Interaction of S-Protein with S-Peptide and with Synthetic S-Peptide Analogs. A Spectroscopic and Calorimetric Investigation*

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ABSTRACT: The interaction of S-peptide, or some S-peptide analogs, with S-protein has been studied by difference spectroscopy, microcalorimetry, difference optical rotatory dispersion, and thermal denaturation experiments. The obtained results indicate that, in the absence of substrate, S-peptide and [Orn^{10}]-S-peptide equally bind to S-protein and that the same number of residues undergo a coil-to-helix conformational transition during the binding process. In agreement with values reported by another laboratory the spectrophotometric dissociation constants for the different ribonuclease S′ are about three orders of magnitude larger than those determined by using recovery of activity as the criterion for association. Some thermodynamic parameters for the process RNase S′ $\rightleftharpoons$ S-protein + S-peptide are also reported. On the contrary S-peptide analogs in which, in addition to the arginine–ornithine substitution, the phenylalanyl residue in position 8 has been replaced either by a glycyl or by a cyclopentylglycyl residue, are unable to bind to S-protein and to undergo the conformational transition. The comparison with our previous data on the conformation of S-peptide analogs in the presence of organic solvents indicates that S-protein acts through specific effects and is thus able to bind and to induce the conformational transition only in those S-peptide analogs possessing particular structural features.

As is well known (Richards and Vithayathil, 1959), limited proteolysis of bovine pancreatic ribonuclease A (RNase A) by subtilisin yields a fully active derivative RNase S. The only change in the covalent structure is the split of one peptide bond between residues 20 and 21 or 21 and 22 (Doscher and Hirs, 1966). The structure of the intact enzyme has been worked out by Kartha *et al.* (1967), while that of RNase S has been solved by Wyckoff *et al.* (1967). When the two forms are compared, it is apparent that, aside from the fanning out of the cut ends of the chain in RNase S, there is indeed little difference between them.

The N-terminal fragment (S-peptide) and the remainder of the molecule (S-protein), which are tightly bound with each other by noncovalent bonds, can be separated by treatment with trichloroacetic acid (Richards and Vithayathil, 1959) or by gel filtration on Sephadex G-75 using 50% acetic acid as the eluent (Doscher and Hirs, 1967).

Neither fragment by itself has biological activity while their equimolar mixture (RNase S′) exhibits nearly the same enzymic activity as RNase A and RNase S.

In our laboratories a rather detailed investigation has been carried out of the conformational and energetic changes accompanying the interaction of the S-protein with the S-peptide as well as with different S-peptide synthetic analogs in dilute aqueous solution (at neutral pH).

In this paper we discuss in detail only the results obtained by studying the S-peptide, the [Orn^{10}]-S-peptide (Scoffone *et al.*, 1966), the [Gly^{8},Orn^{10}]-S-peptide (Rocchi *et al.*, 1968), and the [Cpg^{8},Orn^{10}]-S-peptide[1] (Borin *et al.*, 1971), although the results and conclusions are valid also for the other S-peptide analogs so far examined. The [Orn^{10}]-S-peptide is able to regenerate high ribonuclease activity in the presence of S-protein and is the parent of the [Orn^{10}] series of S-peptide analogs. On the contrary the [Cpg^{8},Orn^{10}]- and [Gly^{8},Orn^{10}]-S-peptide are unable to bind and to activate S-protein.

Natural S-peptide is the standard reference for our measurements.

Different types of experimental approaches have been utilized, namely, (a) difference spectroscopy; (b) ORD differential spectroscopy; (c) microcalorimetry (heats of mixing); (d) melting curves (thermal denaturation measurements).

Measurements of types a and c have permitted direct evaluation of the stability constant for different S-protein–S-peptide (or S-peptide analog) adducts and their enthalpy of formation.

ORD differential spectroscopy allowed us to estimate the increase in the helix content in several partially synthetic RNase S′ analogs in comparison with the S-protein. Finally, the thermal denaturation of RNase S′ analogs has also been studied by means of spectral measurements.

In all cases, moreover, the biological activity of RNase S′ and of RNase S′ analogs was monitored in a systematic fashion, by means of standard catalytic activity assays (Marchiori *et al.*, 1971).[2]

Experimental Section

Materials. RNase A was obtained from Seravac Laboratories Ltd. (Maidenhead-Berks, England) or was prepared by the procedure of Crestfield *et al.* (1963) starting from bovine pancreatic ribonuclease (Fluka A. G. Buchs, Switzerland,

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[1] Cpg is for cyclopentyglycine.

[2] Unpublished results.

four-times crystallized). RNase S, S-protein, and S-peptide were prepared from RNase A by using the proteolytic enzyme designated subtilopeptidase A (Novo Industry AS, Copenhagen, Denmark) essentially by the method of Richards (Richards and Vithayathil, 1959; Gordillo *et al.*, 1962; Marzotto *et al.*, 1964) modified by Doscher and Hirs (1967).

Cytidine 2′,3′-phosphate sodium salt and yeast ribonucleic acid (RNA) were obtained from Schwarz Bioresearch (Orangenburg, N. Y.). RNA was purified by exhaustive dialysis, first against 0.1 M sodium chloride and then against water (Wellner *et al.*, 1963). The different S-peptide synthetic analogs were prepared in this laboratory. For reference, see Table I.

Methods. Solutions of RNase A and S-protein were prepared by dissolving the dry, salt-free powders in 0.01 M sodium phosphate buffer (pH 6.8), containing 0.9% sodium chloride (Sherwood and Potts, 1965a).

S-Peptide and S-peptide analogs were dissolved in water. In some cases the measurements (a) were carried out in β,β-dimethylglutaric acid–sodium hydroxide buffer (DMG, pH 6.0) (Marchiori *et al.*, 1971).[2] All the solutions were passed through a Millipore filter (Bedford, Mass.) pore size 3.0 μ. The pH was measured with a Phillips pH meter equipped with a 4–10 scale expander.

