Helix–Coil Transition of the Isolated Amino Terminus of Ribonuclease*

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ABSTRACT: The helical nature of a peptide comprising the first 13 residues of ribonuclease A is demonstrated by circular dichroism and low ultraviolet absorption spectroscopy. Low temperature, increasing ionic strength, and organic solvents such as trifluoroethanol, methanol, and ethylene glycol favor the helical state. Guanidine hydrochloride and chymotryptic cleavage between residues 8 and 9 favor the coil state. Concentration dependence of ellipticity at 224 nm coupled with sedimentation equilibrium studies showed that the peptide aggregated in ion-free aqueous solution at 1° at higher concentrations, and that this aggregation favors helicity. At an ionic strength μ 0.1 no such aggregation occurs in this concentration range.

This study of a relatively short peptide demonstrates the contribution of short-range interactions to the initiation of folding.

There has recently been an increased interest in the conformational properties of peptides corresponding in amino acid sequence to portions of well-characterized proteins (Shearer *et al.*, 1966; Crumpton and Small, 1967; Scatturin *et al.*, 1967; Epand and Scheraga, 1968; Klee, 1968; Crumpton, 1968; Bodanszky *et al.*, 1969; Brown and Klee, 1969).

Besides the intrinsic interest of such work, these studies are also being carried out in the hope of contributing to the elucidation of the mechanism by which proteins assume their characteristic conformations. The peptides studied to date have a much higher degree of flexibility than the proteins from which they are derived and are, therefore, subject to conformational analysis only with much more difficulty. In our own work, it has been found that the influence of conformational fluctuations can be minimized by working at low temperatures in aqueous media of suitably high ionic strengths, and it has been possible in this manner to deduce conformational characteristics of some peptides of chain lengths in the range 10–20 residues (Brown and Klee, 1969). In this manner, the influence of short-range interactions, perhaps of the type discussed by Kotelchuck and Scheraga (1968), was found to be of major importance in the folding of pancreatic ribonuclease.

We found, in our studies of a series of overlapping peptides from the amino-terminal region of ribonuclease, that those peptides which include the helical segment found in the intact protein (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967, 1970) exhibit a tendency to assume partially helical conformations in solution. This helical tendency was found to be unaffected by the presence of additional, nonhelical regions in some of the peptides studied, but did require a minimum chain length. In these earlier studies, a peptide comprising the first 13 amino acid residues of ribonuclease, peptide 1–13 or, alternatively, C-peptide, was the smallest fragment which could be induced to fold into a helical type of conformation in aqueous solutions. It seemed important to study the conformational transitions of this small and relatively simple peptide in detail since the system is an interesting model for the protein folding process. It would also be useful to be able to characterize the nature of the folded state of this molecule with more certainty. We have accordingly examined in some depth the influence of temperature, solvent, and the state of aggregation on the ultraviolet absorption and circular dichroism of peptide 1–13 and report the results of these studies here.

Materials and Methods

Peptide 1–13 (C-peptide) was prepared by a modification of the cyanogen bromide procedure of Gross and Witkop (1962). In a typical preparation, 504 mg of bovine pancreatic ribonuclease A (Sigma Type XII A) was added to 50.4 ml of 70% formic acid containing 1.25 g of cyanogen bromide (Eastman Organic Chemicals, White Label). This solution, which has a molar ratio of ribonuclease methionine residues: CNBr of 1:80, was stirred slowly in a closed flask at room temperature for 24 hr. The reaction mixture was then diluted with 5–6 volumes of ion-free water, shell frozen, and lyophilized. C-peptide was separated from C-protein by two passages through a Sephadex G-25 (coarse) column, 118 × 6.5 cm, using 0.2 M acetic acid as solvent. Some slight further purification could be effected by a passage through a 1 × 30 cm Sephadex G-10 column using the same solvent.

Amino acid analysis of the lyophilized product, kindly performed for us by Dr. Alan Neims using the automated procedure of Spackman *et al.* (1958), gave the following results, with the theoretical values in parenthesis: lysine 2.17 (2), histidine 0.98 (1), arginine 0.83 (1), threonine 0.70 (1), glutamic acid 3.10 (3), alanine 3.10 (3), phenylalanine 0.85 (1), aspartic acid 0.03 (0), serine 0.01 (0), glycine 0.01 (0), and homoserine plus homoserine lactone 0.52 (1). No other peaks were observed on the chromatograms. Paper electrophoresis of the product at pH 8.5 shows two ninhydrin-positive spots, the faster of which is converted into the slower (less basic) on brief exposure of the peptide to 0.1 N NaOH. Parks *et al.* (1963) have ascribed this behavior of the peptide to the fact that residue 13 exists as an equilibrium mixture of homoserine and its lactone (Armstrong, 1949). Exposure

