Structural Studies of Ribonuclease. XIII. Physicochemical Properties of Tryptic Modifications of Ribonuclease*

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Ribonuclease can be digested by trypsin at 60° to produce two intermediates which were previously fractionated on an IRC-50 column. One component (component IV) was a mixture of two species, each of which had one peptide bond split, either lys 31–ser 32 or arg 33–asp(NH_2) 34, respectively; the other (component III) had both of these bonds split and was, therefore, missing the ser 32–arg 33 dipeptide. In this paper the results of physicochemical studies of components III and IV are reported, both components being similar to native ribonuclease A. The helical content of both derivatives, estimated from b_0 values obtained from optical rotatory dispersion measurements, is 21%, slightly less than that of ribonuclease A. Each of these components contains three abnormal tyrosines, on the basis of spectrophotometric titration data; however, these tyrosines can be exposed at a somewhat lower *p*H than those of ribonuclease A. A significant difference was found in the lower thermal transition temperature, around 40–45° near neutral *p*H, compared to around 60° for ribonuclease A. These results indicate that cleavages of the peptide bonds cited produce little change in conformation, presumably primarily in the region of the bonds which are split; however, the slight loosening of the structure leads to a loss of about 75% of the enzymic activity. Further digestion of component IV at *room temperature* leads to, among others, component 5, which is missing the hexapeptide ser 32–lys 37, and component 1, which is missing this hexapeptide and also the peptides lys 1–arg 10 and thr 99–lys 104. Component 5 also has three abnormal tyrosines; however component 1 has only one abnormal tyrosine.

In a previous paper (Ooi *et al.*, 1963) and in the accompanying paper (Ooi and Scheraga, 1964) it was reported that high-molecular-weight intermediates could be obtained by tryptic digestion of ribonuclease, followed by fractionation on an IRC-50 column. Components IV (a mixture of two species, each of which had one peptide bond split, either lys 31–ser 32 or arg 33–asp(NH_2) 34, respectively) and III (a species with both of these peptide bonds split, i.e., missing the ser 32–arg 33 dipeptide) were obtained by digestion at 60°. Further digestion of component IV at *room temperature* led to several components, of which two are considered here: component 5, which is missing the hexapeptide ser 32–lys 37, and component 1, which is missing this hexapeptide and also the peptides lys 1–arg 10 and thr 99–lys 104. Physicochemical studies were carried out on components IV, III, 5, and 1, and the results were compared to those for native ribonuclease A. It is thus possible to obtain information about the parent molecule from studies of the derivatives in which the positions of the peptide-bond cleavages are known.

Optical rotatory measurements were used to obtain crude estimates of helical content; these were supplemented by studies of thermal denaturation in order to determine the ease of unfolding of the various components.

Since the changes in optical rotation parallel those obtained by ultraviolet difference spectral measurements, as the temperature of ribonuclease A (Hermans and Scheraga, 1961a) and these derivatives (this paper) is raised, the more convenient technique of ultraviolet difference spectra was employed in the thermal denaturation study.

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Native ribonuclease has six tyrosyl residues, three of which titrate abnormally (Tanford *et al.*, 1955), presumably because they are involved in interactions with other amino acid residues of the molecule (Hermans and Scheraga, 1961a,b). Therefore it is of interest to learn whether or not these abnormal tyrosines are normalized in each derivative. Such information would be helpful in determining the folding in various regions of the molecule.

Experimental

Materials.—The derivatives studied (components IV, III, 5 and 1) are those described in previous papers (Ooi *et al.*, 1963; Ooi and Scheraga, 1964). All chemicals were analytical grade where possible.

Measurements.—The *p*H was measured with a Beckman Model GS *p*H meter. Optical rotation measurements were made with a Rudolph Model 80Q precision spectropolarimeter, equipped with an oscillating polarizer. The quartz cell had a diameter of 0.5 cm and a length of 10 cm, and had a jacket through which water was circulated in order to control its temperature. A water-cooled high-pressure General Electric AH-6 mercury arc source provided light of wavelengths (in mμ) 313, 336, 405, 436, 546, and 579.

