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# The Influence of Amino Acid Side-chains on α-Helix Stability: S-Peptide Analogues and Related Ribonucleases S′

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In order to determine the influence of amino acid side-chains on α-helix stability, in relation to the protein folding process, the coil–helix transitions of some synthetic [Orn10]-S-peptide analogues, containing, in position 8, Phe, Tyr, Ile, Ala, cpGly† and Gly, were investigated by the technique of circular dichroism under two different sets of conditions. First, the transitions of the S-peptide analogues in water/trifluoroethanol mixtures were recorded. From the pattern of the transitions and from the ellipticity values in 97% trifluoroethanol, the following increasing order of amino acids as α-helix formers was found: Gly<Tyr≤Phe<cpGly<Ala<Ile. This finding indicates that the conformational parameters (Chou & Fasman, 1974) of the residues in position 8 play an important but not exclusive role in α-helix stability, since the hydrophobicity of the side-chain (Nozaki & Tanford, 1971) of residue 8 exerts a strong influence. From the second approach, studying the capability of the S-peptide analogues to bind to S-protein, the following increasing order was found: (Gly, Ala)<Ile <cpGly<Tyr<Phe. This result reveals that the conformational parameters of the residues in position 8 play no role, whereas their hydrophobic character and side-chain interactions with surrounding residues in the S-protein portion are the determining binding factors. This finding explains the reason for the Phe8 invariance in RNAase A during evolution, and furnishes evidence for the relevant role of long-range interactions in the protein folding process.

## 1. Introduction

It is well known that proteins (Timasheff, 1970) and protein fragments (Moroder *et al.*, 1975), when dissolved in organic solvents (e.g. 2-chloroethanol and trifluoroethanol), tend to assume helical conformations to a high degree. Nozaki & Tanford (1971) suggested that this behaviour is originated by the highly hydrophilic character of the backbone peptide unit.

Using the technique of circular dichroism in order to investigate the ability of neutral amino acid side-chains to support the α-helical conformation, we have

† Abbreviations used: cpGly, cyclopentylglycine; S-peptide, the eicosapeptide obtained from RNAase S, the subtilisin-modified ribonuclease A (Richards, 1958); S-protein, the protein component from RNAase S, sequence 21 to 124; RNAase S′, equimolar mixture of S-peptide and S-protein. The amino acids are of L-configuration.

studied the coil-α-helix transitions of synthetic S-peptide analogues both in water/trifluoroethanol mixtures and in the binding process to S-protein.

The synthetic S-peptide analogues used in this study differ from the natural one in having an ornithyl residue in position 10, while the residues in position 8 are Phe, Gly, Ala, Ile, cpGly† and Tyr.

The naturally-occurring arginine 10 residue was usually replaced by ornithine in the synthesis of S-peptide analogues in our laboratory (Scoffone *et al.*, 1973). This substitution does not affect either the circular dichroism difference spectra that originated from the RNAase S′ formation in solution (Filippi *et al.*, unpublished results) or the S-peptide backbone atomic co-ordinates in crystalline RNAase S′, as determined using the Fourier difference technique (G. Valle, personal communication).

The isolated S-peptide in aqueous solution shows essentially no ordered structure, but when dissolved in trifluoroethanol about ten residues were found in α-helical conformation (Tamburro *et al.*, 1968). In crystalline RNAase S, the sequence of residues 3 to 13 is present as an α-helical structure, as deduced from X-ray data (Richards & Wyckoff, 1971). The conformational transition of the S-peptide induced by the S-protein can be used to follow the formation of the RNAase S′ in solution, by monitoring the enhancement of the helix formation that originated by the addition of increasing quantities of peptides to a constant quantity of S-protein. By plotting the increment, or some related value, *versus* the corresponding peptide to protein molar ratio, a "titration curve" which is a function of the capability of the peptide to bind to S-protein is obtained.

The pattern of the transitions of the S-peptide analogues in water/trifluoroethanol mixtures and in the binding to S-protein can be used to construct two independent scales of the same α-amino acids as α-helix formers. The results will be discussed in relation both to the hydrophobicity data published by Nozaki & Tanford (1971) and to the conformational parameters published by Chou & Fasman (1974). The comparison may be used to explain the invariant character of the phenylalanine residue in position 8 in RNAase A and to explore the influence of long-range interactions in the protein folding process.