Concentrations of RNase A and S-protein solutions were determined by measuring the optical density at 280 nm using values of 0.695 for RNase A (Sherwood and Potts, 1965b) and 0.784 for S-protein (Potts *et al.*, 1964) as the absorbance of a 1-mg/ml solution.

The concentrations of S-peptide and S-peptide analogs were determined by measuring the number of micromoles of amino acids resulting from acid hydrolysis (22 hr, 110°) of an aliquot of the peptide aqueous solution (Spackman *et al.*, 1958). For the spectrophotometric measurements the aqueous solution of the S-peptide, or of the S-peptide analog, was diluted with an identical volume of 0.02 M sodium phosphate buffer containing 1.8% sodium chloride (pH 6.7). Spectrophotometric titrations were performed with a Cary Model 15 recording spectrophotometer, equipped with a 0 to 0.1 optical density unit slide-wire at 20°, with tandem-mix fused quartz cuvets (238 QS) obtained from Hellma GmbH and Co. (Mullheim/Baden, West Germany). The two chambers of these cuvets can contain 1.5 ml of solution and have an identical light path of 4.375 mm.

Samples of 1.0 ml of S-protein solution (concentrations varied from 6×10^{-5} to 7.5×10^{-5} M) were placed in one compartment of both experimental and reference cuvets and 1.0 ml of the solution containing the desired amount of S-peptide, or S-peptide analog, was placed in the other compartment of each cuvet. The absorption cells were placed in the cell compartments of the spectrophotometer, thermostated at 20°, and 15 min was allowed for the equilibration. The base line was then checked and adjusted if necessary, the contents of the experimental cuvet were mixed by inversion, and the difference spectrum was recorded after 5 and 15 min. No time dependence was observed.

Differential ORD measurements have been performed on a Cary 60 spectrophotometer using a differential cell holder and the same tandem-mix fused quartz cuvets employed in the spectrophotometric titrations.

Concentration of S-protein, S-peptide, and S-peptide analogs aqueous solutions were determined as indicated above.

The measurements have been carried out in the following way: the differential cells in the sample holder and in the reference holder contained exactly the same known amounts of identical solutions of S-protein in one compartment and of S-peptide, or S-peptide analog, in the other one. The base line of the spectrum was then recorded, the content of the experimental cuvet was mixed by inversion, and the difference ORD spectrum was recorded. The scanned spectrum showed directly the difference in the ORD spectrum of the S-peptide and S-protein solutions before and after mixing, which reflects the conformational changes occurred in the system as a consequence of the mixing process. In some cases the differential ORD pattern just overlaps the base line, indicating that no conformational transition takes place on mixing the S-peptide analog with S-protein. In other cases the differential ORD pattern exhibits the typical shape of the spectrum of the right-handed, α-helical form, with a minimum at 233 nm. From the $[m']$ value at 233 nm the extent of α-helix formed by mixing different S-peptide analogs with S-protein was calculated. Moreover, from this figure, the number of amino acid residues which underwent the coil-to-helix transition during the mixing process was also estimated.

TABLE I: Maximum S-Protein Activating Capacity of S-Peptide and Some S-Peptide Analogs at the Indicated Molar Ratio. Substrate RNA.

Peptide	Maximum % of the RNase S′ activity (substrate RNA)	Peptide: protein molar ratio
S-Peptide	100	1
[Orn^{10}]-S-peptide[a,b]	45	9
[Gly^{8},Orn^{10}]-S-peptide[c]	0	50
[Cpg^{8},Orn^{10}]-S-peptide[d]	0	50

[a] Scoffone *et al.*, 1966. [b] Marchiori *et al.*, 1971.[2] [c] Rocchi *et al.*, 1968. [d] Borin *et al.*, 1971. Cpg is for cyclopentylglycine.

Calorimetric measurements were carried out at 25° with a LKB 10700 batch microcalorimeter. Weighed amounts of S-protein and S-peptide solution (range of concentration 3×10^{-5} to 2×10^{-4} mole per l.) were poured with syringes in the two cell compartments. After 5–6 hr of equilibration the cell was rotated and the solutions mixed. Corrections for heats of friction were made. No correction for heats of dilution has been made due to the very low contribution of this phenomenon at the concentrations used.

The instrument used, which has a capacity of about 6 ml of reactant solutions, gives an accuracy of the order of *ca.* 2% for a rapid reaction having a heat absorption, or evolution, of 2 mcal. The molar absorbance change (Sherwood and Potts, 1965b) associated with heating solutions of RNase A, RNase S, S-protein, and some RNase S′ analogs in phosphate buffer, or in DMG buffer, were measured by difference spectroscopy at λ 287 nm for RNase A, RNase S′, and partially synthetic analogs and at λ 286 nm for S-protein.

Protein concentrations varied from 8.0×10^{-5} to 6×10^{-5} M for RNase A and S-protein. In the reconstituted enzymes a peptide:protein ratio of about 4:1 for RNase S′ and 10:1, or higher, for RNase S′ analogs was used and the enzyme concentration was considered to be equal to that of the S-protein (from 4.0×10^{-5} to 3.0×10^{-5} M).