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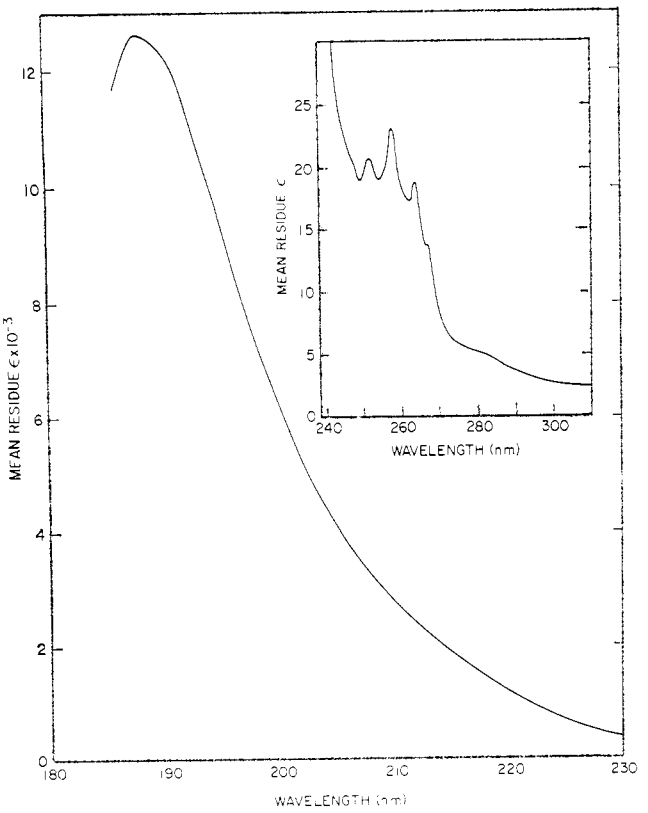

FIGURE 1: Ultraviolet absorption spectrum of peptide 1–13 in water at room temperature. The peptide concentration was 0.126 mg/ml, and the path length was 0.1 cm. The insert shows the near ultraviolet spectrum of the peptide taken at a concentration of 2.51 mg/ml, and a path length of 1.0 cm.

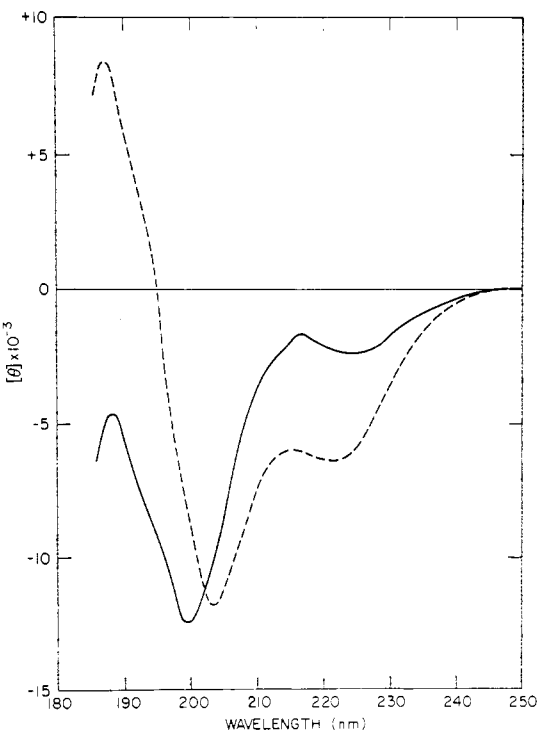

FIGURE 2: Circular dichroism spectra of peptide 1–13 in 0.033 M Na_2SO_4 at 26° (—) and 1° (---). Peptide concentration at 1° was 0.254 mg/ml. Peptide concentration at 26° was 0.254 mg/ml from 186 to 215 nm and 0.507 mg/ml from 215 to 250 nm. Path length was 0.02 cm.

to alkali converts this material quantitatively into the open form. The peptide samples used in the studies described below have been maintained at pH 6 or below in order to eliminate complications due to the potential reactivity of the lactone in basic solution.

The ultraviolet spectrum of the peptide is shown in Figure 1. In the far-ultraviolet range the spectrum takes the form of a relatively featureless broad band with a maximum near 188 nm. This peak, due primarily to the amide chromophore, is characteristic of polypeptides (Imahari and Tanaka, 1959; Rosenbeck and Doty, 1961). The near-ultraviolet spectrum (insert to Figure 1) shows the multibanded pattern which is expected of phenylalanine derivatives. There is, of course, no absorption band near 275 nm due to tyrosine. The origin of the shoulder observed above 280 nm is not clear. A similar shoulder is observed in the spectra of a number of similar peptides from ribonuclease comprising residues 1–8, 1–15, and 1–20 (Richards and Logue, 1962; Klee, 1968). Peptide concentrations were routinely determined by absorption measurements at 258 nm. A molar extinction coefficient at this wavelength of 299 has been determined for peptide 1–13 by dry weight analysis. This result was confirmed by nitrogen and amino acid analysis as well.

The peptide was routinely dissolved in deionized water or in unbuffered salt solutions. The measured pH of these solutions was in the range of 5–6.

Ultraviolet absorption measurements were performed with a Cary 14 spectrophotometer. Temperature was controlled by circulating fluid from a constant-temperature bath through the walls of a hollow brass cell compartment. Sample temperature was measured inside the cell by means of a Yellow Springs Instrument Company thermocouple with an estimated accuracy of ±0.2°. For the low-wavelength measurements the instrument was purged with dry nitrogen.