## 2. Experimental Procedures

The synthesis of [Phe8, Orn10]-S-peptide (Scoffone *et al.*, 1967*a*), [Gly8, Orn10]-S-peptide (Rocchi *et al.*, 1968), [Ala8, Orn10]-S-peptide and [Ile8, Orn10]-S-peptide (Scoffone *et al.*, 1967*b*), [cpGly8, Orn10]-S-peptide (Borin *et al.*, 1971), and [Tyr6, Orn10]-S-peptide (Marchiori *et al.*, 1966) have been already reported. Circular dichroism measurements were performed on a Cary 61 recording spectropolarimeter, at 25°C. Cylindrical fused quartz cells with 0·1 cm and 0·05 cm path-lengths were used for measurements in water/trifluoroethanol mixtures. Twice-distilled water and trifluoroethanol as purchased were used. All measurements were performed at peptide concentrations in the range 0·08 to 0·16 mg/ml. The data are expressed in terms of $[\theta]$, the mean residue molecular ellipticity in deg cm$^2$ dmol$^{-1}$. The binding of the S-peptide analogues to S-protein was followed by measuring the increased ellipticity at 222 nm originated by mixing S-protein and S-peptide analogue solutions in a tandem mixed fused quartz cuvette, according to a procedure described by Filippi *et al.* (1975). The correct concentrations of the peptide solutions were determined by the average recovery of amino acids from an acid hydrolysate of a portion of the solutions. The concentration of S-protein in aqueous solution was determined by measuring the absorbance at 280 nm, using a value of 0·784 as the absorbance of a 1 mg/ml solution.

† See footnote on page 315.

## 3. Results

The circular dichroism spectra of the S-peptide analogues in 97% (v/v) trifluoroethanol are shown in Figure 1(a). A negative band in the 220 to 222 nm region (the typical wavelength of the amide $n \rightarrow \pi^*$ transition of the $\alpha$-helix is 222 nm: Holzwarth & Doty, 1965) is clearly discernible for all the peptides examined. The existence of a negative band at 207·5 nm and the ellipticity values confirm the presence of approximately equal amounts of random coil and $\alpha$-helical conformation, as found for the S-peptide when bound to S-protein, both in solution and in the crystal.

In order to determine whether the side-chain chromophores present in position 8 give appreciable dichroic contributions to the spectra, the circular dichroism values of all the peptides in 97% trifluoroethanol (Fig. 1(a)) were plotted as a percentage of the value of the [Ile8,Orn10] analogue, at corresponding wavelengths, in the 207·5 to 230 nm interval (Fig. 1(b)). The parallelism of the lines resulting for the [Gly8,Orn10], [Ala8,Orn10], [Phe8,Orn10] analogues ensures that no significant contribution comes from these side-chains; more consistent deviations from parallelism are observed for the [Tyr8,Orn10] and [cpGly8,Orn10] analogues, expecially in the 230 nm region. The origin of such behaviour cannot be safely attributed to the aromatic side-chain

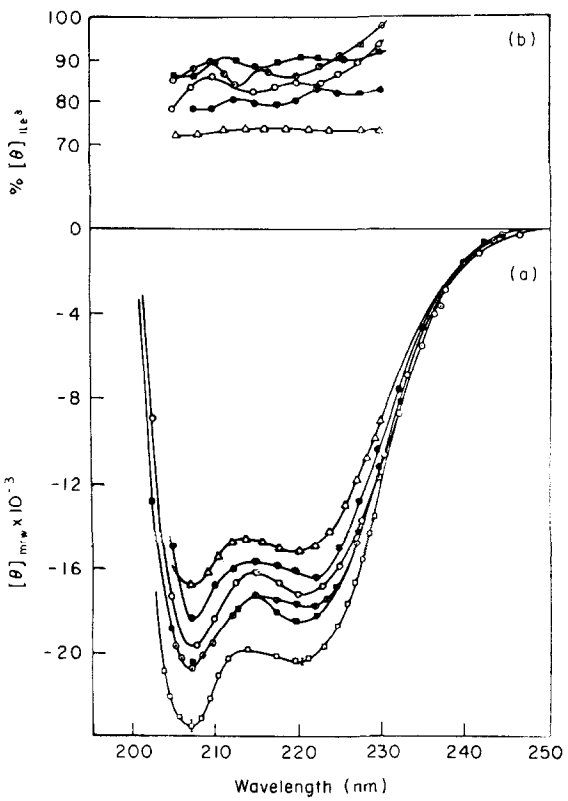


FIG. 1. (a) Circular dichroism spectra in 97% trifluoroethanol of [Gly8,Orn10]-S-peptide (—△—△—); [Ala8,Orn10]-S-peptide (—■—■—); [cpGly8,Orn10]-S-peptide (—⊙—⊙—); [Orn10]-S-peptide (—●—●—); [Tyr8,Orn10]-S-peptide (—○—○—); [Ile8,Orn10]-S-peptide (—□—□—). $[\theta]_{mrw}$ is the mean residue ellipticity, deg cm² dmol⁻¹.

