Conformational Studies of a Series of Overlapping Peptides from Ribonuclease and Their Relationship to the Protein Structure*

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ABSTRACT: Overlapping peptides comprising amino acid residues 1–8, 1–13 (C-peptide), 1–15, and 1–20 (S-peptide) from the amino-terminal region of ribonuclease A were isolated. Circular dichroism spectra in ion-free water and dilute Na_2SO_4 at 26 and 1° showed that the three longer peptides are partially helical and that the helicity increased moderately in water at low temperatures but markedly in salt at low temperatures.

Residues 2–12 are known to be helical in the X-ray crystal structure of ribonuclease. Molar ellipticities of the three longer peptides at 224 nm in 0.033 M Na_2SO_4 were essentially the same at low temperature, thus supporting the concept that the 1–12 region tends to be helical in the isolated peptide as in the intact protein. The 14–20 region is not helical. Such a series of overlapping peptides constitutes a model system for the study of the initiation of folding by short-range interactions.

The conformation of a protein is believed to be a state of minimum energy which is determined uniquely by the linear sequence of its constituent amino acid residues (Anfinsen, 1961; Lumry and Eyring, 1954). The number of pathways a protein chain might take to reach this state of minimum energy must be limited because so little time is needed (Levinthal, 1967). Levinthal has proposed that there exist short-range interactions in some regions of the protein sequence which lead to highly probable conformations in those regions. These structured foci could then direct the general folding process. Phillips (1967) has advanced the similar hypothesis that proteins may start to assume their three-dimensional structure during biosynthesis. Thus, the amino-terminal end might be highly folded before completion of the rest of the chain.

We have attempted to approach these problems experimentally by examination of the conformational properties of a series of peptides derived from the amino-terminal region of ribonuclease. This region is known to contain a segment of α helix in the intact molecule (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967) which encompasses residues 2–12. Earlier work with S-peptide, residues 1–20 (Klee, 1968), has shown that this segment of the ribonuclease molecule can be partially helical in solution. The present studies extend this earlier work to three smaller fragments of this region and to other, more favorable, experimental conditions for demonstrating helicity. The circular dichroism spectra of peptides comprising residues 1–13, 1–15, and 1–20 in dilute salt solution show that each of these peptides has an appreciable helical character. A shorter fragment (residues 1–8) does not exhibit this behavior.

Materials and Methods

The peptides studied were all obtained from bovine pancreatic ribonuclease A (Sigma type XIIA). Peptide 1–20 (S-peptide) was made by a modification (Klee, 1965) of the procedure of Richards and Vithayathil (1959). Peptide 1–15 was prepared from S-peptide by degradation with carboxypeptidase A as described by Potts *et al.* (1963). Peptide 1–13 (C-peptide) was produced from ribonuclease A by a modification of the CNBr cleavage procedure of Gross and Witkop (1962). The methionine 13 is replaced by homoserine or its lactone in this peptide. Peptide 1–8 was a product of the chymotryptic lysis of peptide 1–13 (Marzotto *et al.*, 1964). The peptides were purified by two passages through Sephadex G-25 (118 × 6.4 cm) and sometimes by another 1–2 passages through Sephadex G-10 (31 × 1 cm). The solvent was 0.2 M acetic acid and the peptides were recovered as dry powders by lyophilization. Circular dichroism spectra were obtained with a Cary 6001 attachment to the Cary 60 spectropolarimeter as previously described (Klee, 1968). The estimated errors of our measurements are illustrated on one curve of Figure 3. Mean residue ellipticities were calculated using the relationship $\theta/10 \times MRW/lc$ where θ is the ellipticity in degrees, MRW is the mean residue weight, l is the pathlength in centimeters, and c is the concentration in grams per milliliter. Path lengths were 0.1–1.0 cm.