Ultraviolet difference spectral measurements were carried out with a Cary Model 14 recording spectrophotometer. The temperatures of the measuring and reference cells, respectively, could be separately controlled. The difference spectrum was measured at 286 mμ from 4 to 60°. The reference cell, at 4–6°, and the measuring cell, at a temperature which was varied, were both filled with the same solution. Thermal equilibrium was usually attained upon standing for 20–30 minutes.

Spectrophotometric titrations were carried out at a wavelength of 295 mμ, using a Beckman Model DU spectrophotometer. The *p*H was changed by successive additions of small amounts of a KOH solution.

The buffer used in the optical rotation and ultraviolet difference spectral measurements was 0.01 M sodium citrate, with 0.15 M KCl.

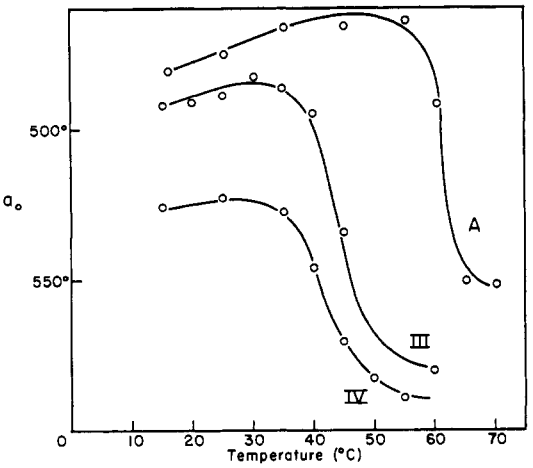

FIG. 1.—Temperature dependence of a_0 for ribonuclease A and components IV and III at *p*H 6.3 in 0.15 M KCl. The concentrations in all cases were 4.0–4.5 mg/ml.

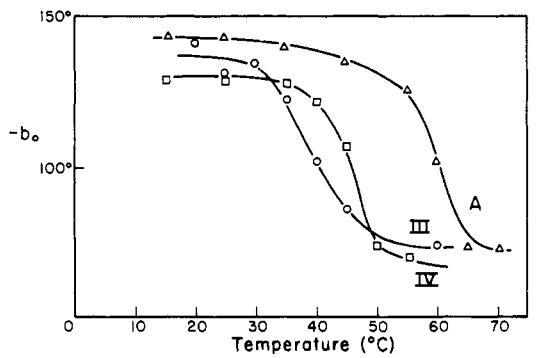

FIG. 2.—Temperature dependence of b_0 for ribonuclease A and components IV and III at *p*H 6.3 in 0.15 M KCl. The concentrations in all cases were 4.0–4.5 mg/ml.

RESULTS

Components IV and III.—The optical rotatory dispersion data may be expressed by the Moffitt (1956) equation:

$$\frac{M}{100}\frac{3}{n^2+2}[\alpha] = \frac{a_0\lambda^2_0}{\lambda^2-\lambda^2_0} + \frac{b_0\lambda^4_0}{(\lambda^2-\lambda^2_0)^2}$$

where λ is the measured wavelength, λ_0 is taken as 212 mμ, n is the refractive index of the solution, M is the average molecular weight of a residue, $[\alpha]$ is the specific rotation, and a_0 and b_0 are constants, the latter being used to estimate the per cent helical content of the molecule (Urnes and Doty, 1961). Plots of $(M/100)\,[3/(n^2+2)][\alpha](\lambda^2-\lambda^2_0)/\lambda^2_0$ versus $\lambda^2_0/(\lambda^2-\lambda^2_0)$ for ribonuclease A and components IV and III at *p*H 6.3 are straight lines at every temperature, the slope and intercept of each line giving b_0 and a_0, respectively. The temperature dependence of a_0 and b_0 are summarized in Figures 1 and 2. The changes of a_0 and b_0 with temperature indicate the existence of a thermal transition in all three proteins, the transition temperatures for components IV and III being lower than that for ribonuclease A. The transition temperatures, estimated from Figure 2, are 40° and 45° for components III and IV, respectively. Recognizing the lack of theoretical justification, we may calculate the following values for the per cent helical content from b_0: 22%, 21%, and 21% for ribonuclease A, component IV, and component III, respectively, at room temperature. At high temperature, the value decreases to about 11%. Since b_0 for ribonuclease A becomes zero in 8 M urea (Jirgensons, 1963), it appears that 11% of a hard core of unfolded protein remains at elevated temperature in the absence of urea.