(b) Ellipticity values of the peptides in 97% trifluoroethanol reported as a percentage of the [Ile8,Orn10]-S-peptide value, at corresponding wavelengths. Symbols as in (a).

of Tyr8, since it is present also in the analogue containing the non-aromatic cpGly8 residue. Gratzer & Beaven (1972) found that the dichroic contribution of an aromatic chromophore, incorporated at a low level in α-helical peptides or copolymers, is small when compared with the peptide Cotton effect and difficult to detect; in fact, by plotting the circular dichroism of a copolymer of L-glutamic acid and L-tyrosine (23:1) in the 210 to 235 nm interval against that of poly-L-glutamic acid, they obtained a straight line.

However, the deviations found here are not sufficiently consistent to invalidate the assumption that the 220 to 222 nm bands of all the spectra in Figure 1 reflect directly the α-helical content of the peptide solutions.

Figure 2 shows the variation of the mean residue molecular ellipticity of the peptides at 222 nm as a function of the solvent composition.

In Figure 3 the capability of the S-peptide analogues to bind to S-protein is represented by the number of residues undergoing the coil–helix transition. The formation of the RNAase S′ complex, as well as the conformational transition involving about half of the S-peptide sequence, also produces a rearrangement of the S-protein, involving both the aromatic and the disulphide chromophores. The dichroic effects produced, clearly evident in the 250 to 320 nm interval, are also present in the peptide absorption region, but casually they have very small values at 222 nm (Filippi *et al.*, unpublished results). On the other hand, there are both experimental and conceptual limits to the interpretation of X-ray data (Saxena & Wetlaufer, 1971; Richards & Wyckoff, 1973) especially near the helical boundaries. When these limits are taken into account, the present results from circular dichroism measurements, indicating the value of 10·4 as the maximum number of residues undergoing the conformational transition in the formation of the RNAase S′ adduct,

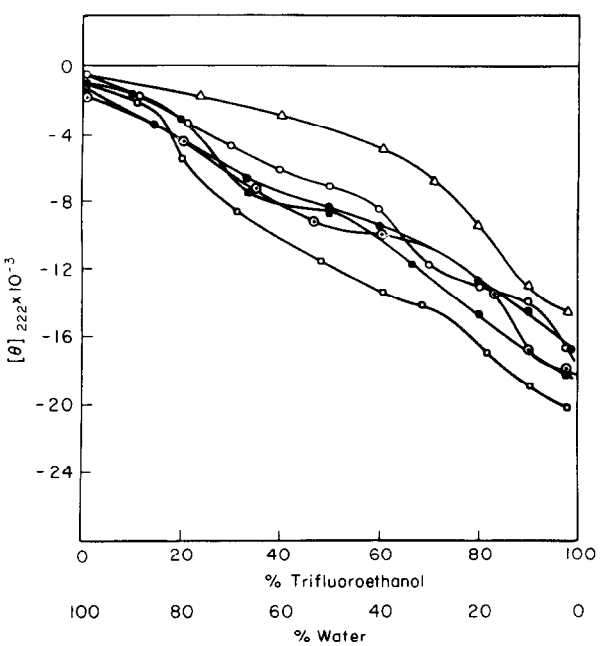


FIG. 2. $[\theta]_{mrw}$ values at 222 nm of the peptides, plotted as a function of solvent composition. Symbols as in Fig. 1(a).