The solutions in water or dilute neutral salt were unbuffered; their pH was 5.5 ± 0.5. Low temperatures were obtained using a Lauda K-2/R bath supplemented by a Neslab cooling bar to circulate fluid through a hollow brass-walled cell holder. Temperature of the sample was measured to ±0.2° with a Yellow Springs Instrument Co. telethermometer. Concentrations of peptide were calculated using an extinction coefficient of 299 for the phenylalanine peak at 258 nm as determined by Kjeldahl nitrogen analysis of C-peptide. This figure compares well with values of 290 and 320 obtained from dry weights of S-peptide (Klee, 1968; Richards and Logue, 1962). Identity and purity of each peptide were confirmed by quantitative amino acid analysis. The ultraviolet spectra of all the peptides were similar to that of S-peptide (Richards and Logue, 1962).

Results

Figure 1 shows that the circular dichroism spectrum of S-

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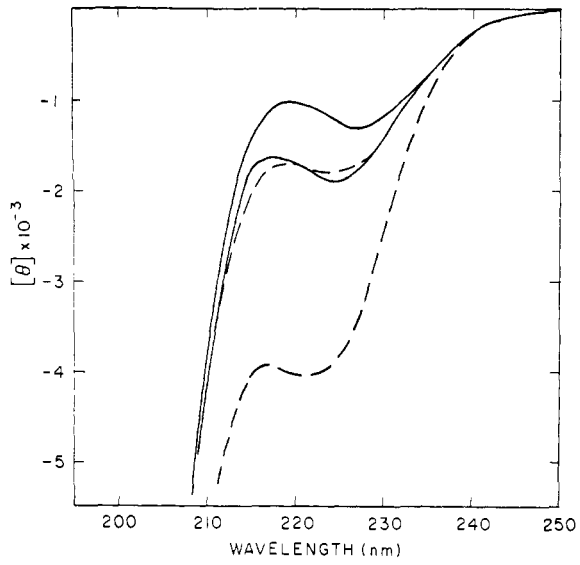

FIGURE 1: Circular dichroism of peptide 1–20 (S-peptide) at 26 and 1° in ion-free water (—) and in 0.033 M Na_2SO_4 (– – –). Peptide concentration was 0.516 mg/ml in water and 0.508 mg/ml in Na_2SO_4. Path length was 0.1 cm. In each case the upper curve is at 26° and the lower curve, 1°.

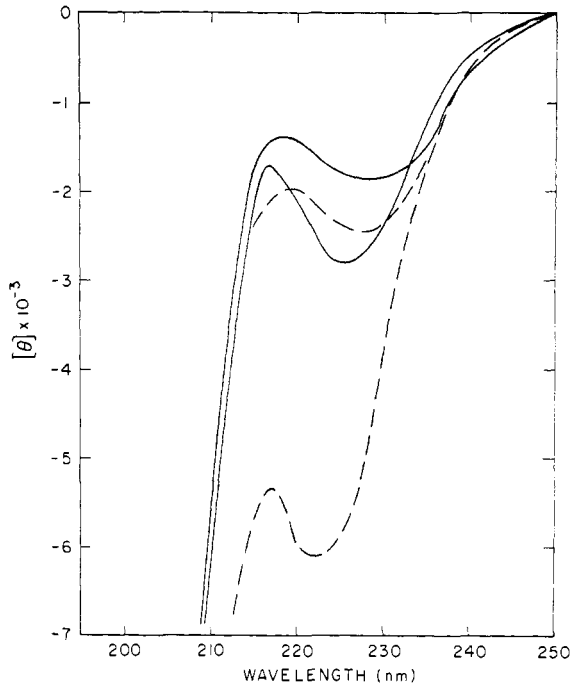

FIGURE 2: Circular dichroism of peptide 1–15 at 26 and 1° in ion-free water (—) and in 0.033 M Na_2SO_4 (– – –). Peptide concentration was 0.261 mg/ml in water and 0.257 mg/ml in Na_2SO_4. Path length was 0.1 cm. In each case the upper curve is at 26° and the lower curve, 1°.

peptide in ion-free water at 26 and 1° is similar to those obtained by Scatturin *et al.* (1967), Klee (1968), Tamburro *et al.* (1968), and Simons and Blout (1968), in solvents of low ionic strength. The negative peak near 225 nm is considered to be due to the $n-\pi^*$ transitions of amide groups perturbed by the α-helical conformation (Holzwarth and Doty, 1965). At low temperature, a modest increase in ellipticity occurs indicating an augmentation in the amount of helix. Figures 2 and 3 show that peptides 1–15 and 1–13, respectively, behave in an analogous fashion in water.