Circular dichroism was measured with the Model 6001 attachment to the Cary 60 spectropolarimeter. Temperature was controlled and measured in the same manner as for the ultraviolet absorption measurements. Also the same quartz cells of path length varying from 0.02 to 5.0 cm were employed in both kinds of measurement. The slit program of the instrument was adjusted so as to maintain a spectral band pass of 1.5 nm. Samples and solvent were run at the same slow scanning rate in the same cells using a 10-sec pen period. Duplicate scans were routinely performed and they superimposed well on one another. Mean residue ellipticity, $[\theta]$, was calculated in the usual manner (Beychok, 1967), using a mean residue weight of 115; the units are deg cm^2 per dmole of amino acid residue.

Molecular weight determinations were carried out by the high-speed sedimentation equilibrium technique of Yphantis (1964). The instrument used was a Spinco Model E ultracentrifuge equipped with the photoelectric scanning device (Schachman and Edelstein, 1966). The wavelength observed was generally 265 nm and initial sample concentrations were of the order of 1 mg/ml. The partial specific volume of the peptide was estimated from its amino acid composition to be 0.72 (Cohn and Edsall, 1965). In those cases where the plot of natural log concentration *vs.* r^2 (r = radius) showed curvature, the molecular weight of the heavy material was estimated by subtracting the extrapolated contribution of the monomeric component from the original trace using the trapezoidal correction method (Yphantis, 1964).

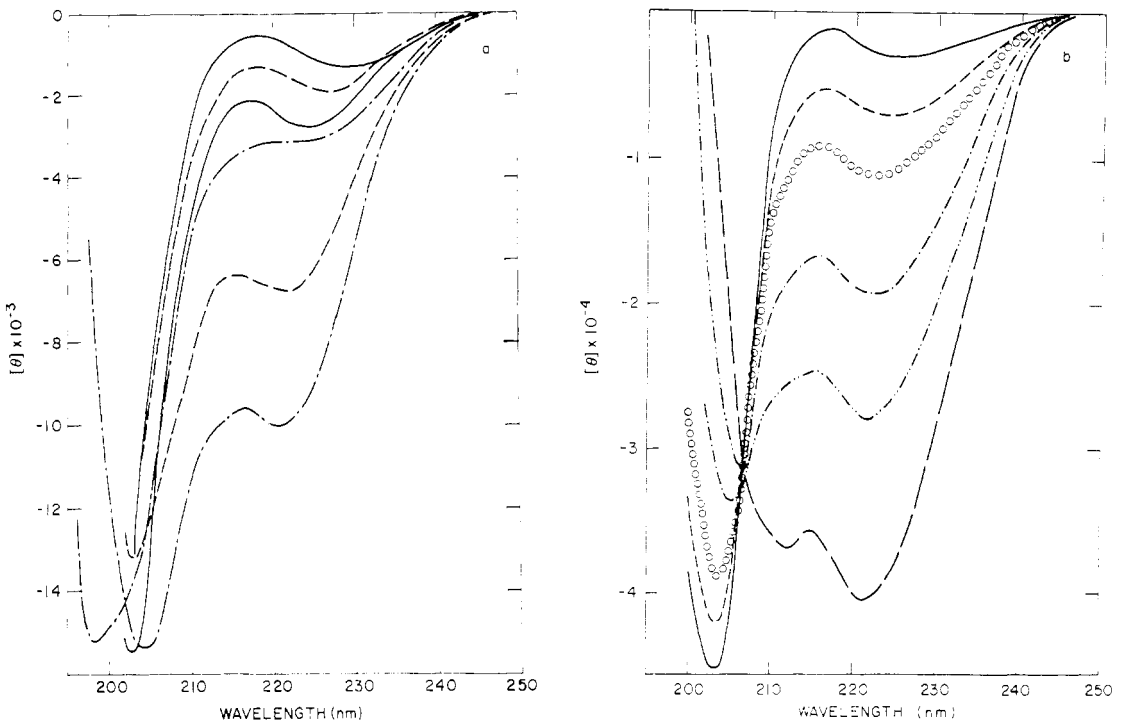

FIGURE 3: Circular dichroism spectra. (a) Circular dichroism spectra of peptide 1–13 at 26 and 1° in water (—), 0.033 M Na_2SO_4 (---), and 0.33 M Na_2SO_4 (— · —). In water, the peptide concentration was 0.0411 mg/ml, and the path length was 1.0 cm. In Na_2SO_4 (0.033 M) the peptide concentration was 0.0405 mg/ml and the path length was 1.0 cm. In Na_2SO_4 (0.33 M) the peptide concentration was 0.174 mg/ml, and the path length was 0.1 cm. In each case, the upper curve is at 26° and the lower curve is at 1°. (b) Calculated circular dichroism spectra for various proportions of α helix and random coil derived from the data of Holzwarth and Doty (1965) for helical and random poly-L-glutamic acid. Curves for 10% (—), 20% (---), 30% (OOO), 50% (– · –), 70% (– · · –), and 100% (— —) α helix are shown.