Identical concentrations were placed in three 1.0-ml, quartz-stoppered cuvets in the Model 15 Cary recording spectro-

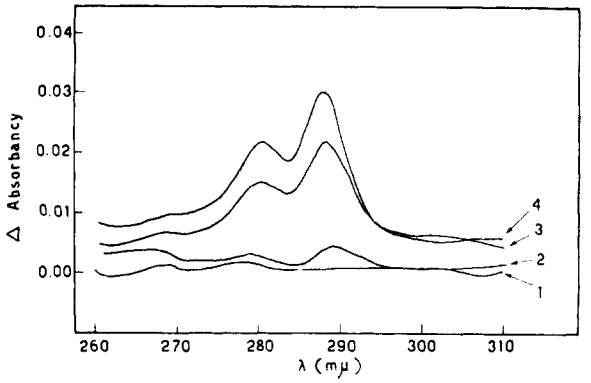

FIGURE 1: Ultraviolet difference spectra for the reassociation of S-peptide and S-protein. Curve 1 is for the base line; curve 2 is for a molar ratio of S-peptide to S-protein of 0.198; curve 3, 0.794; and curve 4, 1.72. See text for conditions.

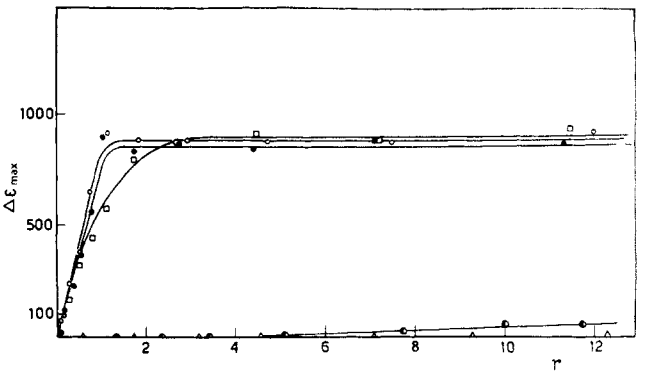

FIGURE 3: Spectrophotometric titration curves for the titration of S-protein with S-peptide and three S-peptide analogs. The maximum absorbance change in the 287- to 289-nm region, in experiments similar to those shown in Figures 1 and 2, are expressed as molar extinction changes as a function of the peptide:S-protein molar ratio, *r*. Unless otherwise indicated the measurements are carried out in 0.01 M sodium phosphate buffer (pH 6.8) containing 0.9% sodium chloride. —●— S-peptide; —○— [Orn[10]]-S-peptide; —◐— [Cpg[8],Orn[10]]-S-peptide; —△— [Gly[8],Orn[10]]-S-peptide; —□— [Orn[10]]-S-peptide in DMG buffer (pH 6.0). See text for conditions.

photometer mentioned before. The reference cell and one of the experimental cuvets were kept at a constant temperature of 15° by circulating water through the jacket of the reference chamber and the jacketed cuvet holder. The temperature of the other sample solution was regulated by a circulating water bath connected with the jacketed cuvet and measured by a mercury thermometer just at the exit of the drain pipe from the cuvet holder. After the desired temperature was reached there was a delay of 15 min to eliminate any possible time-dependent change in the absorbance. The base line was then checked, and adjusted if necessary, and the difference spectra finally recorded.

Results

Prior to displaying the results of measurements of the types a, b, c, and d mentioned above it is useful to summarize some information on the various synthetic peptides examined in this work.

The S-peptide analogs and the molar ratios to S-protein at which their maximum catalytic activity against RNA was observed are reported in Table I.

Another point relevant for the final discussion is that, using the activity assay to monitor the association of S-protein with [Orn[10]]-S-peptide, a dissociation constant, K_d, of the order of 10^{-7}–10^{-8} has been calculated[3] on the basis of the procedure of Richards and Vithayathil (1959).

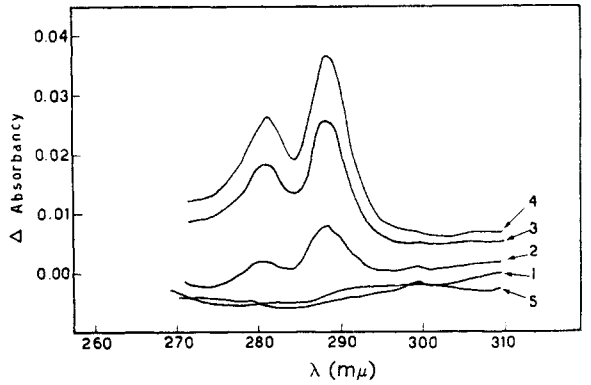

FIGURE 2: Ultraviolet difference spectra for the reassociation of [Orn[10]]- and [Gly[8],Orn[10]]-S-peptide with S-protein. Curve 1 is for the base line; curve 2 is for a molar ratio of [Orn[10]]-S-peptide to S-protein of 0.326; curve 3, 0.778; and curve 4, 1.15. Curve 5 is for a molar ratio of [Gly[8],Orn[10]]-S-peptide to S-protein of 20.8. See text for conditions.

(*a*) *Difference Spectroscopy Studies.* When S-peptide analogs capable of yielding RNase S′ analogs having catalytic activity were examined by the spectrophotometric procedure, typical dual-peaked, tyrosine difference spectra were recorded (Woodfin and Massey, 1968). On the contrary, interaction of catalytically inactive peptides with S-protein did not result in any measurable spectral change in the aromatic region. For example, the difference spectra obtained with the S-peptide, [Orn[10]]-S-peptide, and with [Gly[8],Orn[10]]-S-peptide in phosphate buffer, respectively, are shown in Figures 1 and 2.[4] The total $\Delta\epsilon$ in the 287- to 289-nm region, where the optical density difference exhibits a maximum, was estimated to be about 900 M^{-1} cm^{-1}, in reasonable agreement with values found by others (Woodfin and Massey, 1968; Sherwood and Potts, 1965b).