The figures also show that in 0.033 M Na_2SO_4 (μ = 0.1) these peptides increase somewhat in ellipticity at 26°, but that at 1°, the increase is much more marked. The salt effect can probably be ascribed to the suppression of charge interactions (Craig, 1967) which might hinder helix formation. Note also that in Figure 3 the large negative peak at 199–200 nm in salt shifts to 203–204 nm with low temperature. This shift is another characteristic occurrence during changes from random to helical conformation (Holzwarth and Doty, 1965) and occurs in all three peptides.[1] Figure 4 shows the change in circular dichroism on cooling of peptide 1–13, as measured by direct subtraction of the two curves. The calculated difference curve has the same shape and sign as the circular dichroism curve of helical poly-L-glutamic acid (Holzwarth and Doty, 1965), thus corroborating our interpretation of these curves. Qualitatively identical difference curves have been calculated for peptides 1–15 and 1–20. The decreased random coil contribution to

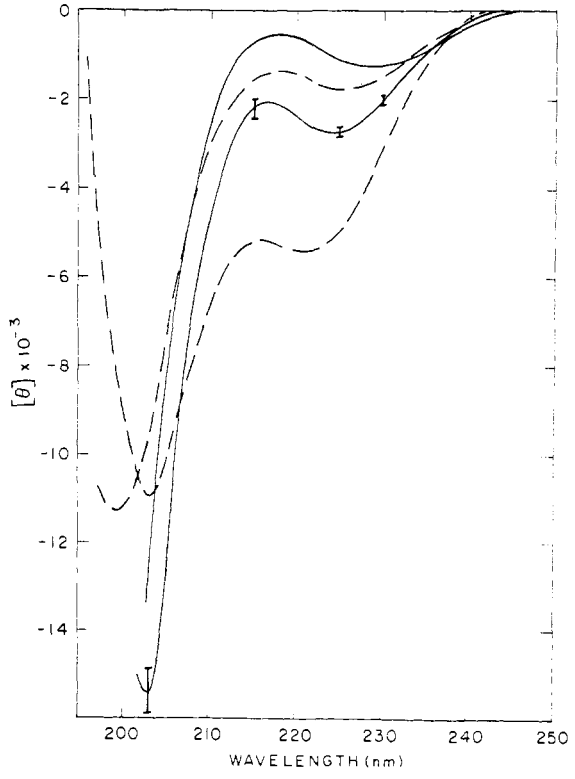

FIGURE 3: Circular dichroism of peptide 1–13 (C-peptide) at 26 and 1° in ion-free water (—) and in 0.033 M Na_2SO_4 (– – –). Peptide concentration was 0.0612 mg/ml in water (path length = 1.0 cm) and 0.221 mg/ml in Na_2SO_4 (path length = 0.1 cm). In each case the upper curve is at 26° and the lower curve, 1°.

[1] The circular dichroism spectra of peptides 1–13 and 1–20 are converted to that of a random coil (see Figure 5) in the presence of high concentrations of guanidine hydrochloride or after chymotrypsin hydrolysis of the peptide bond between residues 8 and 9. Conversely, it is possible to increase the helicity of these peptides by further raising the ionic strength, by adding methanol or trifluoroethanol to the solvent, and by further decreasing the temperature. In no case has it yet been possible to exceed a mean residue ellipticity of about 15,000 at 224 nm. Peptide 1–15 has not been studied under these conditions, however.