Results

In our study of the conformational transitions of peptide 1–13 the primary analytical technique has been the measurement of circular dichroism spectra. It may be seen in Figure 2 that the peptide undergoes a large conformational alteration on changing the temperature of a dilute aqueous salt solution from 26 to 1°. The change in the form of the circular dichroism curve on lowering the temperature is that to be expected on increasing the mean helicity of a polypeptide (Holzwarth and Doty, 1965). Thus the magnitude of this ellipticity near 222 cm increases by a factor of 3 and the large peak which is near 200 nm at ambient temperatures is shifted appreciably towards the 208-nm position found in fully helical polypeptides.

The conformation of peptide 1–13 is highly dependent

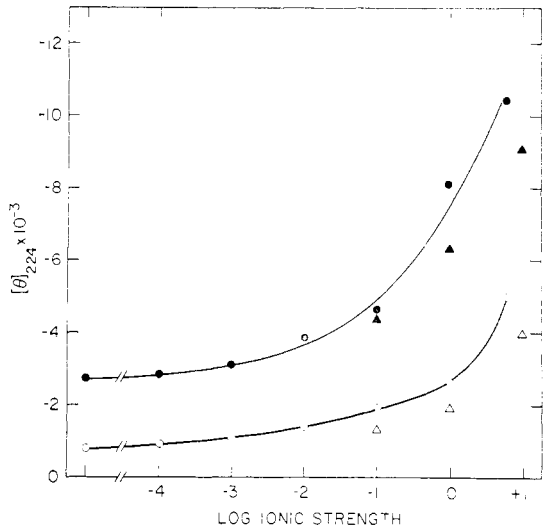

FIGURE 4: The effect of ionic strength on the ellipticity of peptide 1–13 at 224 nm at 26° (open symbols) and 1° (closed symbols) in either Na_2SO_4 (circles) or $(NH_4)_2SO_4$ (triangles). The peptide concentration was 0.0125 mg/ml in Na_2SO_4 and 0.0120 mg/ml in $(NH_4)_2SO_4$. The path length was 2.5 cm.

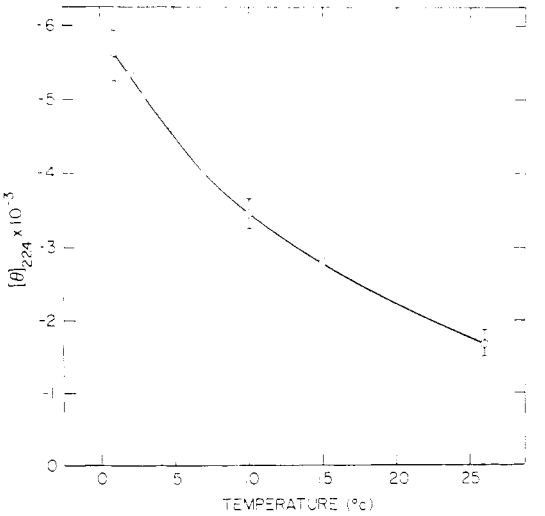

FIGURE 5: The effect of temperature on the mean residue ellipticity of peptide 1–13 at 224 nm in 0.033 M Na_2SO_4. The peptide concentration was 0.738 mg/ml. The path length was 0.1 cm.

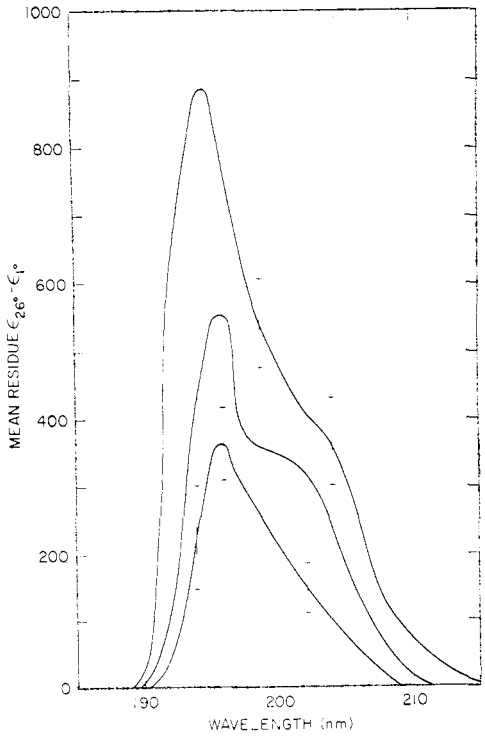

FIGURE 6: Calculated difference in the far-ultraviolet absorption of peptide 1–13 on going from 26 to 1° in water (lower curve), 0.033 M Na_2SO_4 (middle curve), and 0.33 M Na_2SO_4 (upper curve). Each curve represents the average of two measurements, each on different peptide solutions. For the lower curve, the peptide concentration of each solution was 0.126 mg/ml. For the middle curve, the peptide concentrations were 0.106 and 0.109 mg per ml and for the upper curve, they were 0.082 and 0.494 mg per ml. The path length was 0.1 cm for all measurements except that made on the solution of 0.494 mg/ml, where it was 0.02 cm. The error bars represent the range of experimental values.