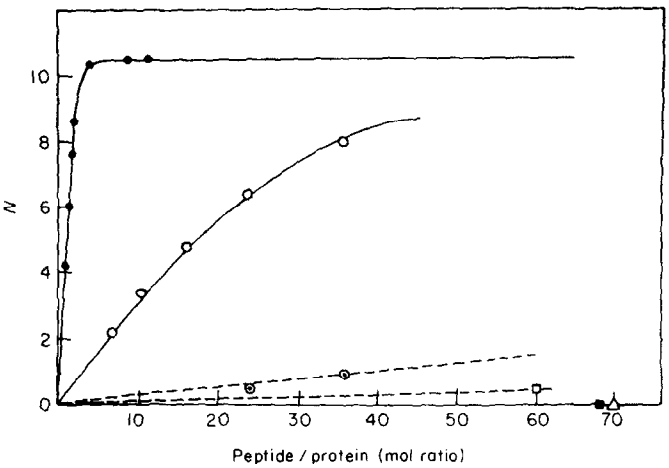


FIG. 3. Titration curves of S-protein with S-peptide analogues at 25°C. The number of residues ($N$) undergoing the coil-to-helix transition, calculated from the molar ellipticity changes at 222 nm, using −36,000 as mean residue α-helical ellipticity, are plotted as a function of the peptide/protein molar ratios. The molar ellipticities are calculated assuming the enzyme concentrations to be equal to that of the S-protein (2·81 μM), in 0·01 M-$NaPO_4$ containing 0·9% (w/v) NaCl at pH 6·8: [Orn10]-S-peptide (—●—●—); [Tyr8,Orn10]-S-peptide (--○--○--); [cpGly8,Orn10]-S-peptide (○); [Ile8,Orn10]-S-peptide (□); [Ala8,Orn10]-S-peptide (■); [Gly8,Orn10]-S-peptide (△).

can be accepted as being in excellent agreement with the X-ray diffraction estimates from 11 residues.

## 4. Discussion

Comparison of the transition profiles shown in Figure 2 suggests that, in the conditions employed here, isoleucine is the most efficient residue of this series in stabilizing the α-helix conformation of the peptide, and that glycine is the least efficient. It is difficult to establish a precise scale among the remaining residues, since the lines representing the transitions intersect at many points; with some reservations it may be observed that in solutions of high water content tyrosine is the least effective, and that at low water content alanine is the most effective. If we consider both the values at 97% trifluoroethanol and the transition patterns, the following α-helixphilic scale results:

$$\text{Gly} < \text{Tyr} \leq \text{Phe} < \text{cpGly} \leq \text{Ala} < \text{Ile}. \qquad (1)$$

The inner α-helix conformational parameters, $P_{\alpha_i}$ (Chou & Fasman, 1974, Table II) and the hydrophobicity of amino acid side-chains (Nozaki & Tanford, 1971, Table IX) are reported in scales (2) and (3), respectively:

$$\text{Gly} < \text{Try} < \text{Phe} < \text{Ile} < \text{Ala} \qquad (2)$$

$$\text{Gly} < \text{Ala} < \text{Tyr} < \text{Phe} < \text{Ile}\dagger \ . \qquad (3)$$

† The position of Ile is deduced by the values reported by Tanford (1962, Table 1(a)). Since the data used were estimated to be of relatively low precision, the location of Ile in relation to that of Phe is somewhat uncertain.

The location of Gly in the lowest position in the α-helixphilic scale (1) is in accord both with the behaviour of the amino acid in protein (scale (2)) and with its hydrophilic character (scale (3)). On the opposite side we find Ile; this residue shows a high helical potential when located in the central region of the helix but clearly less than Ala (Chou & Fasman, 1974, Table II). To explain the results reported in scale (1) in addition to the conformational parameters, a further factor, very likely hydrophobicity, should be taken into account; in fact, the larger non-polar side-chain of Leu in comparison with that of Ala was assumed to be responsible for the higher stabilization of the helix in linear polypeptides, through a hydrophobic stabilization (Chou *et al.*, 1972).

The comparison of the transition patterns (Fig. 2) of the Tyr8 and Phe8 peptides shows that in mixtures with relatively high water content, the amount of the former peptide in helical form is consistently less than that of the latter. This behaviour is in accord with the conformational inner helical parameters of the two amino acids (Tyr = 0·58, Phe = 1·14). In solutions enriched in trifluoroethanol, the helical content of the two peptides becomes almost the same, as if the hydrophobicity of the side-chains, which are very similar, had become the determining parameter for helix content. For the unnatural cpGly amino acid, no data of hydrophobicity and (obviously) of conformational parameter are reported. Its spectrum (Fig. 1(a)) shows that the residue may be located in an α-helical region and (Fig. 2) its α-helixphilicity is equal to that of Ala. If we assume that each carbon atom of the non-aromatic side-chain cyclopentyl ring makes a contribution to hydrophobicity of the same order as each carbon atom side-chain of Val and Ile (Nozaki & Tanford, 1971), the ratio of cpGly/Ala hydrophobicities is between 4 and 5. The fact that in the α-helixphilic scale (1), Ala and cpGly are located in the same position, suggests that the hypothetical conformational inner helix parameter in Table II of Chou & Fasman for cpGly should be much less than that of Ala.

