Studies on the Conformation of Ribonuclease S-Peptide*

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ABSTRACT: Ribonuclease S-peptide is the amino-terminal eicosapeptide segment of bovine pancreatic ribonuclease A (*i.e.*, residues 1–20). In the intact ribonuclease molecule a large portion of this segment is found to be helical (Kartha, G., Bello, J., and Harker, D. (1967), *Nature 213*, 862; Wyckoff, H. W., Hardman, K. D., Allewell, N. M., Inagami, T., Johnson, L. N., and Richards, J. M. (1967), *J. Biol. Chem 242*, 3984).

The circular dichroism of S-peptide in dilute aqueous solution has been measured over a broad range of pH (1–11.5) and temperature (4–79°). These measurements show negative circular dichroism peaks near 200 and 225 mμ which indicate the presence of both helical and random coil structures in S-peptide throughout this range of conditions. In 5 M guanidine hydrochloride the peptide behaves as a random coil whereas 2-chloroethanol induces a high degree of helicity. These results are taken to indicate that, in the course of its conformational fluctuations, S-peptide often assumes a structure which is similar to that of the amino-terminal segment of intact ribonuclease.

S-Peptide is a fragment of bovine pancreatic ribonuclease, 20 amino acid residues in length, corresponding to the amino terminus of the molecule (*i.e.*, residues 1–20) (Richards, 1958). This study of the conformational properties of S-peptide in solution was undertaken not only because of the intrinsic interest of a polypeptide of this intermediate size, but also as a result of the recently reported structure determinations of ribonuclease A (Kartha *et al.*, 1967) and ribonuclease S (Wyckoff *et al.*, 1967), based on X-ray diffraction data; these agree in placing a sizeable segment of α helix in this part of the protein molecule. The model of Wyckoff *et al.* (1967) shows residues 2–12 of RNase S to be in a helical stretch comprising some 2.5 turns. One general problem of protein chemistry is the question of the extent to which the preferred conformation of particular segments of a protein is determined by interactions with nearby amino acid residues in the linear sequence as opposed to interactions with amino acids far removed in the polypeptide chain. Studies of the properties of isolated small segments of protein molecules can provide a direct route toward its solution. It is, therefore, clearly of interest to inquire to what extent the helical nature of S-peptide is conserved in solution in the absence of the remainder of the ribonuclease molecule (S-protein). Furthermore, since the conformation of the isolated S-peptide has recently been investigated from a theoretical point of view by energy minimization procedures (Gibson and Scheraga, 1967), data pertaining to its conformation in solution are needed to test such predictions. Finally, these studies may also be relevant to the question of the mechanism by which polypeptide chains acquire the three-dimensional structure characteristic of native proteins. It has been argued that the folding of polypeptide chains is not a random process but proceeds sequentially from the amino terminus (Phillips, 1967) or is directed by highly probable local interactions which serve to provide pathways for folding (Levinthal, 1967). The sequential mechanism proposed by Phillips implies that a polypeptide segment derived from the amino terminus of a protein will tend to assume a conformation similar to that found in the complete protein. This work then provides a preliminary test of this hypothesis.

The present study has focused on the far-ultraviolet circular dichroism of S-peptide under a variety of conditions. The data are consistent with a time-average conformation for S-peptide which has both helical and random coil contributions. The tendency of S-peptide to exhibit a partially helical character is only slightly affected by temperature and pH. High concentrations of guanidine hydrochloride convert the material into an apparently random coil, whereas 2-chloroethanol stabilizes a highly helical conformation.

Experimental Section

S-Peptide was obtained from salt-free RNase S prepared according to the method of Richards and Vithayathil (1959) with some minor modifications (Klee, 1965). S-Peptide was prepared by trichloroacetic acid precipitation of RNase S and purified according to Richards and Vithayathil (1959). The material was completely free of S-protein as attested by enzymatic assay (which could detect less than 0.1% contamination) and by spectral analysis (see Results). Samples were prepared by dilution of stock solutions of 1 or 5 mg/ml in deionized water.

Guanidine hydrochloride was prepared from the carbonate by the method of Anson (1940). 2-Chloroethanol (Eastman White Label) was treated with anhydrous Na_2CO_3 immediately before use.