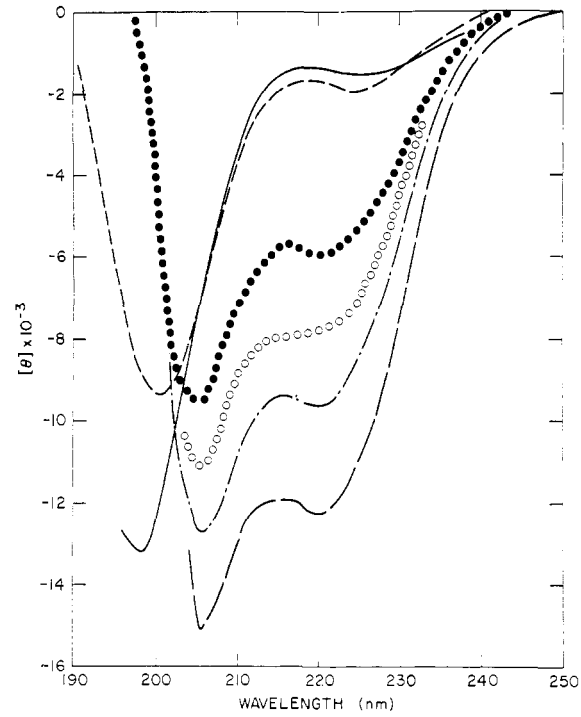

FIGURE 7: The effect of 2,2,2-trifluoroethanol on the circular dichroism of peptide 1–13 at 26°. Curves representing peptide in water (—), 10% (--), 30% (●●●), 50% (○○○), 70% (-·-), and 90% (— —) trifluoroethanol are shown. Peptide concentration was 0.141 mg/ml. Path length was 0.1 cm.

on the ionic strength of the solution, as is also true for some larger peptides (Brown and Klee, 1969). This result is documented in detail in Figures 3a and 4. Figure 3a shows the circular dichroism curves of peptide 1–13 at 26° and 1° and at each of three different values of added ionic strength. The family of curves so generated shows the general trend of increased ellipticity near 222 nm with both increasing ionic strength and decreasing temperature. Thus, at 26°, the ellipticity near 222 nm increased progressively as the ionic strength of the medium is increased from 0 to 0.1 to 1 M, and a similar, but larger, change is seen at 1°. Figure 4 summarizes a great many more experiments in which the ellipticity at 224 nm was measured as a function of ionic strength at the two different temperatures. The lines have been drawn to fit the points obtained with sodium sulfate as the neutral salt (circles). They are parallel to one another at low ionic strengths but diverge at ionic strengths of 0.1 M and above as we have already seen. Note that ammonium sulfate (triangles) gives similar but not identical results, and also that there is no indication of a leveling off of the salt effect even at very high ionic strengths.

It is instructive to compare the experimentally determined curves of Figure 3a with the set of calculated curves shown in Figure 3b. The calculated curves are the result of the appropriately weighted averaging of the circular dichroism curves of helical and random poly-L-glutamic acid taken from the data of Holzwarth and Doty (1965). The curves represent the expected circular dichroism of molecules which are helical to the extent of 10–100%, the remainder being random coil. The calculated curves bear a strong qualitative resemblance to the experimental curves of Figure 3a although the two sets of curves differ by a factor of about 3 in the ordinate scale.[1]

The temperature dependence of the conformation of peptide 1–13 can only be studied over a limited range in aqueous solvents. We have found that raising the temperature to values well above 26° has very little effect on the ellipticity of the peptide. The temperature dependence of ellipticity in the accessible range below 26° is shown in Figure 5. The data shown refer to an ionic strength of 0.1; qualitatively similar results were obtained at both lower and higher ionic strengths. There is no evidence of a leveling off at the lowest accessible temperatures. Experiments performed in methanol–water and ethylene glycol–water mixtures allowed us to make measurements at temperatures as low as −13°. Even in these cases, however, although the mean residue ellipticity reached values of −15,000 at 224 nm there was no leveling off of the effect.

It has been demonstrated that the helical conformation of polyamino acids and proteins induces a marked hypochromicity of the amide absorption band near 190 nm (Rosenbeck and Doty, 1961). We have been able to observe

[1] The absence of a clear isosbestic point in Figure 3a might be taken to imply that structures other than random coil and α helix are present. It should be pointed out however that the data below 210 nm are subject to relatively large error and the curves are steep enough in this region that the presence of an isosbestic point cannot be ruled out at this time. It is also possible that end effects or solvation changes with temperature or salt concentration are playing a role.

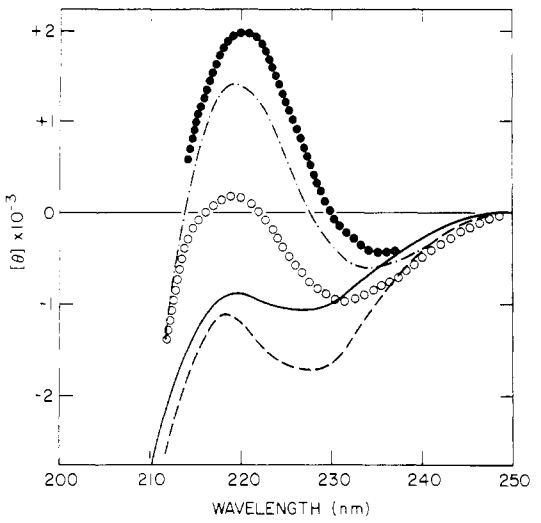

FIGURE 8: The effect of guanidine hydrochloride on the circular dichroism of peptide 1–13 at 26°. The curves represent water (—), 1 M (---), 3 M (OOO), 5 M (–··–), and 8 M guanidine hydrochloride (●●●). Peptide concentration varied from 0.316 to 0.421 mg per ml. Path length was 0.1 cm.