Recently, by using a somewhat different approach, Arfmann *et al.* (1975) have studied the influence of amino acid side-chains on α-helix stability and, for the amino acids common to their studies and ours, the following α-helix stabilizing power was found

$$\text{Gly} < \text{Phe} < \text{Ala} < \text{Ile},$$

which is in accord with our results.

The observed discrepancy between the results obtained and those expected from the conformational parameters is not surprising, since Chou & Fasman (1974) have already pointed out that such utilisation may lead to wrong results when applied to isolated protein fragments in water, while in chloroethanol a good agreement was found between the helical content of horse cytochrome *c* fragments and the ordered structures in the native protein from X-ray data (Toniolo *et al.*, 1975). On the other hand, the conformational parameters (related to amino acids in proteins) and the *s* values (determined for amino acids in linear polypeptides) were in good agreement for residues with highly hydrophobic side-chains such as Leu (Chou & Fasman, 1974), or else they were in good agreement when the *s* values were obtained from linear polypeptides in a less aqueous environment, e.g. for glutamic acids, $P_\alpha = 1{\cdot}53$ (Chou & Fasman, 1974), $s = 1{\cdot}19$ in aqueous solution and 1·43 in 20% (v/v) ethanol (Hermans, 1966). The results from the values extrapolated to 100% trifluoroethanol in Figure 2 do not completely agree with those expected from the conformational

parameters of the residues in position 8: a better agreement is reached if the hydrophobicities of their side-chains are also taken into account.

Since the sequence in α-helix of the S-peptide is bound to the S-protein by its hydrophobic face while the hydrophilic face is exposed to water, the limits of this approach may be removed by utilising the well-known property of S-peptide and S-protein to recombine giving a non-covalent complex (RNAase S′), whose structure is indistinguishable from RNAase S (M. Mammi, personal communication). From the known tertiary structure of RNAase S, it can be seen that the side-chain of the invariant Phe8 residue is located in a hydrophobic pocket of the S-protein portion (Richards & Wyckoff, 1971). Both S-peptide and S-peptide analogues assume an α-helical conformation (residues 3 to 13) in the binding process, so that the formation of the complex, which may be correlated with the tendency of the peptides to undergo the conformational transition, allows the correct use of the conformational parameters, since we are dealing with a protein. Furthermore, this system allows a direct estimation of the influence of side-chain–side-chain interactions which are present in tertiary structure. Due to the modifications afforded in position 8 (Fig. 3), the binding force of the S-peptide analogues to S-protein increases in the order

$$(\text{Gly, Ala}) < \text{Ile} < \text{cpGly} < \text{Tyr} < \text{Phe}.$$

The location of the strong α-helix former, alanine, at the same level as the strong α-helix breaker, glycine, is surprising when the fact is considered in the light both of the α-helixphilic scale 1 and of the conformational parameter values. This finding may be explained by assuming that the hydrophobic side-chains, constituting the hydrophobic pocket in which Phe8 is inserted in the natural protein, need a big group to interact with. A second reason lies in the energetically favourable transfer of a more hydrophobic side-chain into a less polar environment. These hypotheses are supported by the greater binding force to S-protein of the Ile8 analogue, when compared with that of Ala8. Isoleucine is a much weaker α-helix former than alanine but a much more hydrophobic residue.

The Phe8 analogue shows the greatest ability in binding to S-protein. We can reasonably assume that this behaviour does not result from the conformational parameter of Phe, which is only a little stronger as an α-helix former than Ile and much weaker than Ala, but must result mainly from the formation of the largest hydrophobic interactions between the phenylalanine benzene ring and the surrounding side-chains in the reconstituted RNAase S′. Having established that the conformational parameters play only a marginal role, the decreased binding constant due to the Phe/Tyr replacement should be attributed mainly to the presence of the phenolic hydroxyl group. The association process of the Tyr8 analogue to S-protein implies the burying of the Tyr8 side-chain in a hydrophobic pocket without hydrogen bonding (B. Filippi *et al.*, unpublished results), and is characterized by two energetically unfavourable situations, the destruction of the phenol–water hydrogen bond and the transfer of the hydroxyl group in an apolar environment. The hydrophobic scale presented by Nozaki & Tanford (1971) was determined from extrapolated values of solubility measurements in water/ethanol and water/dioxane solutions, that is, in conditions where the phenolic hydroxyl group is hydrogen-bonded. This may explain the slight difference in the free energy of transfer of Phe and Tyr side-chains into a hydrophobic environment. Chignell & Gratzer (1968) have correctly pointed out the energetic cost of burying a tyrosyl side-chain in the interior of a protein

without hydrogen bonding. The result is that the Nozaki & Tanford (1971) hydrophobic scale can be applied correctly to the transitions induced by trifluoroethanol on the isolated peptides, but not to the binding of the Tyr8 analogue to S-protein. The dissociation constant of the [Tyr8,Orn10]-RNAase S′, determined by applying a modification of the method of Berger & Levit (1973) is 12 times greater than for the [Phe8,Orn10]-RNAase S′ ($8 \times 10^{-6}$ M against $0{\cdot}6 \times 10^{-6}$ M).