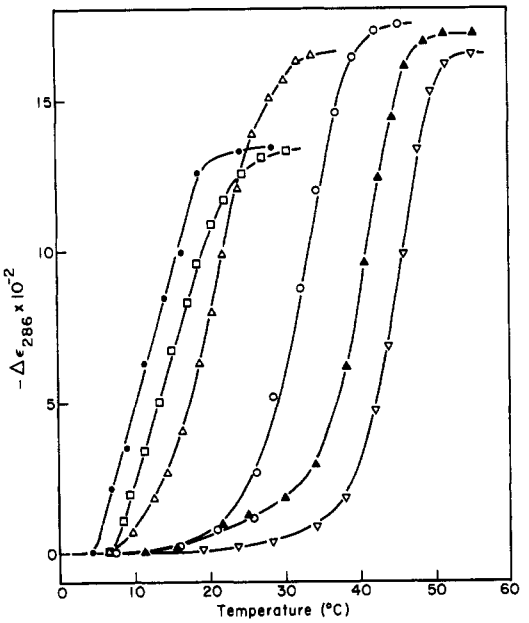

FIG. 3.—Temperature dependence of the molar ultraviolet difference spectrum of component IV at 286 mμ. The reference is the same solution (1.17 mg/ml) at the same *p*H at low temperature. ▽, *p*H 6.52; ▲, *p*H 4.15; O, *p*H 3.51; △, *p*H 2.81; □, *p*H 2.00; ●, *p*H 1.60.

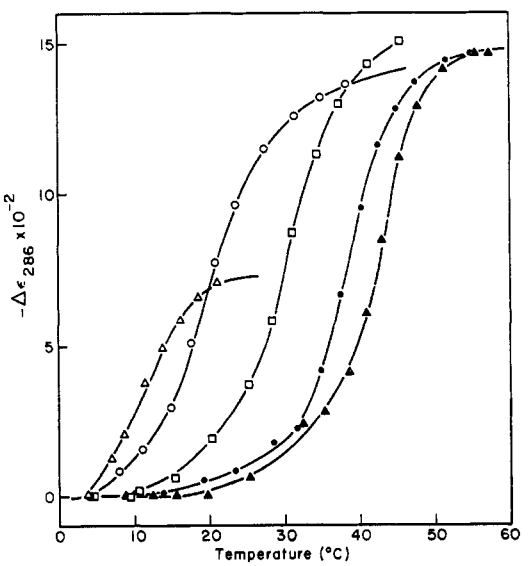

FIG. 4.—Temperature dependence of the molar ultraviolet difference spectrum of component III at 286 mμ. The reference is the same solution (0.38 mg/ml) at the same *p*H at low temperature. ▲, *p*H 5.42; ●, *p*H 4.28; □, *p*H 3.48; O, *p*H 2.70; △, *p*H 1.23.

Since components IV and III undergo a thermal transition, as does ribonuclease A, these components appear to retain most of the native structure of ribonuclease A. However, since they have a lower transition temperature, their structure must be somewhat less stable than that of ribonuclease A; this is consistent with the reduced enzymic activity of these components compared to ribonuclease A (Ooi *et al.*, 1963).

In order to obtain more details about the thermal transition in these components, the temperature de-

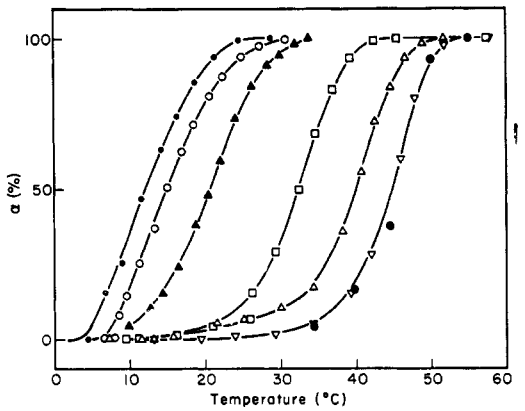

FIG. 5.—Temperature dependence of α for component IV, computed from the data of Fig. 3. The large solid circles on the *p*H 6.52 curve were obtained from b_0 data; all other data are from ultraviolet difference spectra measurements.