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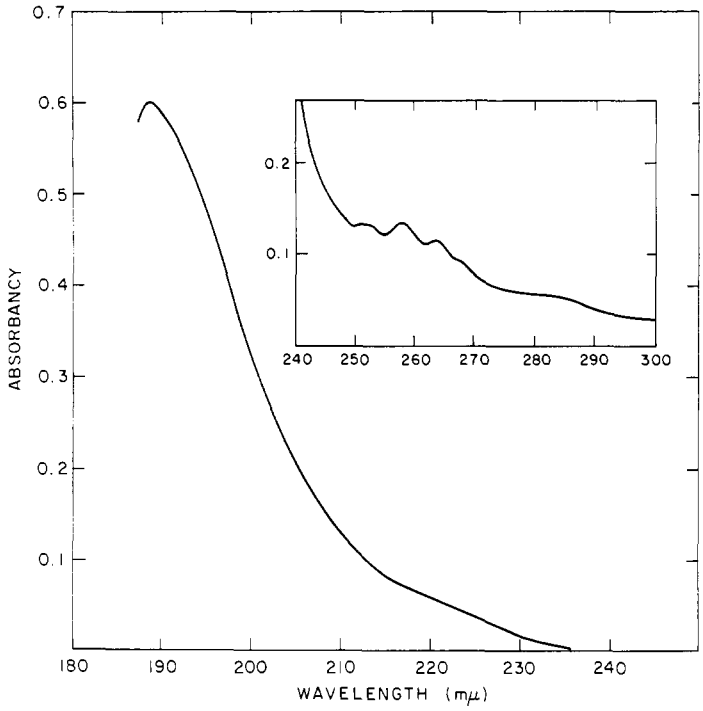

FIGURE 1: The ultraviolet absorption spectrum of S-peptide. The spectrum was taken of a 0.1-mg/ml solution in a 1-mm path-length cell (solvent, water). Insert: 1 mg/ml in water using a 1-cm path length.

Circular dichroism measurements were performed with a Cary 6001 attachment to the Cary Model 60 spectropolarimeter. Samples were examined at concentrations from 0.05 to 1 mg/ml and at path lengths of 1 and 10 mm. Beer's law was followed by the material in all cases showing the absence of aggregation and instrumental artifacts under these conditions. The slit was programmed to maintain a spectral band pass of 15 Å. Temperature was controlled by means of a hollow-walled brass cell holder through which water was circulated from a Lauda refrigerated water bath. Temperatures were measured inside the cell by means of a Yellow Springs Instrument Co. thermocouple and was constant to within better than 0.2° during the run.

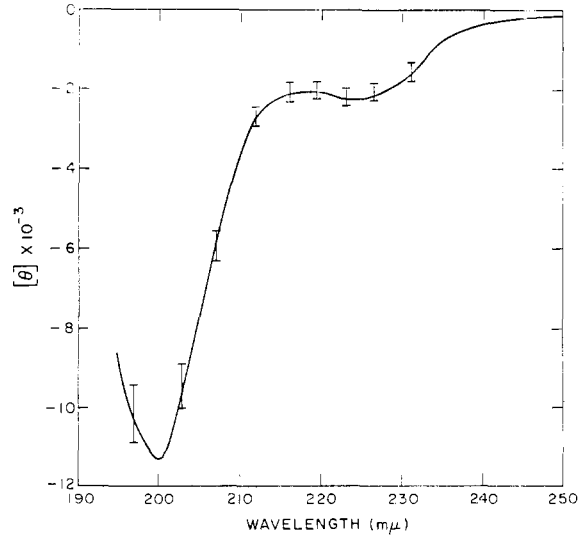

FIGURE 2: Circular dichroism of S-peptide in 0.005 M Na_3PO_4 (pH 11.5). The temperature was 4°. The bars indicate the maximum estimated uncertainty of the data and apply to the data shown in Figures 3–6 as well.

The data are expressed as mean residue ellipticity values $[\theta]$ defined as follows. $[\theta] = (\theta/10)(MRW/lc)$, where θ is the measured ellipticity in degrees, l is the path length of the solution in cm, c is concentration in g/cm^3, and MRW, the mean residue weight, is taken as 107.5 for S-peptide. $[\theta]$ has the dimensions of deg (cm^2/dmole). The instrumental calibration was monitored with *d*-10-camphorsulfonic acid.

pH when reported is that of the solvent, and was measured with a Radiometer TTTlc pH meter at 25°. The concentrations of peptide used in these studies were too low to change the pH of the buffers used appreciably.

