a combination of many different physical and enzymatic studies by Scheraga and coworkers (summarized in Burgess and Scheraga, 1975). These models are not basically in conflict and they agree in regarding the processes of unfolding and refolding as a fixed series of reversible steps, the refolding process being the reverse of the unfolding.

The more detailed model for the unfolding is that of Burgess and Scheraga (1975). The ^1H NMR description of unfolding (Benz and Roberts, 1975; Roberts and Benz, 1973) is compatible with that of Burgess and Scheraga, and indeed the ^1H NMR data were included by the latter in their pool of information. The model regards the unfolding as taking place in six overlapping stages that can be described approximately but do not consist of six sharply defined intermediate steps. We consider the temperature dependence of the Raman spectra in the framework of these six stages.

Stage I (Native RNase A up to 35 °C). Our basis is a native structure with about 30 residues whose geometry as defined by the ϕ,ψ angles[3] approximates within about ±35° the strict α-helical angles of −58°,−47°; about 40 residues that fit within ±35° of the β-pleated-sheet angles of −120°,+135°;

[2] Sulfate-free RNase A (from Worthington Biochemical and from Schwarz/Mann) was found to aggregate more readily at elevated temperatures, thus making quantitative spectroscopic studies more difficult.

[3] Definitions of the torsional angles ϕ and ψ and related geometrical parameters are given in the report of the IUPAC-IUB Commission on Biochemical Nomenclature (1970).

and the remainder (~54 residues) taken to have intermediate geometry (Wyckoff et al., 1970; Richards et al., 1973). It has been proposed (R. C. Lord, to be published) that a correlation exists between the amide III frequency and the angle ψ which results in a parallel correlation between the intensity contour in the amide III region and the distribution of residues among the values of ψ. In the case of RNase A, this distribution leads to the observed doublet contour in the amide III region. So far as the Raman spectrum is concerned this structure is maintained up to 35 °C. In particular, there is no detectable change in the tyrosine doublet, from which the simplest conclusion is that Tyr-92 retains its strong H bonding to the $-CO_2^-$ group of Asp-38.

Stage II (35–45 °C). The Raman intensity at 1250 cm^{-1}, where the amide III frequency of peptide linkages with intermediate geometry is centered, increases very slightly in the range 40–45 °C. It seems possible that this results from changes in the geometry of residues 17–25, which are specified by Burgess and Scheraga (1975) to fold out from the rest of the protein at this stage. Most of these residues already have ψ angles in the intermediate range, except Thr-17 and Asn-24 (both in the β range) and Ser-22 and Tyr-25 (both α like). If these four residues (and perhaps one or two others elsewhere in the structure) convert to intermediate geometry in stage II, the observed increase of about 4% of the total change at 75 °C might be explained. There is still no observable change in the tyrosine doublet, which suggests that the three strongly bonded Tyr residues have not altered their bonding.

Stage III (40–50 °C). In this stage the segments Asn-27 to Asn-34 and Ser-75 to Ser-80 are postulated to unfold. The former contains seven residues of approximate α-helical geometry, and the latter, four residues of β-sheet geometry. In the amide III region there is a clear-cut increase at 1250 cm^{-1} (20% of the total change at 75 °C) which suggests the creation of as much as 8–10 residues of new intermediate geometry. However, the relative amounts of α and β conformation appear to decline uniformly and perhaps not all of the residues with α-helical geometry are changed to intermediate geometry by the unfolding. Tyr-76, the only Tyr residue that would be directly affected by the postulated unfolding, is known to be weakly bonded in the native protein at room temperature (Li et al., 1966) and thus stage III should produce no change in the Tyr doublet ratio, in agreement with observation. However, it is found that the C–S frequency at 657 cm^{-1}, probably due to gauche,gauche methionine, begins to change between 45 and 50 °C, which would be expected because the sequence 27–34 contains Met-29 in the gauche,gauche form. Transformation of some of the Met-29 to trans,gauche or trans,trans with increasing temperature would produce the observed decrease at 657 cm^{-1}.

Stage IV (50–60 °C). Substantial changes in the Raman spectrum occur in this range. An increase in the amide III intensity indicates that about half of all residues converted to intermediate conformation at 75 °C have been converted by 60 °C. Quantitatively this means that some 20 new intermediate residues are formed between 30 and 60 °C, so that about 10–12 residues are converted in stage IV. Burgess and Scheraga (1975) postulated that segments 51–60 and 104–124 unfold in this range. The first contains six residues of α-helical geometry and the second nine residues of β conformation. From the spectrum the apparent decrease in α and β residues is more nearly 5 and 7 but our method of estimation (R. C. Lord, results to be published) is probably no more reliable than this. Therefore, the change in the amide III contour is in approximate quantitative agreement with the proposed segmental unfolding for this stage.