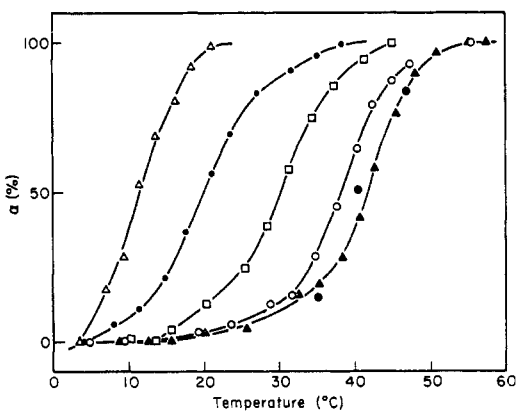

FIG. 6.—Temperature dependence of α for component III, computed from the data of Fig. 4. The large solid circles on the *p*H 5.42 curve were obtained from b_0 data; all other data are from ultraviolet difference spectra measurements.

pendence of the ultraviolet difference spectra was measured, and the results are shown in Figures 3 and 4. In both cases the transition temperature decreases with decreasing *p*H, the same behavior previously observed for ribonuclease A (Hermans and Scheraga, 1961a). The molar difference extinction coefficients at 286 mμ of components IV and III above around *p*H 2 are 1700 and 1500, respectively. However these values are lower below *p*H 2, as was also observed for ribonuclease A (Hermans and Scheraga, 1961a). From these data it seems very likely that the behavior of the abnormal tyrosines of components IV and III is the same as that of ribonuclease A (also see spectrophotometric titrations below). As was done previously (Hermans and Scheraga, 1961a), the data of Figures 3 and 4 were converted to values of α, the per cent unfolded (see Figs. 5 and 6). The ultraviolet difference spectra data reflect a change in the environment of tyrosyl residues caused by a change in conformation. When the per cent unfolding, obtained from the b_0 values, is plotted against temperature, the points lie on the same curve as do those from the spectral measurements (see Figs. 5 and 6), indicating that the tyrosyl residues of components IV and III are exposed as the molecules are unfolding, as found for ribonuclease A (Hermans and Scheraga, 1961a).

Recently it was shown that curves such as those of Figures 5 and 6 may be regarded as the summation of

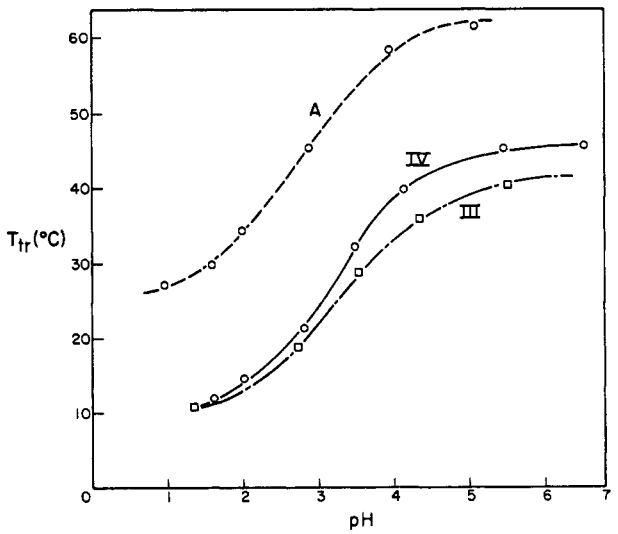

FIG. 7.—*p*H dependence of average transition temperature, T_{tr}, for ribonuclease A and components IV and III.