If the maximum absorbance changes recorded in the 287- to 289-nm region, in experiments similar to those shown in Figures 1 and 2, are plotted as molar extinction changes against the peptide:protein molar ratio, the curves of Figure 3 are obtained. The curves obtained for the S-peptide and for the [Orn[10]]-S-peptide, in phosphate buffer, reach a maximum at a peptide:protein molar ratio between 1 and 2. For the [Cpg[8],-Orn[10]]-S-peptide the molar absorbance change becomes detectable only at higher molar ratios, between 4 and 5, and at a molar ratio of 10, $\Delta\epsilon$ 287, is only about 10% of the maximum molar absorbance change obtained with the S-peptide. Finally, the [Gly[8],Orn[10]]-S-peptide shows a difference spectrum completely flat up to a molar ratio of about 20.

If one assumes that the absorbance change in the aromatic region monitors the extent of re-formation of RNase S′, the peptides examined can then be divided roughly in two groups: those which are able to bind completely S-protein in a peptide:protein molar ratio between 1 and 2, in phosphate buffer, and

[3] Unpublished results from this laboratory.

[4] No appreciable absorbance increase was observed above 300 nm in the difference spectra and, consequently, no correction for light scattering was made.

TABLE II: Dissociation Constants (K_d) of Different S-Protein S-Peptide (or S-Peptide Analogs) Adducts Calculated by Spectrophotometric Technique. See Text for Conditions.

Peptide	K_d	Buffer (M)	pH
S-Peptide	2×10^{-5} [a,b]	0.1 acetate	4.5
S-Peptide	7×10^{-5} [b]	10^{-3} sodium phosphate	
S-Peptide	1×10^{-5} [c]	10^{-2} sodium phosphate	6.8
[Orn[10]]-S-peptide	0.2×10^{-5} [c]	10^{-2} sodium phosphate	6.8
[Orn[10]]-S-peptide	2.0×10^{-5} [c]	DMG	6.0

[a] Richards and Logue, 1962. [b] Woodfin and Massey, 1968. [c] This work.

those which do not bind to S-protein appreciably even at a molar ratio 10 to 20 times higher.

A specific stabilizing effect of phosphate ions (Sela and Anfinsen, 1957; Sela *et al.*, 1957) is suggested by the fact that with the [Orn[10]]-S-peptide the re-formation of the abnormal tyrosine residue was obtained with a peptide:protein molar ratio of less than 2 in phosphate buffer and between 3 and 4 in DMG buffer (Figure 3).

The dissociation constant (K_d) values for the S-peptide, and for those S-peptide analogs which are able to bind S-protein, were calculated according to the procedure suggested by Woodfin and Massey (1968). Our values appear to be, within the limits of the method, of the same order of magnitude as those found by these authors, by difference spectroscopy, for the S-peptide–S-protein system or those calculated by the same authors from the data of Richards and Logue (1962). Some of these K_d values are reported in Table II.

It is to point out that the K_d values determined by us for the [Orn[10]]-RNase S′ (see above), and by Richards and Vithayathil (1959) for the RNase S′, using the activity assay, are not in agreement with the spectroscopic K_d values, the former being distinctly smaller than the latter.

(*b*) *Difference ORD Spectra.* With this technique it has been possible to detect the increase in helix content when S-protein was mixed with S-peptide, or with an S-peptide analog, even when the number of amino acid residues in the α-helical conformation was very small.

As an example Figure 4 shows the ORD differential spectra obtained for the recombination of S-protein with S-peptide and [Gly[8],Orn[10]]-S-peptide in 1:1 and 2:1 molar ratio, respectively. The number of residues undergoing the coil-to-helix conformational transition, the peptide:protein molar ratio, and the per cent of helical increase in the reconstituted enzymes are shown in Table III for [Orn[10]]- and [Gly[8],Orn[10]]-S-peptide analogs in comparison with S-peptide. One can reasonably assume that the coil-to-helix conformational variation deals mainly with amino acid residues contained in the S-peptide portion and consequently that the helical content of S-protein does not change markedly by adding S-peptide. If this is true it appears clear that the S-peptide analogs which possess potential catalytic activity and are able to bind to S-protein, as judged by the re-formation of the "abnormal" tyrosine residue, are also able to undergo the coil-to-helix conformational transition, but the presence of S-protein does not induce the disordered form–ordered form transition in those analogs which neither bind nor activate S-protein.

These findings, which fully agree with the data obtained by difference spectroscopy, confirm the hypothesis that S-protein can act only through very specific effects and is thus able to recognize, to bind, and to change the conformation of only some S-peptide analogs, although all the analogs examined possess the structural requirements which are needed to undergo the solvent-induced conformational transition, as demonstrated by the comparison of the CD spectra in water and in presence of organic solvent (Tamburro *et al.*, 1968; Scoffone *et al.*, 1971).

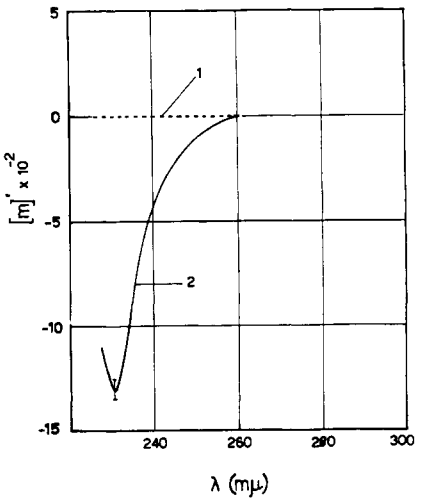

FIGURE 4: Differential ORD spectra obtained for the recombination of S-protein with [Gly[8],Orn[10]]-S-peptide (curve 1, peptide:protein molar ratio 2:1) and S-peptide (curve 2, peptide:protein molar ratio 1:1). See text for conditions.

(*c*) *Microcalorimetry (Heats of Mixing).* The results of the microcalorimetric measurements are shown in Figure 5. It is evident from these data that interaction of the S-protein with the S-peptide, as well as with the [Orn[10]]-S-peptide, in dilute aqueous solution is accompanied by a large decrease in enthalpy, ΔH, of the system. The ΔH attains a limiting value of approximately -44 kcal/mole (kcal per mole of the maximum stoichiometric amount of RNase S′ present in solution) at a molar ratio $r > 2$ at 25°. Figure 5 also shows, however, that the ΔH values in the case of the S-peptide are systematically higher than in the case of the [Orn[10]]-S-peptide.