Ultraviolet spectra were obtained on a Cary Model 14 spectrophotometer with N_2 purging at an ambient temperature of 23°, using the same cells as those used in the circular dichroism measurements.

Results

The ultraviolet absorption spectrum of S-peptide is shown in Figure 1. The insert shows the spectrum of a 1-mg/ml aqueous solution of S-peptide which exhibits the typical multiband pattern of phenylalanine derivatives. The molar extinction coefficient calculated from these data for the peak at 258 mμ is 290, in satisfactory agreement with the value of 320 found by Richards and Logue (1962).[1] The spectrum shows further that there is very little contamination of the S-peptide sample by tyrosine-containing derivatives (Wetlaufer, 1962). The apparent peak near 285 mμ is seen also in the spectrum of Richards and Logue (1962) and is most probably a real feature of the S-peptide spectrum. The spectrum of this material at lower wavelengths was taken at a concentration of 0.1 mg/ml in a 1-mm path-length cell. There are indications of peaks near 210 mμ and in the 220–225-mμ region, but a more detailed study of the spectrum in this region is clearly needed for interpretation. The peak near 190 mμ has an extinction coefficient of 6450, on a mean residue weight basis, a result which is between the random coil and helical absorbancies of both poly-L-glutamic acid and poly-L-lysine (Rosenheck and Doty, 1961), but which is seen to be abnormally low when the side-chain absorbancy is taken into account. It will be shown below that the ellipticity of S-peptide is also very low when compared with polyamino acid models.

A typical circular dichroism curve of S-peptide in dilute aqueous solution is shown in Figure 2 for the

[1] It is likely that the lower value obtained in this study is due to the presence of small amounts of water in the peptide preparation since the sample was not vigorously dried. Since the solutions used in these experiments were prepared by weight, it is possible that the values of ellipticity reported are systematically low by 10%. Uncertainties of this order of magnitude can, however, in no way affect the arguments presented.

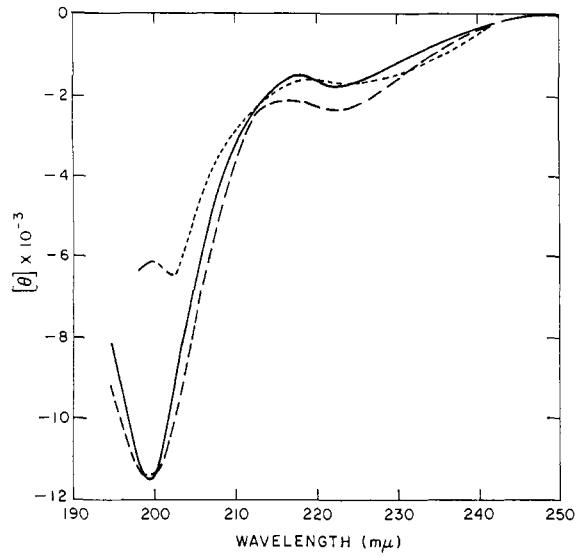

FIGURE 3: Circular dichroism of S-peptide in 0.005 M Na_3PO_4 (pH 11.5) at several temperatures. The temperatures are (– – –) 4°, (——) 26°, and (· · · ·) 79°.