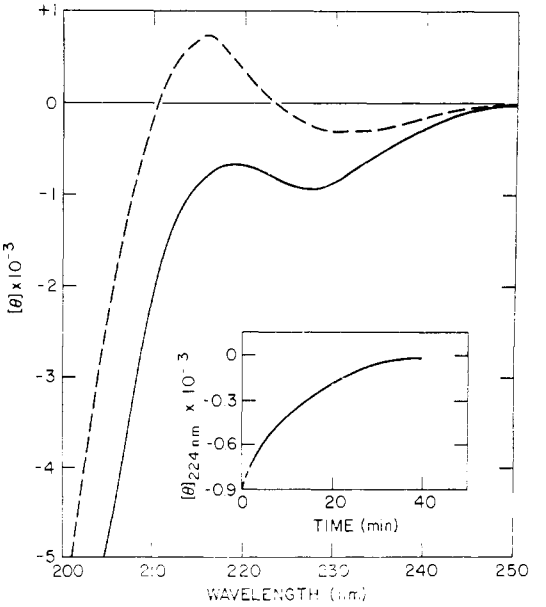

FIGURE 9: The effect of chymotryptic hydrolysis of the Phe-8–Glu-9 bond on the circular dichroism of peptide 1–13 at 26°. The curves are before (—) and 40 min after (---) exposure to chymotrypsin. Peptide concentration was 0.50 mg/ml in 0.05 M potasium phosphate, pH 7.4. Path length was 0.1 cm. The insert shows the time course of the chymotrypsin reaction as measured by the change in ellipticity at 224 nm. At time 0, chymotrypsin was added to the peptide solution described above at a concentration of 0.0013 mg/ml.

a similar hypochromic effect on the absorption spectrum of peptide 1–13 when its helicity is increased on lowering of the temperature. Figure 6 shows the difference in absorptivity of the peptide between 26° and 1° at solvent ionic strengths of 0, 0.1, and 1 M. The data show that the peptide bond absorption decreases on lowering the temperature by an amount which is proportional to the ionic strength of the medium, as would be predicted by the circular dichroism data discussed previously. The extent of the hypochromicity at the highest ionic strength is about 25% of that observed by Rosenbeck and Doty for the helix–coil transitions of poly-L-glutamic acid and poly-L-lysine. Interestingly, the ellipticity of peptide 1–13 at the same ionic strength and temperature (Figure 3a) is also about one-fourth that of helical polyglutamic acid. Thus, the two independent types of optical measurement give essentially identical results.

It has been demonstrated that trifluoroethanol is a solvent which promotes the acquisition of helical structures in polypeptides (Goodman *et al.*, 1963). Peptide 1–13 is no exception as may be seen in Figure 7. The figure shows the circular dichroism spectrum of the peptide in mixtures of trifluoroethanol containing up to 93% by volume of the organic solvent. The curves are of interest because of their similarity to Figure 3a and may be taken as evidence in favor of our interpretation of the circular dichroism spectra of the peptide in aqueous salt solutions.

Although the circular dichroism spectrum of the peptide does not change markedly on raising the temperature above 26°, there are many reasons to believe that even at ambient temperatures its conformation is not that of a random coil. The generally accepted characteristics of the circular dichroism spectra of randomly coiled polypeptides are as follows: (a) a weak positive band near 218 nm, (b) a strong negative band near 200 nm, and (c) a very weak negative band near 235 nm. The circular dichroism spectrum of peptide 1–13 at 26° (Figure 2) is clearly very different. Furthermore, addition of guanidine hydrochloride to the peptide results in large changes in ellipticity at this temperature and at high concentrations of this denaturant (5–8 M), the circular dichroism spectrum of the peptide shows the characteristics expected of a random coil (Figure 8). A very similar change in the circular dichroism of the peptide can be effected by the chymotrypsin-catalyzed hydrolysis of the peptide bond between phenylalanine at position 8 and glutamic acid at position 9. Figure 9 shows the circular dichroism of pattern of native and chymotrypsin-digested peptide. The similarity of the curve of the fragmented material to that of the unfolded peptide in guanidine hydrochloride is clear. The insert to Figure 9 shows that the ellipticity at 224 nm can be used to follow the time course of the proteolysis.

It is important in interpreting the results of conformational studies to ascertain the state of aggregation of the material under investigation. This is particularly true of peptides of intermediate chain length, many of which display marked tendencies to aggregate in aqueous solutions (Epand and Scheraga, 1968). We have, therefore, been careful to assure ourselves that the results presented earlier refer to monomeric peptide. That this is true is demonstrated in the following series of experiments in which we show that although the peptide can form polymeric aggregates, it does so only at concentrations which are appreciably higher than those used in our optical studies.