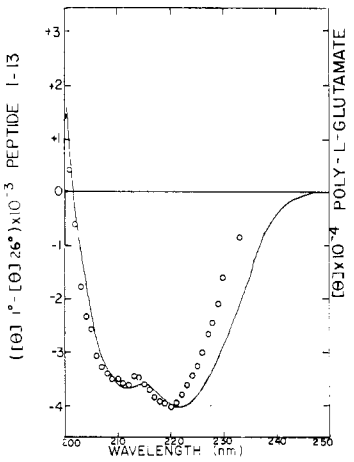

FIGURE 4: Calculated difference circular dichroism curve of peptide 1-13 on going from 26 to 1° (open circles). The solid line is the circular dichroism spectrum of helical poly-L-glutamic acid taken from Holzwarth and Doty (1965).

these spectra on cooling is neglectably small because of the relatively small ellipticity of the random coil above 205 nm. It is possible to produce calculated circular dichroism curves which superimpose on those of Figures 1–3 by adding the circular dichroism curves of helical and random coil polyglutamic acid in suitable proportions and subsequently normalizing them.

Figure 5 shows the circular dichroism spectrum of peptide 1–8 in water at 26 and 1° to be quite different from the longer peptides. The weak negative peak at 230–235 nm, the positive peak at 215 nm, and the large negative peak at about 200 nm are characteristic of the random coil (Holzwarth and Doty, 1965). At 1° the positive peak at 215 nm doubled in size; this effect may be due to some as yet undefined change from one extended state to another. In 0.033 M Na_2SO_4, the curves for peptide 1–8 at 26 and 1° are superimposable on the ones shown for ion-free water. This lack of a salt effect may be related to the smaller number of charged groups on this peptide or a length too short to maintain any but an extended structure.

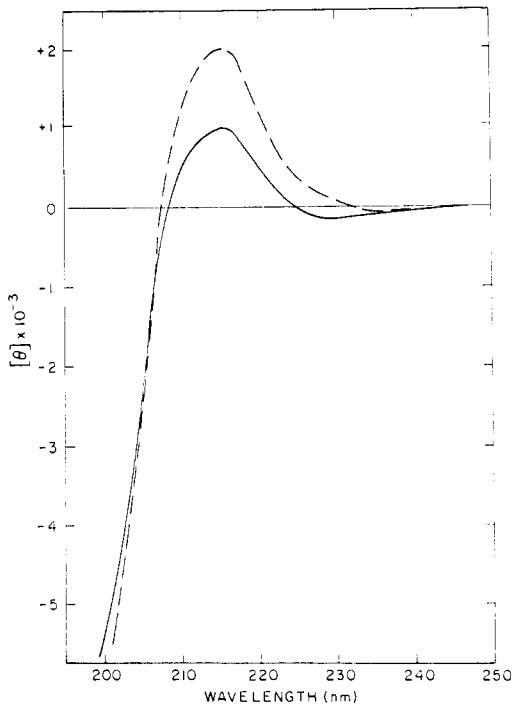

FIGURE 5: Circular dichroism of peptide 1–8 in ion-free water at 26 (—) and 1° (– – –). Peptide concentration was 0.0610 mg/ml and path length was 1.0 cm. The curves in 0.033 M Na_2SO_4 are essentially the same at the respective temperatures.

TABLE I: Molar Ellipticity of Peptides from Ribonuclease.[a]

Peptide	Molar Ellipticity × 10^{-3} at 224 nm	
	26°	1°
1–8	+0.7 ± 0.1	+3.7 ± 0.6
1–13	−22 ± 3	−71 ± 11
1–15	−35 ± 5	−93 ± 14
1–20	−33 ± 5	−77 ± 12

[a] The data are the average of values obtained over a broad range of peptide concentrations (0.008–0.520 mg/ml) in 0.033 M Na_2SO_4 where no deviations from Beer's law were noted. Sedimentation equilibrium experiments with peptides 1–13 and 1–20 show that there is no aggregation in this concentration range. Molar ellipticities were calculated by multiplying mean residue ellipticity times the number of residues. Rotatory strength of the n–π^* transitions was calculated for peptides 1–13, 1–15, and 1–20, and again the values were similar for the three peptides.