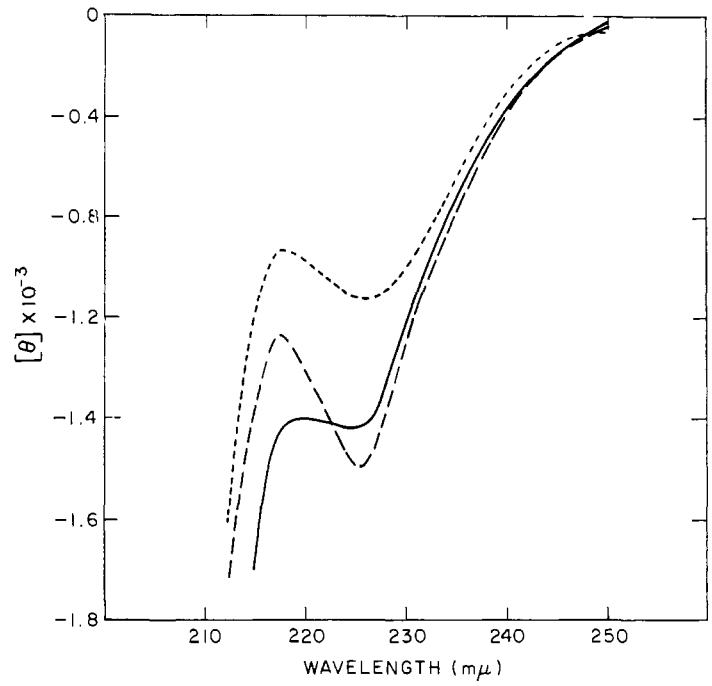

FIGURE 4: Circular dichroism of S-peptide at several pH values, all at 26°: (– – – –) pH 1 (0.1 M H_2SO_4); (· · · ·) pH 6.5 (0.004 M NaH_2PO_4); (——) pH 11.5 (0.005 M Na_3PO_4).

spectral range 250–290 mμ. The characteristic features of the curve are (1) a weak negative peak in the 222–225-mμ region; (2) a much stronger negative peak near 200 mμ; and (3) typically, a shoulder near 208 mμ. The region around 215 mμ generally shows some kind of notch or flattening between the two main negative peaks. The particular curve shown in Figure 2 has been obtained at 4° and pH 11.5, which conditions were chosen so as to enhance the contribution of the 222-mμ peak to the total circular dichroism.

Figure 3 shows the temperature dependence of the circular dichroism of S-peptide at pH 11.5 over the range 4–79°. Although there is a clear decrease in the magnitude of the 225-mμ peak on going from 4 to 26°, the change in this spectral region is negligible at temperatures above 26°. There is, however, a dramatic decrease in the ellipticity near 200 mμ on heating above 26°. Although not shown, data taken at temperatures intermediate between 26 and 78° give intermediate circular dichroism curves. Although the conformation of S-peptide does exhibit some dependence on pH, the changes observed in the region between pH 1 and 11.5 are small and primarily show changes in the magnitude of the ellipticity peaks without affecting the numbers or positions of the peaks appreciably (Figure 4). It is seen in the figure that the 225-mμ peak is stronger both at high and at low pH than in neutral solutions. The curve at pH 6 is unchanged in the absence of added salt.

The circular dichroism spectra of S-peptide in solutions containing varying amounts of guanidine hydrochloride (at neutral pH) are shown in Figure 5. It is remarkable that the curve in 1 M guanidine hydrochloride is very similar to that in dilute buffer at neutral pH (represented by the open circles). An increase in the concentration of guanidine hydrochloride results in a gradual change of the circular dichroism spectrum of S-peptide to one whose characteristics no longer bear any resemblance to that shown in the earlier figures but which is characterized by a positive dichroism with a maximum near 218 mμ. These experiments show that the circular dichroism of aqueous S-peptide is reflecting conformational rather than primarily compositional properties of the material.

Figure 6 shows the circular dichroism of S-peptide dissolved in 90% (v/v) 2-chloroethanol, a solvent which has often been shown to favor very highly helical structures (Doty *et al.*, 1958; Weber and Tanford, 1959; Gratzer *et al.*, 1968). The spectrum shown in the figure is indeed compatible with such a structure for S-peptide in this solvent.

Discussion

The circular dichroism of S-peptide exhibits a remarkably stable negative peak near 225 mμ[2] which is believed to be a characteristic of α-helical structures (Holzwarth and Doty, 1965). The shoulder usually seen near 208 mμ is also expected of helices. However, the much larger negative peak near 200 mμ is a characteristic of randomly coiled structures (Holzwarth and Doty, 1965). Thus, the circular dichroism of S-peptide indicates that the material exhibits a mixed conformation which contains elements of helical and of

[2] The position of this peak has been found to vary between 222 and 227 mμ depending upon the conditions of solvent and temperature. In this discussion this peak, which is believed to represent the $n-\pi^*$ transition of peptide bonds in a helical conformation, is called for convenience the 225-mμ band. It has been observed both with model compounds (Nielsen and Schellman, 1967) and polypeptides (Sage and Fasman, 1966; Quadrifoglio and Urry, 1967; Schellman and Lowe, 1968) that the wavelength of this transition may vary over a wide range.