The first indication that the peptide can undergo aggregation emerged from a study of the concentration dependence of its ellipticity at 224 nm. Figure 10 shows that there is no concentration dependence of ellipticity at 0.1 ionic strength at either low or moderate temperatures up to a peptide concentration of at least 0.7 mg of peptide per ml. Thus the data obtained at this ionic strength almost certainly pertain to the monomeric peptide, a conclusion which is confirmed by the ultracentrifugal analysis presented below. The data obtained in salt-free water, on the other hand, tell a different story. In the absence of added salt, and at low temperature, the ellipticity of the peptide rises to a plateau value at con-

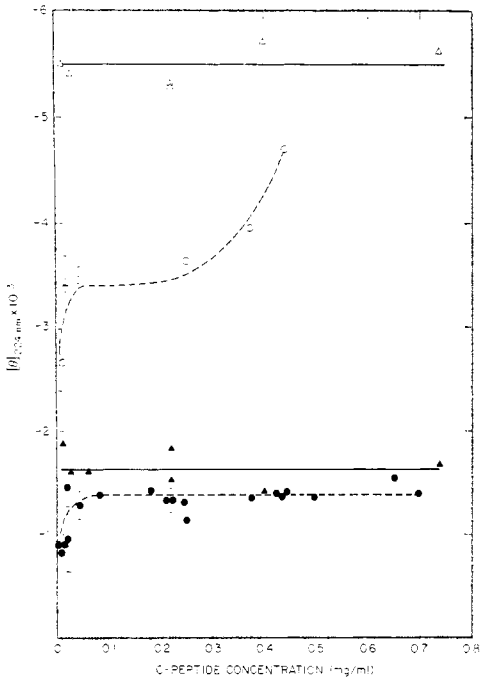

FIGURE 10: The concentration dependence of the mean residue ellipticity of peptide 1–13 at 224 nm at 26° (lower 2 curves) and 1° (upper 2 curves) in water (circles) and Na_2SO_4 (0.033 M) (triangles). Path lengths ranged from 0.1 to 5.0 cm.

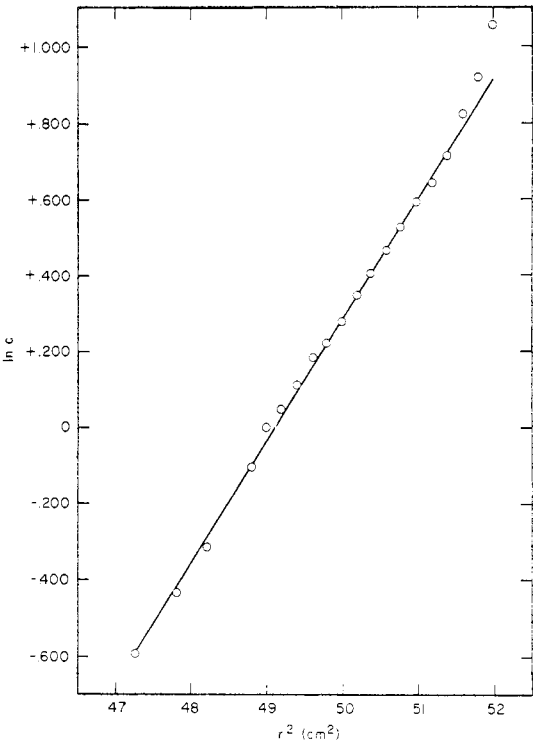

FIGURE 11: Sedimentation equilibrium of peptide 1–13 in 0.033 M Na_2SO_4 at 26°. A sample of 1.25 mg/ml had been maintained at 60,000 rpm for 20 hr. The cell was scanned at 265 nm. The logarithm of peptide concentration in centimeters of the chart is shown as a function of the square of the distance from the center of rotation (in square centimeters). The molecular weight calculated from this data is 1430. The theoretical molecular weight is approximately 1500.

centrations near 0.05 mg/ml and then starts to increase again at concentrations above 0.2 mg/ml. At 26°, the second increase is not observed. The two different effects of concentration observed most likely are (a) a primary salt effect at the lowest concentrations in which the peptide itself along with its counterions is supplying a significant ionic strength to the medium, and (b) aggregation at the higher concentrations and lower temperature.

Sedimentation equilibrium studies allow a direct determination of the particle weight of the peptide as a function of its concentration. These studies have all been carried out in 0.1 M ionic strength solutions. At temperatures near 26° there is no evidence for aggregation of the peptide at concentrations up to at least 4 mg/ml. Thus the data in Figure 11 show the presence of a single component of mol wt 1430 up to very high peptide concentrations at 26°. At 1° (Figure 12) the peptide is still monomeric (mol wt 1500) at concentrations up to 2–3 mg/ml but does form what is apparently a large aggregate of molceular weight somewhere between 10,000 and 30,000 at higher concentrations. Figure 12 shows that the ultracentrifugal data at 1° can be analyzed into two straight lines whose slopes correspond to monomer and to high polymer. Thus it is clear that the optical experiments described in Figures 1–9 refer to the monomeric state of the peptide since they were performed at concentrations below that necessary for aggregation to occur.