The curves shown in Figures 1–3 are very similar to one another and differ primarily in the magnitudes of the dichroic bands. The data shown in the figures have been expressed as mean residue weight ellipticities in order to facilitate comparison with values from the literature. This method of calculation does not, however, allow one to compare the absolute amount of helical structure in a mole of each peptide. Such a comparison is made in Table I where the molar ellipticity of the peptides studied is presented. The table demonstrates that at 1° the molar ellipticities of peptides 1–13, 1–15, and 1–20 are the same, within experimental error, when compared in dilute salt solution. Thus, the actual helical content of these peptides and hence the number of amino acid residues in each peptide which take part in helix formation is constant even though the chain length varies. It may also be inferred from the table that residues 14–20 do not take part in the helical structure and that the presence of homoserine lactone in position 13 of peptide 1–13 is not appreciably affecting the measured ellipticities.

Discussion

This work demonstrates that in the case of the isolated amino terminus of ribonuclease, short-range interactions suffice to produce a structure which is very close to that of the amino terminal region of the intact protein. Peptides from this region assume a partially helical character in solution. The helical region of these peptides comprises the same amino

acids in solution as it does in the protein crystal. Amino acid residues 14–20, which are not part of a regular structure in crystalline ribonuclease, appear to be largely random also when present on peptide 1–20 in solution. The nature of the short-range forces responsible for these effects is still somewhat obscure, although the recent calculations of Kotelchuck and Scheraga (1968) suggest that side-chain backbone interactions with a peptide unit may play a dominant role.

The stability of the helices is much lower in the peptides which we have studied than it is in the protein. This instability is shown by the relatively low ellipticities found. The amount of helicity present may be only of the order of 10–20%. The peptide conformations are highly mobile as indicated by the strong influence of ionic strength on ellipticity. Thus, long-range interactions in the protein must increase the stability and decrease the conformational fluctuations of this helical region. Similar conclusions were drawn from studies of peptides isolated from myoglobin (Epand and Scheraga, 1968). It would seem desirable to study the conformation of peptides at low temperatures in order to minimize the loss of stability due to the absence of long-range interactions.

Peptide 1–8 might be expected to show some weak tendency to form helix since it contains those amino acid residues which form the first half of the helix found in the longer peptides. We have not been able to demonstrate any such tendency, however, and conclude that this peptide is simply too short to allow even a marginally stable helix to form. This finding is consistent with the observation that a minimum number of amino acid residues, generally nine or more, is necessary before helicity can be demonstrated in synthetic polyamino acid systems (Goodman *et al.*, 1963).

It will be of interest to study the conformational properties of peptides, especially overlapping peptides from various regions of protein molecules. The fact that a number of empirical proposals for correlating amino acid composition and sequence with helicity (see Low *et al.*, 1968, for references) are at least partially successful indicates that suitable peptides from any helical region of a protein may tend to become helical in isolation. It is pertinent to recall here that Shearer *et al.* (1966) found that a peptide which comprises residues 38–61 of oxidized ribonuclease has an appreciably helical character in solution. This peptide includes the region 50–58, which is found to be helical in the ribonuclease crystal. Thus the potential centers of initiation of folding proposed by Levinthal (1967) will very likely be found. On the other hand, the recent observation of Kato and Anfinsen (1969) that disordered S-protein (Richards, 1958) will not refold properly in the absence of S-peptide is consistent with a temporally ordered pathway as suggested by Phillips. Perhaps both mechanisms are operative in concert.

However, another more general mechanism has been suggested by the work of Kotelchuck and Scheraga (1968). Their calculations show that each peptide unit has a preferred conformation which is determined by the side-chain backbone interactions and is relatively unaffected by neighboring side chains. These short-range interactions will result in a preferred time-average conformation even for the unfolded molecule. This somewhat more stable conformation would then simplify the final folding by long-range interactions. Thus the foci of folding are not limited to certain regions of the molecule but each peptide unit has a preferred conformation. Such a series of overlapping peptides as we have examined here constitutes an experimental system for studying this intermediate stage of protein folding which is stabilized by short-range interactions.

Acknowledgments

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