TABLE III[a]

	Peptide:protein ratio[a]	% helix	No. of residues undergoing coil–helix transition (±0.8)
S-Peptide	1	8.6	10.7
	2	8.6	10.7
[Orn[10]]-S-peptide	2	8.6	10.7
[Gly[8],Orn[10]]-S-peptide	2	0	0

[a] Per cent of helix formation and number of residues undergoing the coil-to-helix transition by mixing S-protein with S-peptide and two S-peptide analogs, in the indicated molar ratio, as determined by differential optical rotatory dispersion. Concentration of S-protein, 1.689 mg/ml in 0.01 M sodium phosphate buffer (pH 6.8) containing 0.9 M sodium chloride.

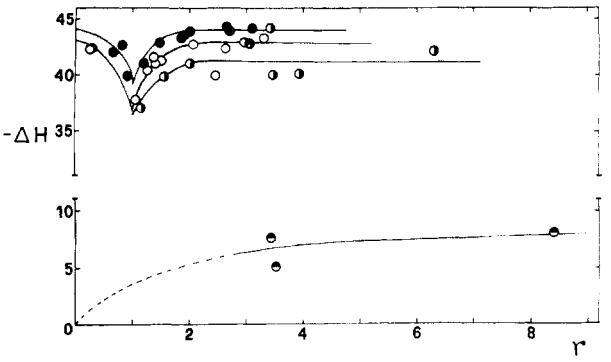

FIGURE 5: Enthalpy change for the recombination of S-protein with S-peptide and two of its analogs as a function of the peptide:S-protein molar ratio, *r*. Unless stated otherwise the measurements are carried out in 0.01 M sodium phosphate buffer (pH 6.8) containing 0.9% sodium chloride. ΔH is in kilocalories per mole of RNase S′ or RNase S′ analog. The enzyme concentration is calculated on the basis of the peptide or S-protein concentration assuming complete recombination. —●— S-peptide; —◑— [Orn[10]]-S-peptide; —◓— [Cpg[8],Orn[10]]-S-peptide; —○— S-peptide (in water).

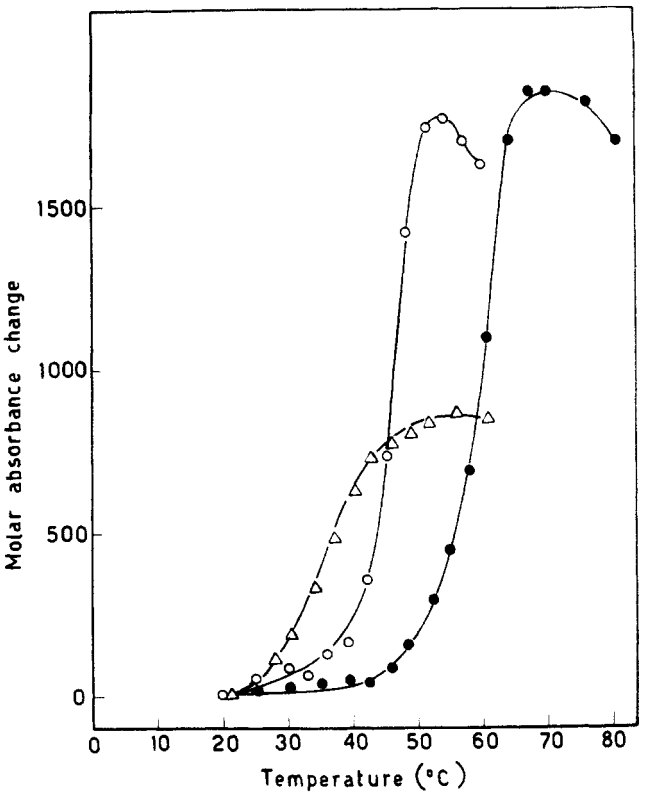

FIGURE 6: The molar absorbance changes associated with heating solutions of RNase A, [Orn[10]]-RNase S′, and S-protein in 0.01 M sodium phosphate buffer (pH 6.8) containing 0.9% sodium chloride. The values were obtained by difference spectroscopy at λ 287 for RNase A and [Orn[10]]-RNase S′ and at λ 286 for S-protein. See text for conditions. —●— RNase A; —○— [Orn[10]]-RNase S′; —△— S-protein.

This difference may be tentatively attributed to the lack of the suggested interaction between glutamic acid 2 and arginine 10 in the S-peptide (Hofmann *et al.*, 1970). With the (Cpg[8],-Orn[10])-S-peptide, however, our microcalorimetric data indicate much weaker interactions with S-protein, since only *ca.* 8 kcal/mole are released when this peptide is mixed with S-protein at molar ratios higher than 8. From our ΔH data for the S-peptide–S-protein system it is possible to derive, according to the procedure outlined by Bjurulf *et al.* (1970), for the equilibrium constant, K_d, of the process

$$\text{RNase S}' \rightleftarrows \text{S-protein} + \text{S-peptide} \qquad (1)$$

a mean value of 0.62×10^{-5} (standard deviation of the mean 0.15×10^{-6}) at 25°. This leads to a free-energy change, $\Delta G°$, for the dissociation reaction 1 of *ca.* 7.1 kcal mole^{-1} at 25°.