According to these facts, the higher binding constant of the cpGly analogue, when compared with the Ala and Ile analogues, is easily explained by the energetically favourable transfer of the cpGly side-chain in an apolar medium and by the formation of larger hydrophobic bonds of the cyclopentyl ring with the surrounding side-chains.

Dunn & Chaiken (1975) have compared the binding properties to S-protein of the natural S-peptide with the [Leu9]- and [Gly9]- (1-15)-S-peptide analogues and have found that the binding constant decreased 3·05 and 21·2 times, respectively. In the RNAase A of 24 species of which the N-terminal amino acid sequence has been determined in position 9, Glu is found 17 times, Gln 6 times and Lys once (Welling *et al.*, 1975). According to the Chou & Fasman (1974) conformational parameters, Glu is a strong α-helix former, Gln is an α-helix former and Lys a weak α-helix former. The frequency and the nature of the three side-chain types suggests a tendency for RNAase A to have a hydrophilic strong α-helix-forming residue in this position. In crystalline RNAase S, the Glu9 side-chain is exposed to the solvent, so that an unfavourable interaction between the hydrophobic leucyl side-chain and water can strongly contribute to the three times decreased binding constant caused by the Glu/Leu replacement.

Concerning Phe8, the two approaches we have used lead to the following conclusions. The transitions of the isolated peptides induced by trifluoroethanol suggests that the conformational parameter of the residue in position 8 plays an important but not exclusive role in α-helix stability, since a strong positive influence derives from the interaction between the hydrophobic side-chain and the backbone. In the binding process of the S-peptide analogues to S-protein, the conformational parameter of residue 8 plays no apparent role. Since the application of the conformational parameters in locating helix, β and coil regions in proteins is highly successful, the results we have obtained related to position 8 in RNAase S′ may constitute an exception, because of the prevailing role of side-chain–side-chain interactions. It is just this exception that allows us to explain the presence of the invariant phenylalanine residue in position 8. In the bovine pancreatic RNAase A, the hydrophobic interactions with the benzene ring of Phe8 side-chain involve the residues Val47, Val54, Val57, Val108, Ile81, Ile106, Leu51, Met79, Pro117 and Phe120: such a large number of residues, distributed along three fifths of the protein, must have exerted an extraordinary pressure against any change.

This invariance seems strictly related to the folding process of RNase A. Regarding this problem, it is generally suggested (Anfinsen & Scheraga, 1975; Fasman & Chou, 1974) that short- and medium-range interactions play a more prevalent role than long-range interactions. The folding process is believed to start from one or more backbone nucleation sites, determined by short-range interactions, which acquire additional stabilization from medium-range interactions; through the formation of β-turns, also depending on short- and medium-range interactions, the initial ordered structures are oriented in such a way as to allow the constitution of long-range interactions and the minimization of the energy of the whole structure. It was

shown by Anfinsen & Haber (1961) that fully reduced RNAase A can be reoxidized in air to reconstitute the native protein with full enzymic activity. On the contrary, the reduced S-protein may be correctly reoxidized with a yield of only 20%; when the reduced S-protein is reoxidized in the presence of S-peptide, a much higher activity is found (Kato & Anfinsen, 1969); this finding clearly indicates that for the correct folding of the enzyme, conformational information is located in the S-peptide fragment, and that this information is active in the folding process in spite of the lack of the peptide bond between residues 20 and 21. The results shown in Figure 3 exclude the possibility that short- and medium-range interactions are responsible for this conformational information in S-peptide and unambiguously point to long-range interactions. As a consequence, the formation of the hydrophobic core of a protein can be the driving force of the folding process. The balance between short-, medium-, and long-range interactions is actually not determined. The RNAase S′ complex is a good candidate for such an investigation, since many S-peptide analogues, still able to activate S-protein, have been synthesized (Scoffone *et al.*, 1973).

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