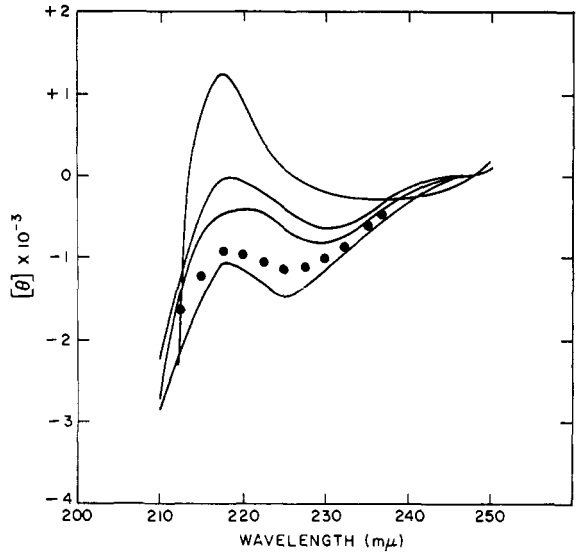

FIGURE 5: The effect of guanidine hydrochloride on the circular dichroism of S-peptide. The curves represent (from top to bottom) 5, 3, 2, and 1 M guanidine hydrochloride. The open circles are data taken in water. Temperature 26°, pH 7.

random-type structures. The spectra in Figures 2–5 are indeed qualitatively very similar to that of poly-L-ornithine at pH 11.7 which is also believed to exist in a mixed random and helical conformation (Grouke and Gibbs, 1967).

The 225-mμ peak of S-peptide is strengthened somewhat at temperatures near 0, but is surprisingly insensitive to temperature in the range 26–79°. It is also remarkable that 1 M guanidine hydrochloride, a concentration sufficient to cause large conformational changes in many proteins, is almost without effect on the circular dichroism of S-peptide. Higher concentrations of this reagent do, however, result in the gradual conversion of the circular dichroism spectrum of S-peptide into that of a randomly coiled material with a positive rather than negative dichroism near 220 mμ. The high concentrations of reagent required for the conversion testify to the remarkable stability of the structure seen in S-peptide in dilute salt solutions.

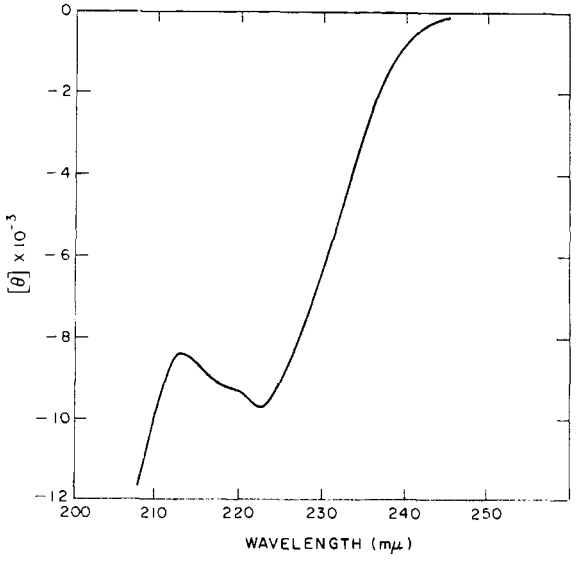

FIGURE 6: Circular dichroism of S-peptide dissolved in 90 vol % 2-chloroethanol at 26°.

S-Peptide contains neither tyrosine nor tryptophan residues. This circumstance together with the fact that the other amino acid side chains have absorption bands only at wavelengths above 240 mμ or below 210 mμ (Wetlaufer, 1962) strongly indicates that the ellipticity peak at 225 mμ is not primarily a reflection of side-chain absorption.[3,4]

The considerations of the previous paragraph cannot rule out the contribution of β-type structures to the observed circular dichroism of RNase. It is important to point out in this regard that no concentration dependence has been observed for the circular dichroism of S-peptide over a tenfold range, a result which makes intermolecular β structures unlikely. It is, however, not possible to rule out the existence of some conformations of S-peptide which result in β-type dichroism (Townsend *et al.*, 1966). Any such contributions to the circular dichroism would be masked by the relatively larger helical and random coil contributions.