Neglecting the small heat of dilution effects, and thus assuming as a first approximation that our ΔH data are standard state values, $\Delta H°$, one readily estimates, for the above mentioned process, a value of 124 eu (in phosphate buffer). The marked increase in entropy connected with the process 1 must, evidently, be largely determined by the increased conformational freedom both of S-protein and especially of S-peptide when separate in dilute aqueous solution. In the case of the [Orn[10]]-S-peptide–S-protein system it is difficult to evaluate a reliable K_d value on the basis of our calorimetric data. However the order of magnitude of this K_d value should not be too different from that obtained for reaction 1.

Very recently, Hearn *et al.* (1971) have published the results of a detailed investigation on process 1, carried out by means of equilibrium and calorimetric measurements over a wide temperature range. These authors report the following thermodynamic data for the dissociation reaction in 0.3 M NaCl (pH 7.0) at 25°: $\Delta H = 39.8$ kcal mole^{-1}; $\Delta G° = 10.5$ kcal mole^{-1}; $\Delta S° \simeq 98$ eu. The ΔH value was interpolated from a set of batch microcalorimetry data for different temperatures, and the $\Delta G°$ was extrapolated using equilibrium constant values for reaction 1 derived by means of enzyme assays at 35, 40, and 45°.

The same authors also report the results of a set of flow calorimetry experiments according to which no trend is discernible in ΔH with varying concentration of the reactants, which indicates that the reaction between S-peptide and S-protein is complete at all concentrations used. The mean value of ΔH, +33.6 ± 0.9 kcal mole^{-1}, (flow calorimetry) agrees fairly well, in the opinion of Hearn *et al.*, with the value found by means of batch calorimetry. By the same token one might argue that also our calorimetric, limiting, ΔH value for reaction 1, + 44 kcal mole^{-1}, is in fairly good agreement with the value determined by Hearn *et al.* (1971) with the batch calorimeter. However it must be pointed out that: (a) the calorimetric data reported in Figure 5, through the evident curvature of the ΔH against *r* plots, clearly indicate that reaction 1 is not completely shifted to the left at all concentrations used (which are close to those used by Hearn *et al.*); (b) the difference between our limiting ΔH value and that given by Hearn *et al.* is furthermore beyond our experimental errors.

(d) "Melting" Curves. When RNase A, RNase S′, or different partially synthetic RNases S′, and S-protein were heated, dual-peaked difference spectra characteristic of tyrosine normalization were recorded (Wetlaufer, 1962).

In Figure 6 the melting profiles obtained for RNase A, [Orn[10]]-RNase S′, and S-protein, *i.e.*, the plots of the molar absorbance changes (at 287 nm for RNase S′; at 286 for S-protein) against temperature, are reported. Consistently, a maximum absorbance change, based on repeated determinations, of −1800 ± 100 for RNase A and of −850 ± 50 for S-protein, respectively, was obtained, in reasonable agreement with previous observations (Bigelow, 1961; Hermans and Scheraga, 1962; Sherwood and Potts, 1965b). Similarly, a

TABLE IV: Spectrophotometric Data for the Thermal Transition of RNase A and Various of Its Derivatives in 0.01 M Sodium Phosphate Buffer (pH 6.8) Containing 0.9% Sodium Chloride and in 0.3 M Sodium Chloride (pH 7.0).

	pH 6.8 phosphate buffer[a]		pH 7.0 0.3 M NaCl[b]	
Substance	T_m[c] (°C)	ΔH_{vH} (T_m), kcal mole^{-1}	T_m[c] (°C)	ΔH_{vH} (T_m), kcal mole^{-1}
RNase A	59	70		
RNase S			46.2[d]	72[d]
RNase S′[e]	48.5	69		
[Orn^{10}]-RNase S′[f]	46.5	69.8		
S-Protein	37	40	36[d]	40.3[d]

[a] This work. [b] Hearn *et al.*, 1971. [c] Temperature at which the change is half-completed, the so-called melting temperature. [d] Mean values. [e] S-Peptide:S-protein molar ratio, *ca.* 4. [f] [Orn^{10}]-S-peptide:S-protein molar ratio, *ca.* 10.

molar absorbance decrease of −1800 but a marked difference in the midpoints of the thermal denaturations, was observed when S-protein was mixed with those S-peptide analogs which are able to bind to S-protein as judged by the spectrophotometric and the heat of mixing data.

In Figure 7 the extent of denaturation, expressed as the percentage of the maximum change in the absorbance at 287 nm, is plotted as a function of temperature for RNase A, RNase S′, and [Orn^{10}]-RNase-S′, respectively.

The data obtained with RNase A and RNase S′ are in good agreement with those reported by Takahashi *et al.* (1969). Finally, in Figure 7 the extent of denaturation for the [Orn^{10}]-RNase S′ in phosphate buffer and in DMG buffer are compared. It is evident that the melting curves are very similar but that the transition temperatures are slightly different, *i.e.*, 46.5° and 44.5°, respectively.

The finding that the transition temperature is a little lower in DMG than in phosphate buffer appears consistent with the observed influence of the buffer on the dissociation constants. In summary our data show that the melting temperature (T_m) of the different adducts depends, in the same buffer, on the peptide–protein affinity. T_m is in fact seen to assume the values 48.5°, 46.5°, and 44.5°, approximately, passing from RNase S′, to [Orn^{10}]-RNase S′ (in phosphate buffer), to [Orn^{10}]-RNase S′ (in DMG buffer). The melting curves and the transition temperatures obtained for [Gly^{8},Orn^{10}]- and [Cpg^{8},-Orn^{10}]-RNase S′ are instead very similar to those obtained for S-protein. Our S-protein T_m value is *ca.* 37°, in agreement with that recently reported by Tsong *et al.* (1970).

The standard enthalpy change (ΔH_{vH}) of the denaturation process has been calculated from the slope of the α *vs.* T plot (melting curves of Figure 7) at the temperature of half-conversion, T_m, by formal application of the van't Hoff equation $d \ln K/dT = \Delta H^\circ/RT^2$.