In this range the intensity ratio of the Tyr doublet changes enough to implicate one of the three buried tyrosines. The Raman spectrum cannot say which, but there is much other evidence (Bigelow, 1961; Scott and Scheraga, 1963; Rupley and Scheraga, 1963; Li et al., 1966) that Tyr-25 and Tyr-97 are "exposed" only with great difficulty, leading to the conclusion that Tyr-92 must go from strong to weak H bonding at this stage. The S–S frequency has completed about half of its total change (Figure 6a), presumably because of alteration of the geometries of cystine bridges 28–84 and 58–110. The C–S frequency at 657 cm^{-1} is also half-way to completion of its total change, which is rather surprising since three of the four methionines (29, 30, and 79) should have been affected. This probably means that the cystine C–S vibrations are also contributing somewhat at 657 cm^{-1} in the spectrum of the partially unfolded protein.

A large change in the binding of sulfate ions also occurs between 50 and 60 °C, although more than half of the bound ions is still held at the higher temperature. If it is presumed that sulfate ion is bound to His-12 and His-119 (Kartha et al., 1967, 1968; Wyckoff et al., 1970) this would imply that these residues begin to unfold at this stage. For the latter, at least, the NMR evidence (Benz and Roberts, 1975; Roberts and Benz, 1973) shows that its unfolding is mainly above 60 °C.

Stage V (55–65 °C). In this range the postulated event is the unfolding of the first 16 residues of the N-terminal part of the sequence. These include eight or nine residues of helical geometry. Substantial differences appear in the amide III contour in the range 60–65 °C, and qualitatively the maximum at the α-helical peak (1265 cm^{-1}) seems to broaden more than the β-conformation peak at 1240 cm^{-1}. The change in shape suggests that the residues with new geometry formed by the unfolding of this segment possess ψ angles that are borderline in value to the α-helical range. When the Raman spectrum of the S-protein (RNase A minus the first 20 residues) is examined as a lyophilized powder (M. C. Chen, unpublished results), the shoulder at 1265 cm^{-1} is noticeably reduced in intensity whereas that at 1240 cm^{-1} has the contour shown in the 60 °C spectrum. This supports the interpretation of the change in the amide III region from 60 to 65 °C as showing a slight loss in α-helical geometry.

Other observations in this range include the near completion of the S–S changes, which shows that all S–S bridges except one (probably 65–72) have undergone considerable alteration from their native geometry. However, as stated earlier, this geometry is still all in the gauche,gauche,gauche form. The intensity ratio of the Tyr doublet shows the unbonding of one Tyr over this temperature range, which is probably Tyr-25, since it is bonded in the native form to Asp-14.

Stage VI (60–70 °C). The remaining segments that might unfold in this stage are 35–50, 62–74, and 81–102. Burgess and Scheraga (1975) suggest, however, that some of these retain their native conformation even at 70 °C in solutions of pH 6.8. The amide III region, which still shows considerable β structure at 70 °C and pH 5, is consistent with this suggestion. The segments 62–74, 81–87, and 96–102, whose backbone geometries are approximately those expected for β conformations, may be responsible for this. However, it must also be noted that there is evidence that a number of residues (about 10–12) of near α-helical geometry also persist at 70 °C. We cannot say where these are and perhaps they represent simply the number of unfolded residues that happen to achieve values of the ϕ,ψ angles appropriate to the α helix.

The third strongly bonded tyrosine, presumed to be Tyr-97,

is still not entirely exposed even at 75 °C, as shown by the intensity ratio of the tyrosine doublet (Figure 5b) (compare Bigelow, 1964). Finally the S–S and C–S lines at 516 and 657 cm^{-1} have very nearly reached level values, indicating that the unfolding is nearly complete at 70 °C so far as the reversible process is concerned (Figure 6).

General Comments on Raman Spectroscopic Results in the Context of the Unfolding Scheme. A number of comments about the thermal unfolding of RNase A at pH 5 can be made in the light of the preceding detailed discussion. One is that while the unfolding may proceed in more or less well-defined stages, these are not directly apparent in the temperature dependence of the Raman spectrum. The overall impression is that of a rather smooth change from the native to the unfolded structure. When several quite different residues are used as probes to follow the course of the change, closely similar temperature dependences and values of T_m are found (for example, compare the intensity ratio of the tyrosine doublet in Figure 5b with the disulfide frequency in Figure 6a). The actual value of T_m from the various Raman lines is very near to 62 °C, in agreement with a large number of other kinds of studies.