The qualitative appearance of the circular dichroic spectra of S-peptide is quite consistent with the simultaneous presence of helical- and coil-type structures in this material. It is remarkable, however, that the magnitudes of the Cotton effects are smaller by almost a factor of 10 than those of the model polyamino acids which are commonly used as standards. It is not clear to what extent the chain length of the sample (20 amino acids only for S-peptide) is responsible for this effect, but it has been shown on theoretical grounds that the $\pi-\pi^*$ transition of polypeptide helices (at 190 and 208 mμ) will reflect chain length fairly strongly (Woody and Tinoco, 1967). In this connection, it is of interest that S-peptide, even in 2-chloroethanol, where it should be largely helical, exhibits a 222-mμ dichroic peak with an amplitude only one-fourth or so that of

[3] There does appear to be a series of weak negative dichroic bands near 267, 261, and 255 mμ which apparently are due to the phenylalanine residue at position 8 of S-peptide. These bands were not observed by Simons and Blout (1968) but are only clearly visible with concentrated solutions (5 mg/ml, 1-cm cell) and at high sensitivity settings.

Legrand and Viennet (1965) have shown that L-phenylalanine exhibits a series of *positive* dichroic bands in aqueous solution at a series of pH values. These are located at 218 mμ (strong) and near 252, 257, and 263 mμ (all very weak). Although the 218-mμ band may be contributing appreciably to the dichroic spectrum of S-peptide it is not possible to be sure of either the sign or the magnitude of this contribution.

[4] In a recent note, Scatturin *et al.* (1967) reported measurements of the circular dichroism of S-peptide in neutral aqueous solution and in 8 M urea. The data are in agreement with those presented here; their 8 M urea curve is approximately equivalent to the curve shown in Figure 5 for 3 M guanidine hydrochloride and therefore represents incomplete unfolding. In contrast to the interpretation proposed in this paper, Scatturin *et al.* (1967) conclude that S-peptide is completely random.

Simons and Blout (1968) have also recently published a circular dichroism spectrum of S-peptide (in the wavelength region 210–250 mμ). These data are also in reasonable agreement with the curve shown in Figure 2 at neutral pH.

helical polylysine or polyglutamic acid. It may be the case then that the n–π^* transition at 225 mμ also reflects the length of the helix, at least when it is very short. The cyclic decapeptide, gramicidin S, which appears to be largely helical in conformation exhibits circular dichroic bands of normal intensity (Quadrifoglio and Urry, 1967) so that the great diminution seen in the S-peptide dichroic curves may result from relatively large free end effects which would be absent or negligible in the case of most proteins and polyamino acids as well as in cyclic peptides.

It is not possible to estimate the amount of helicity of S-peptide with any degree of assurance. On making the usual assumptions of helix and random coil ellipticities of −40,000 and +3000, respectively (at 222 mμ) (Holzwarth and Doty, 1965), S-peptide is seen to be 10–15% helical in aqueous buffers. It is possible to calculate somewhat higher values using other models, but these numbers will suffice as a first-order approximation. Schellman (1958) has, happily, treated the case of a partially helical polypeptide of precisely 20 amino acid residues in great detail and with a high degree of rigor. His results predict that there will be a broad distribution of helical lengths for such a polypeptide when the average helicity is of the order of 10–20%. The predicted distribution shows that more than 80% of the chains will be in the random coil state at any given time with the remainder distributed fairly evenly among all species having from 5 to 20 residues in a helical conformation. The data presented in this paper, which show a preponderance of random coil together with low helical content, are consistent with this model. Schellman's treatment ignored any specific effects of side-chain interactions. It is now known that these may well be important in determining the probability that any given segment of a polypeptide be helical. In particular, it may be argued that it is unlikely that the carboxyl-terminal segment of S-peptide, which includes the sequence -Ser-Ser-Thr-Ser-, will assume a helical conformation (Blout *et al.*, 1960; Davies, 1964). If this view is correct, then those residues of S-peptide which tend to be helical in free solution are the same as those which are found to be so in the protein (*i.e.*, residues 2–12).