Table IV shows the spectrophotometric values obtained for RNase A, and various of its derivatives, in phosphate buffer at pH 6.8 in comparison with the values calculated by Hearn *et al.* (1971) in 0.3 M NaCl at pH 7.0.

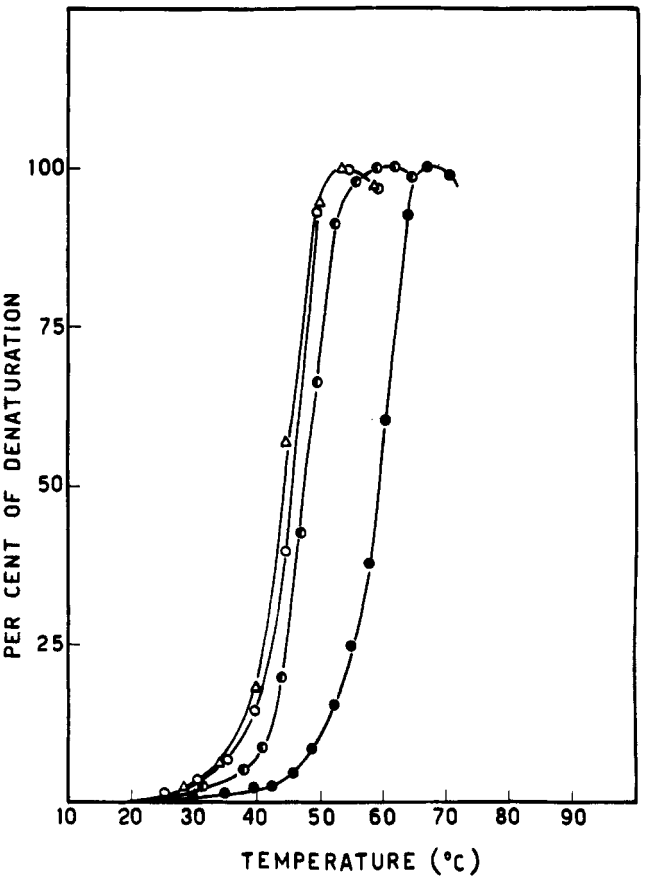

FIGURE 7: Transition temperatures of RNase A, RNase S′, and [Orn^{10}]-RNase S′. Unless stated otherwise the measurements are carried out in 0.01 M sodium phosphate buffer (pH 6.8) containing 0.9% sodium chloride. The extent of denaturation is expressed as the percentage of the maximum absorbance change at 287 nm for respective enzymes. See text for conditions. —●— RNase A; —◐— RNase S′; —○— [Orn^{10}]-RNase S′; –△– [Orn^{10}]-RNase S′ in DMG buffer (pH 6.0).

Discussion

The whole body of the data obtained by different experimental techniques and presented in this communication, indicate that, in absence of the substrate, the S-peptide and the [Orn^{10}]-S-peptide equally bound to S-protein.

Moreover the number of residues undergoing the coil-to-helix conformational transition during the binding process is the same in both peptides. These findings indicate that the substitution of arginine in position 10 with an ornithyl residue does not affect the ability of the S-peptide to bind to S-protein, when the substrate is absent. On the contrary S-peptide analogs in which, in addition to the arginine–ornithine substitution, the phenylalanyl residue in position 8 has been replaced, either by a glycyl or by a cyclopentylglycyl residue, are unable to bind and to undergo the conformational transition. The importance of phenylalanine in position 8 in the S-peptide–S-protein association process has been already discussed (Borin *et al.*, 1971).

In this connection it is important to recall that addition of organic solvents to an aqueous solution of the S-peptide brings about a coil-to-helix conformational transition of the latter (Tamburro *et al.*, 1968). A similar behavior is shown by the S-peptide analogs (Scoffone *et al.*, 1971). The extent of this conformational transition depends on the amino acid sequence of the peptide considered but both the potentially active and

inactive S-peptide analogs so far examined are able to assume a partially helical structure in the presence of appropriate organic solvents. It has been already suggested (Scatturin *et al.*, 1967) that with regard to the S-peptide conformation, the effect afforded by the addition of S-protein to an S-peptide aqueous solution is comparable to the solvent modification obtained adding organic solvents.

Our present results suggest however that, differently from organic solvents, the S-protein acts through specific effects, as a "template" solvent and is able to induce the conformational transition only in those S-peptides analogs possessing particular structural features. Further studies are needed for an understanding of the mechanism through which the recombination process takes place. At this stage it can only be reemphasized that phenylalanine in position 8 has to play a very important role in the binding process.

As already pointed out, according to our data, there is no difference in the capacity of the S-peptide and [Orn^{10}]-S-peptide to bind to S-protein in the absence of the substrate. However when activity assays are used to monitor the formation of RNase S′, RNase S′ analog, the effect of the arginine–ornithine substitution becomes significant (see Table I).

In addition, in the presence of substrates, the dissociation constant values of the adducts result about three orders of magnitude smaller than the spectroscopic and calorimetric ones. The latter set of data for the [Orn^{10}]-S-peptide–S-protein system would lead one to predict that, at the very low concentrations used in the catalysis experiments (10^{-7}–10^{-8} M) negligible amounts of RNase S′ analog should form, even at molar ratios of 9 or higher, in contradiction to the high catalytic power observed for the dilute mixture. It is at present difficult to try to account for this discrepancy.