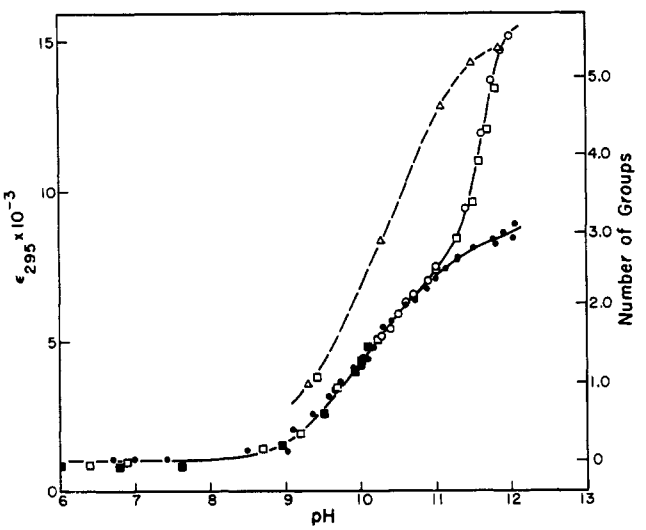

FIG. 8.—Spectrophotometric titrations at room temperature. □ and ○, independent runs on component IV (0.79 mg/ml); △, back titration of component IV from *p*H 11.9; ●, ribonuclease A (0.70 mg/ml).

two independent transitions in each molecule (Scott and Scheraga, 1963). When the curves of Figures 5 and 6 are treated in this manner, and Van't Hoff plots are constructed, the following values are obtained for the enthalpy change in denaturation: 58 kcal and 117 kcal for component IV, and 41 kcal and 69 kcal for component III. These may be compared to the values 60 kcal and 126 kcal obtained for ribonuclease A. These values are independent[1] of *p*H. The ΔH data support the above conclusion that components IV and III and ribonuclease A have similar conformations, but that successive splitting of these specific peptide bonds loosens the structure a little.

[1] These data may, at first sight, appear to be at variance with those of Scott and Scheraga (1963). However, Scott and Scheraga made their measurements at the maximum wavelength, which varied with temperature, while we have followed the procedure of Hermans and Scheraga (1961a,b) in using the same wavelength of 286 mμ at each temperature. While it is difficult to decide which procedure is valid, all our samples were studied by the same procedure; therefore the comparison between samples is made on a consistent basis.

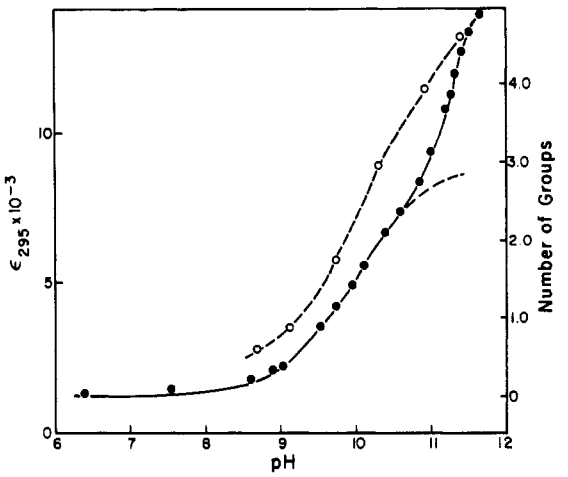

FIG. 9.—Spectrophotometric titrations at room temperature. ●, component III (0.57 mg/ml); O, back titration of component III.

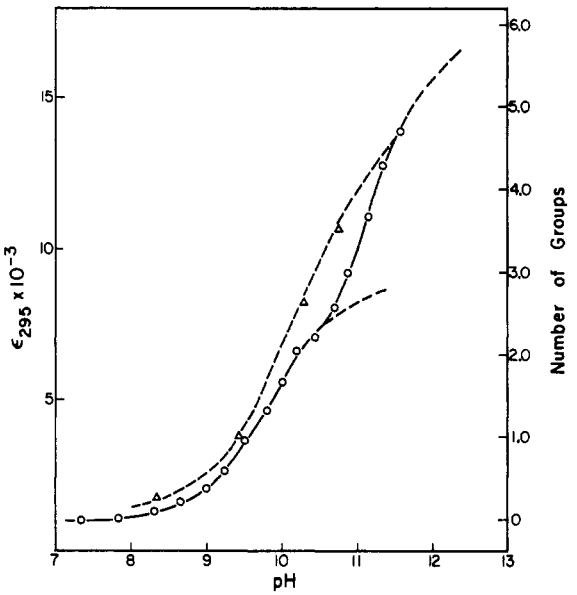

FIG. 10.—Spectrophotometric titrations at room temperature of component 5 (0.91 mg/ml). O, forward titration; △, back titration.