It should be apparent that it is difficult or impossible to monitor the behavior of a given individual residue by the Raman effect when more than one of that kind are present. Hence one could not elaborate a scheme of unfolding like that of Burgess and Scheraga (1975) on the basis of Raman data alone. However, their proposal has quantitative implications, as discussed above, for the temperature dependence of the conformations of various residues and these implications can be checked by the Raman effect. While Raman spectra at present cannot do more than show that a proposed scheme is or is not consistent with the data, at least a direct conflict between the spectra and the proposal would cast serious doubt on the latter.

We have shown above that the detailed course of unfolding postulated by Burgess and Scheraga (1975) shows no conflict with the Raman data except for the unfolding of tyrosines. Possibly their scheme proposes the occurrence of certain events at slightly lower temperature than is indicated by the spectra, but this point is not easy to nail down because the overlapping of the various stages makes quantitative assessment difficult. Also, we have used solutions in which the effective concentration of sulfate ion in 7% RNase A is about 0.03 M as determined spectroscopically (~5 SO_4^{2-}/RNase A) and this amount of sulfate may be sufficient to retard the unfolding slightly. Finally, no buffer was used in the solutions and the pH might vary somewhat at higher temperatures from the value of 5 measured at 32 °C. In any case the T_m measured from Figures 4–6 of about 62 °C is what would be expected from other work in this pH range.

Finally, a comment of the reversibility of the unfolding needs to be made. So far as the Raman effect is concerned, the unfolding by temperature is reversible provided the temperature does not exceed about 70 °C for a long period of time (1 h or more). This can be shown from the curves of Figures 4–6, which can be repeated quantitatively with increasing or decreasing temperature. If the sample is held for several hours at 75 °C, the curves obtained on cooling do not return to the original native plateau at room temperature but show hysteresis as the result of substantial irreversible denaturation. The departure of the cooling curve from the heating curve is a measure of the extent of permanent damage to the enzyme. This is of special interest with the line at 982 cm^{-1} due to sulfate ion, which shows clearly that irreversible denaturation destroys the ability of the enzyme to bind sulfate tightly.

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Erythrocyte Spectrin. Purification in Deoxycholate and Preliminary Characterization†

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ABSTRACT: Erythrocyte spectrin, isolated by aqueous extraction of erythrocyte ghosts, may be freed from contaminating membrane lipids and small amounts of other proteins by gel chromatography in 5 or 10 mM deoxycholate. The purified protein, in deoxycholate, is a mixture of monomers and dimers, both highly asymmetric molecules. The hydrodynamic properties of the dimer closely resemble those of muscle myosin, and spectrin and myosin also have similar circular dichroism spectra. The proportion of dimer to monomer in the purified protein varies from one preparation to another, an observation for which there is no simple explanation. In the absence of deoxycholate, spectrin associates beyond the dimer stage, possibly by loose end-to-end aggregation involving hydrophobic forces.

The term "spectrin" was originally coined (Marchesi and Steers, 1968) to designate the protein that is readily solubilized from erythrocyte ghosts by dilute EDTA[1] or other aqueous media in which the concentration of inorganic cations is kept very low. Polypeptide chain analysis by sodium dodecyl sulfate gel electrophoresis shows that the principal constituents of this material appear as two bands corresponding to apparent molecular weights of about 220 000 and 200 000 (Trayer et al., 1971), but numerous other polypeptide components are present as well, in amounts depending on the precise conditions of solubilization (Reynolds and Trayer, 1971). More recent usage is to employ the term "spectrin" to refer exclusively to the two major electrophoretic bands (Fairbanks et al., 1971), and this is the definition adopted in this paper, with the understanding that this does not necessarily imply the presence of only two kinds of chemically distinct polypeptide chains. An alternative name for the same protein is "tektin A" (Clarke, 1971). This name derives from the solubilization procedure described by Mazia and Ruby (1968), which is less selective than the aqueous extraction method.

There is strong evidence suggesting that the proteins soluble in aqueous media play a central role in the maintenance of the shape of the erythrocyte membrane (Rosenthal et al., 1970; Steck, 1974; Singer, 1974), and that they may serve as a site of attachment for trans-membrane proteins, limiting their freedom of motion (Nicolson and Painter, 1973; Elgsaeter and Branton, 1974; Pinto da Silva and Nicolson, 1974). It is not known, however, whether this structural function is ascribable

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[1] Abbreviations used are: DOC, sodium deoxycholate; EDTA, ethylenediaminetetraacetic acid.