The buffer system appears to influence the K_d (spectroscopic) values, as is shown in Table II. But, as already pointed out by Woodfin and Massey (1968), the influence of the substrate must be much more important in altering in substantial way the apparent binding affinity of S-peptide, or S-peptide analogs, with S-protein. Furthermore different substrates may exhibit quantitatively different effects, as shown by the fact that the maximum activity of RNase S′ against RNA is reached at a peptide:protein molar ratio near unity, while against cytidine 2′,3′-cyclic phosphate the maximum is only reached at a higher molar ratio (Richards and Vithayathil, 1959; Marchiori *et al.*, 1971[2]). Further support to the influence of the substrate was given by Winchester *et al.* (1970) who showed, by differential thermal analysis, that, for RNase A at pH 5.5, the formation of a 1:1 nucleotide–enzyme complex raises the transition temperature from 62 to 71.5° and the energy of activation for the thermal denaturation, at pH 4.2, from about 79 to 114.5 kcal mole^{-1}. These findings seem to indicate that the substrate plays a cooperative role, in the peptide–protein complex stabilization, and that this cooperativity is affected by the substitution of amino acid residues in the sequence of one of the two partners. In other words a chemical puzzle with three components requires a higher precision than one with only two components does. The whole body of reported results can only explain some discrepancies in the dissociation constant values obtained from activity assays carried out in different experimental conditions. However, the differences between the enzymic and spectrophotometric, or calorimetric, K_d values are not interpretable unless some basic assumptions of the kinetic scheme are not valid for the examined system.

In our opinion this is a very interesting point which has to be further investigated.

Finally it must be pointed out that Hearn *et al.* (1971) have recently evaluated, on the basis of catalytic activity data, that $\Delta G°$ for reaction 1 should be 10.5 kcal mole^{-1}, which leads to a K_d value of about 2×10^{-8}, in reasonable agreement with our K_d figure estimated by the same experimental technique. However the 25° K_d value of Hearn *et al.* (1971) was extrapolated from data obtained at temperatures in the range 30–40°, in the upper limit of which extensive denaturation of RNase S′ takes place. This fact makes, in our opinion, somewhat uncertain the above-mentioned K_d value.

But, more important, the opinion held by Hearn *et al.*(1971) that their K_d value at 25° is qualitatively substantiated by calorimetric data appears to us highly questionable. In fact, their assumption is based on a set of flow-microcalorimetric data which are to scattered to allow the conclusion that ΔH for reaction 1 is indeed invariant with reactant concentration (Hearn *et al.*, 1971). Incidentally Marzotto *et al.* (1970) have recently reported also a few data on the heat of recombination of S-protein with S-peptide, according to which $\Delta H = -14$ kcal mole^{-1} for the recombination process at 30°. This figure, in the light of our results as well as of those of Hearn *et al.* (1971) is doubtless wrong. The paper of Marzotto *et al.* (1970) does not offer any rational basis to explain this difference.

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Human Serum Lipoproteins. Evidence for Three Classes of Lipoproteins in S_f 0–2*

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ABSTRACT: The lipoprotein composition of the S_f 0–2 lipoproteins from the serum of individuals and from pooled serum was studied. S_f 0–2 was found to contain three classes of lipoproteins: high density lipoproteins (HDL_1), low density lipoproteins (LDL_3), and a lipoprotein which shares antigenic determinants with LDL, LDL-a-1. HDL_1 has a sedimentation coefficient at d 1.002 g/cm^3 ($s_{1.002}$) of 4.6 S, and a molecular weight by Agarose gel chromatography of 0.5×10^6. The electrophoretic and immunological properties of HDL_1 are similar to those of HDL_2. Delipidated HDL_1 and HDL_2 were found by polyacrylamide gel electrophoresis to contain the same polypeptides. LDL_3 has electrophoretic and immunological properties similar to those of LDL isolated from the density range 1.030–1.040 g/cm^3, and $s_{1.002}$ = 8.4 S. Its molecular weight is 1.8×10^6. LDL-a-1 has $s_{1.002}$ = 12.7 S, and a molecular weight of 5.4×10^6. LDL-a-1 is also present in the density range 1.060–1.075 g/cm^3; when isolated from that density range, LDL-a-1 was found to have $s_{1.002}$ = 13.4 S. Both LDL-a-1 fractions have a pre-β mobility by Agarose gel immunoelectrophoresis. Anti-LDL-a-1 antisera were absorbed with LDL (d 1.030–1.040 g/cm^3). The absorbed antisera precipitated with LDL-a-1 but not with LDL.

Serum lipoproteins S_f 0–2 are normally present in low concentrations, approximately 15–35 mg/100 ml. Elevated levels have been reported to be associated with high levels of very low density lipoproteins (Nichols, 1967).

There is some uncertainty in the literature about the types of lipoproteins which occur in S_f 0–2 (d 1.050–1.060 g/cm^3) and in the density range 1.060–1.075 g/cm^3. Both low and high density lipoproteins[1] have been found by immunochemical methods in the density range 1.050–1.063 g/cm^3 (Alaupovic, 1968; Lee and Alaupovic, 1970) and in the density range 1.063–1.110 g/cm^3 (Ayrault-Jarrier *et al.*, 1963; Alaupovic, 1968). The major questions raised by these observations are as follows. (1) Are HDL and LDL of different hydrated density but occur in these density ranges due to incomplete separation during centrifugation? (2) Are HDL and LDL which occur in these density ranges of similar hydrated density but nonetheless distinct molecular species? (3) Are the lipoproteins which occur in these density ranges hybrid molecules containing both HDL and LDL antigens? Answers to these questions are essential before we can proceed to study the details of polypeptide composition of the lipoproteins of these hydrated density ranges.

We have determined the types of lipoproteins which occur in S_f 0–2 (d 1.050–1.060 g/cm^3) and in the density range 1.060–1.075 g/cm^3. Lipoproteins were characterized by sedimentation and flotation analyses in the analytical ultracentrifuge, isopycnic density gradient centrifugation, Agarose gel chromatography, polyacrylamide gel electrophoresis, and immuno-

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[1] Abbreviations used are: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; the polypeptides are designated by their carboxyl-terminal amino acids as R-Thr, R-Gln, R-Glu, and R-Ala.