Discussion

In our earlier studies we concluded that peptide 1–13 is the smallest of our series of fragments of the amino terminus which tends to assume the helical structure found in that region of crystalline ribonuclease (Brown and Klee, 1969). This conclusion is considerably strengthened as a result of the studies reported here which show in more detail that the optical properties of the peptide at low temperature and moderate ionic strength are those which have characteristically been ascribed to α helical structures. The peptide contains all of the amino acid residues which make up the short segment of α helix found at the amino terminus of the molecule (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967) and the ease with which it may be induced to take up a helical structure is of great interest with regard to the problem of the mechanism of protein folding.

The data presented here show that the peptide may be induced to exhibit a fairly high degree of helicity simply by supplying an ionic strength of 0.1 M or greater. It is a reasonable supposition that the effect of added salts is simply to neutralize electrostatic interactions within this highly charged peptide. Similar observations on the sometimes dramatic effects of neutral salts on peptide conformation have been made by Craig (1967) on the basis of membrane diffusion measurements. McDiarmid and Doty (1966) have made such observations with polyamino acid models.

The helical state of peptide 1–13 does not have the stability associated with helical structures in native proteins. Clearly, as has been pointed out before, long-range interactions among distant segments of the polypeptide chain are necessary to stabilize short helices in proteins (Epand and Scheraga, 1968). The point which we wish to emphasize, however, is that structures of only marginal stability under physiological conditions, such as the helix in peptide 1–13, can serve to direct the folding process of polypeptide chains into efficient and productive channels.

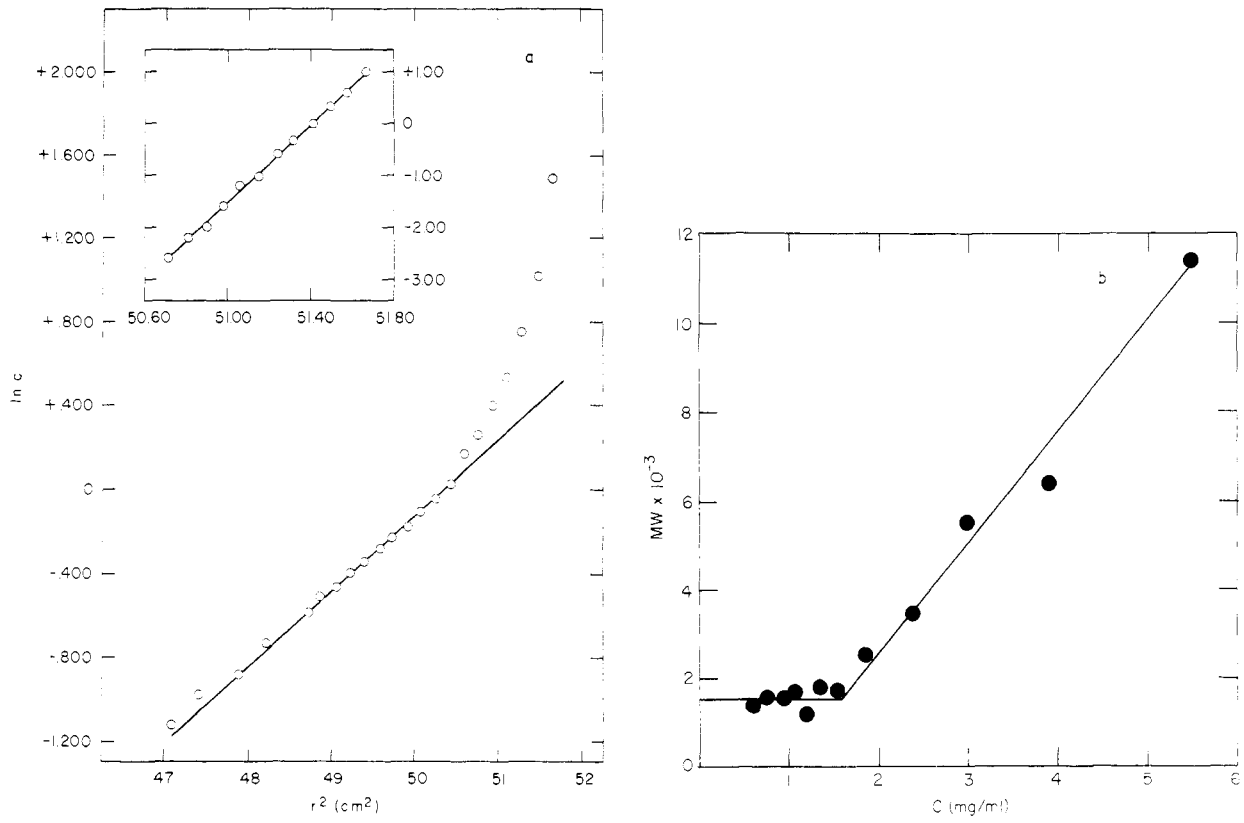

FIGURE 12: (a) Sedimentation equilibrium of peptide 1–13 in 0.033 M Na_2SO_4 at 1°. The sample is the same as the one described in the legend to Figure 11. The insert shows the calculated distribution of the high molecular weight aggregate near the cell bottom. The molecular weight of the monomer is 1500 in this experiment and that of the aggregate is approximately 16,000. (b) The relationship between the concentration of the peptide and the weight-average molecular weight, calculated from the data shown in a.

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