Another way of treating the data of Figures 5 and 6 is to assign an average transition temperature at $\alpha = 50\%$, without decomposing the curves into two independent transitions. The *p*H dependence of such transition temperatures is shown in Figure 7. All three substances show a similar dependence of T_{tr} on *p*H, indicating that the interaction between tyrosyl and carboxyl groups in a hydrophobic region in ribonuclease A (Hermans and Scheraga, 1961a,b) is unchanged by the splits of certain specific peptide bonds of components IV and III. The lower transition temperatures reflect the general loosening of the structure, to which reference has already been made.

Spectrophotometric titrations of components IV and III confirm that three abnormal tyrosines are present in these molecules, as in ribonuclease A; i.e., the *reversible* portion of the titration curves (from neutral *p*H to around *p*H 10.5) corresponds to three groups (see Figs. 8 and 9). This is in agreement with the observation (Ooi and Scheraga, 1964) that components IV and III cannot be digested by chymotrypsin at room temperature; i.e., the buried tyrosines of these components are inaccessible to chymotryptic

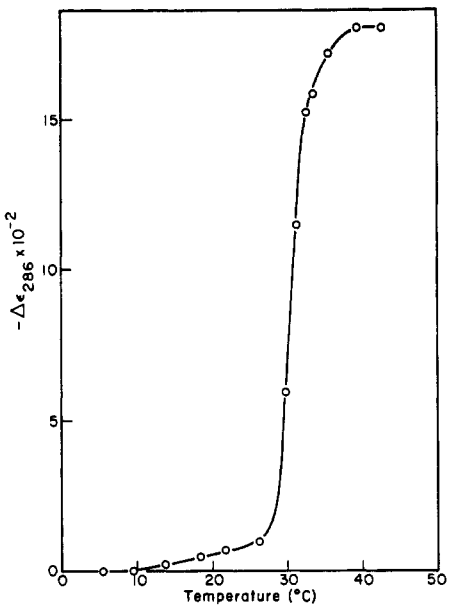

FIG. 11.—Temperature dependence of the molar ultraviolet difference spectrum of component 5 at 286 mμ at *p*H 6.3. The reference is the same solution (0.66 mg/ml), at the same *p*H, at lower temperature.

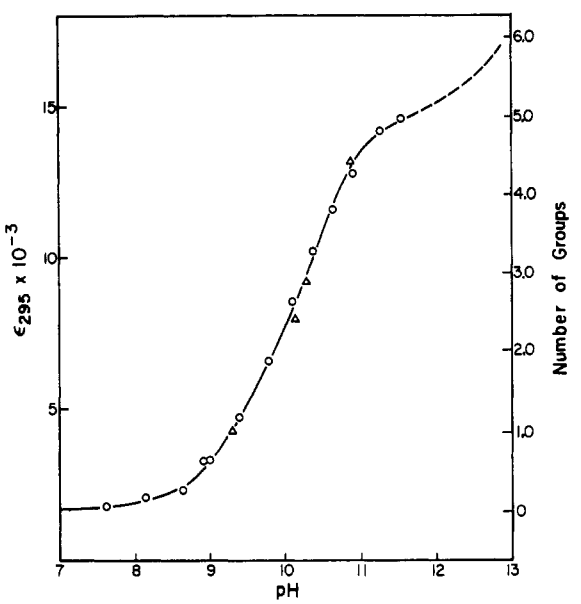

FIG. 12.—Spectrophotometric titrations at room temperature of component 1 (0.71 mg/ml). O, forward titration; △, back titration. It can be seen that the titration is a reversible one.

attack, as are the buried tyrosines of native ribonuclease A. Once these three abnormal tyrosines are normalized at high *p*H, they are not titrated reversibly, i.e., back titration of components IV and III from *p*H 11–12, where the abnormal tyrosines of *ribonuclease A* are still buried, gives optical densities higher than those observed in the forward titration. The *p*H where normalization begins to occur is lower for components IV and III than for ribonuclease A; the values are *p*H 11.3 and 11 for components IV and III, respectively, compared to a value greater than 12 for ribonuclease A.

Summarizing these results, we may conclude that the conformations of components IV and III are similar to that of ribonuclease A except for some loosening due to the splitting of peptide bonds between

cys 26 and cys 40; this loosening leads to a loss of about 75% in the enzymic activity (Ooi *et al.*, 1963).