Gibson and Scheraga (1967) found, by energy minimization calculations, that there are a large number of local minima in the energy landscape of S-peptide. This result is compatible with the foregoing discussion of its conformation in solution which centers about a continuous fluctuation of structure. It is also noteworthy that one of the most stable structures found by Gibson and Scheraga has a short segment of helix in the first half of the molecule whereas the completely helical structure is much less probable energetically. These results are also in accord with the data discussed here.

It would therefore appear that, in the course of its conformational fluctuations, the isolated S-peptide often assumes a structure which is quite similar to that of the S-peptide portion of intact ribonuclease. It is perfectly clear, however, that a large part of the conformational stability of this structure in the intact protein is the result of interactions between S-peptide and S-protein. Thus, in answer to the question posed in the introduction to this paper, both near-neighbor and distant interactions determine the conformation of a protein but in the case of the amino-terminal region of ribonuclease near-neighbor interactions alone are sufficient to result in a reasonable approximation to the final conformation. This interpretation is compatible with the proposal of Phillips (1967) which states that proteins may fold into their native conformation during the course of their biosynthesis starting from the amino-terminal end. The present findings may also provide an example of the kind of highly probable local interaction which Levinthal (1967) proposes to be necessary for the correct folding of a protein chain within a reasonable time.

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Studies on Rabbit Muscle Enolase. Evidence for Two Identical Polypeptide Chains and Two Substrate Binding Sites in the Active Enzyme*

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ABSTRACT: Rabbit muscle enolase, an enzyme containing two polypeptide chains of 41,000 molecular weight/active unit of 82,000 molecular weight, was treated with trypsin and with cyanogen bromide, and the number of peptides produced in each treatment was estimated from the number of spots of peptide maps and one-dimensional electrophoretograms, respectively. The extent of the trypsin digestion was assessed by the liberation of carboxyl-terminal lysine and arginine residues susceptible to attack by carboxypeptidase B, and the extent of the cyanogen bromide reaction was determined by methionine analyses. Both the total ninhydrin-positive peptides and the arginine-containing peptides (visualized with Sakaguchi reagent) were counted. All the results are consistent with a unique polypeptide sequence of one-half the molecular weight of the active enzyme. It is thus concluded that the two polypeptide chains in rabbit muscle enolase are identical. The number of substrate binding sites in rabbit muscle enolase was determined by gel filtration techniques, using the substrate analog [^{14}C]glycolic acid phosphate as the ligand. Analog (2 moles) was bound per 82,000 g of enzyme, with an average dissociation constant of 2×10^{-4} M. This agrees reasonably well with the K_I (competitive) value of 7×10^{-4} M for glycolic acid phosphate.

Earlier studies on rabbit muscle enolase (2-phospho-D-glycerate hydrolyase, EC 4.2.1.11) have established that the active enzyme (mol wt 82,000) is made up of two polypeptide chains of 41,000 molecular weight (Winstead and Wold, 1965). Both polypeptide chains have an amino-terminal *N*-acetylalanine and the carboxyl-terminal tripeptide sequence lysyl-alanyl-lysine (Winstead and Wold, 1964). In view of these similarities, the studies reported in this paper were undertaken in an attempt to establish whether or not the entire amino acid sequence is identical in the two chains.

To this end, the enzyme was degraded with cyanogen bromide and trypsin and the number of resulting peptides was determined by peptide mapping. In order for this approach to have any validity, it is essential that both the extent and the specificity of the peptide cleavage be evaluated. This is readily accomplished for the cyanogen bromide reaction, since an amino acid analysis of the acid-hydrolyzed reaction product will give the extent of the reaction in terms of conversion of methionine into homoserine (and homoserine lactone) (Gross and Witkop, 1962) and at the same time, indicate the specificity, in terms of the recovery of other potentially reactive amino acids. For the tryptic digestion, no such direct assay is available, however. In this work we took advantage of the specificity of carboxypeptidase B for basic amino acid residues, and monitored the trypsin digestion both for liberation of carboxyl-terminal lysine and arginine susceptible to attack by carboxypeptidase B, and for free lysine and arginine liberated directly by trypsin. With this type of information at hand, the total number of peptide bonds broken, and thus also the total number of peptides expected if the chains were either identical or different, can readily be estimated quite accurately.

When the results from the peptide counts showed that rabbit muscle enolase consists of two identical polypep-

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