Components 5 and 1.—Component 5, which is missing the ser 32–lys 37 hexapeptide, also has three abnormal tyrosines, as deduced from the spectrophotometric titration curve shown in Figure 10. These tyrosines are normalized above *p*H 10.7, a value which is lower than that observed for components IV and III. Once these tyrosines are normalized at alkaline *p*H the back titration is irreversible.

The temperature dependence of the ultraviolet difference spectrum of component 5 at *p*H 6.3 is shown in Figure 11. While this curve appears to be of the type observed for the other components studied here, it was not reversible, in contrast to the reversibility observed for the other components. Above 30° there was a large decrease in optical density, with a long time being required to obtain constant readings. Upon cooling, hysteresis was noted; these results indicate that there is an irreversible change of conformation in the thermal transition which leads to normalization of the tyrosines of component 5. This irreversibility is also consistent with the change in the spectral maximum from 278 mμ before heating to 275 mμ after cooling.

The experiments with component 5 indicate that removal of the ser 32–lys 37 hexapeptide produces little change in conformation, but that the structure is a bit looser than that of ribonuclease A at room temperature. This is consistent with the observed loss of about 90% in the enzymic activity (Ooi and Scheraga, 1964).

Spectrophotometric titration data on component 1, which is missing not only the ser 32–lys 37 hexapeptide but also the peptides lys 1–arg 10 and thr 99–lys 104, indicate that five tyrosines titrate reversibly with an apparently normal *pK*, and one tyrosine is still abnormal (see Fig. 12). Therefore the removal of the extra sixteen amino acids from component 5 causes drastic changes in the conformation of the molecule. Since this component is missing part of the N-terminal tail and has a different conformation from ribonuclease A, it is not surprising that it has no enzymic activity (Ooi and Scheraga, 1964). Thus, while components IV, III, and 5 are similar in conformation to ribonuclease A, and thus have some enzymic activity, the changes brought about by trypsin in the formation of component 1 are so drastic that enzymic activity is lost.

Discussion

The results with components IV and III show that these molecules have essentially the same conformation as ribonuclease A, with a slightly looser structure, as indicated by the lower transition temperatures. The looseness between cys 26 and cys 40 in component IV permits it to be attacked by trypsin at room temperature, whereas native ribonuclease A had to be heated above its transition temperature (60°) in order to be digested by trypsin. The conformation of component 5 is similar, but even looser. The observed enzymic activities of these components is consistent with this interpretation. Presumably, the side chains of the amino acid residues in the ser 32–lys 37 hexapeptide interact with the rest of the molecule, in ribonuclease A, to give it full activity.[2] The splitting of peptide bonds in this hexapeptide disrupts these side-chain interactions somewhat, without producing significant changes in conformation. However the small changes produced are sufficient to change the spatial relationships of the groups in the active site and thereby reduce the enzymic activity. Furthermore, since component 5 is *irreversibly* denatured upon heating, the hexapeptide must play a role in stabilizing the native conformation of ribonuclease A.

From the observations with component 1, in which two of the three abnormal tyrosines are normalized, it appears that the N-terminal tail plays some role in the interactions leading to the abnormality of these two normalized tyrosines. This conclusion is compatible with the observation of Richards and Vithayathil (1960) that S-protein (which contains no N-terminal tail) has only one abnormal tyrosine. In other words, the N-terminal tail is essential for maintaining the native conformation of ribonuclease A.

Two of the abnormal tyrosines are residues 25 and and 97 (Cha and Scheraga, 1963). The one abnormal tyrosine of component 1 is probably not residue 25 since chymotrypsin can remove the peptide from lys 1 to tyr 25 at 60° to leave a product which still contains one abnormal tyrosine[3] (Rupley and Scheraga, 1963). This abnormal tyrosine is probably buried in the hard core whose existence is inferred from the optical rotatory measurements. It is not yet possible to state whether this tyrosine is residue 97 or a third, as yet unidentified, tyrosine.

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[2] Since component 5, which lacks the hexapeptide, still contains three abnormal tyrosine residues, no tyrosine can be involved in the interactions between the hexapeptide and the rest of the molecule.

[3] If residue 25 is normal in component 1, this derivative should be digestible by chymotrypsin at room temperature. Unfortunately, a sufficient amount of component 1 was not available to carry out this experiment.