Thermodynamics of the Binding of S Protein to Agarose–S Peptide to Form Agarose–Ribonuclease S′*

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ABSTRACT: The thermodynamics of the dissociation of derivatized ribonuclease S′ into its component polypeptides, derivatized S peptide (residues 1–20) and derivatized S protein (residues 21–124), have been studied using special conditions which allow direct determination of binding at low concentrations of the two interacting components. The special conditions, based on the use of S peptide covalently attached to Agarose and S protein labeled with [^{3}H]acetate (0.76 or 8.9 moles per mole), permitted direct determination of free S protein in equilbirium with Agarose–ribonuclease S′ complex in terms of radioactivity. The binding studies were conducted at S-protein concentrations from 2×10^{-7} to 1×10^{-5} M at temperatures from 3–50°, at pH 7.5, and at an ionic strength of 0.1. The resulting van't Hoff plots were linear only at low temperatures; the deviation from linearity at higher temperatures appears to be a direct consequence of a thermal transition in S protein. Using only the linear temperature range, it was found that the equilibrium constant for the dissociation of Agarose–ribonuclease S′ (into Agarose–S peptide + S protein) was $2.5 \pm 1 \times 10^{-6}$ M under all the following experimental conditions: using S protein containing 0.76 mole of of acetate per mole (this derivative retained 95% of normal enzymatic activity in the presence of S peptide); using S protein containing 0.76 mole of acetate/mole, in the presence of saturating concentrations ($100 \times K_I$) of a mixture of the 2′ and 3′ isomers of cytidylate; and using S protein containing 8.9 moles of acetate/mole (this derivative retained less than 2% of normal enzymatic activity in the presence of an excess of S peptide). The thermodynamic parameters for the dissociation at 10° were $\Delta G = +7.5$ kcal mole^{-1}, $\Delta H = +1.3$ kcal mole^{-1}, and $\Delta S = -22$ cal deg^{-1} mole^{-1}, suggesting that the binding process is entirely entropy driven. These values are very different from those obtained in a recent study by Hearn *et al.* (*Biochemistry 10*, 806 (1971)). The sources of the large differences are not understood, but some aspects of the ribonuclease S′ dissociation have been discussed in an attempt to qualitatively relate differences in experimental conditions to the large variation in results obtained in different laboratories.

We have shown that the S-peptide–S-protein interactions can be studied very readily when S peptide is covalently bound to Agarose (Gawronski and Wold, 1972). The primary purpose of the present study was to determine the thermodynamic parameters of the S-protein–S-peptide interaction using this Agarose–S-peptide derivative. At the same time we also wished to evaluate the effect of derivatization of S protein both on ribonuclease S′ stability and activity and to investigate the use of radioactive labels as a means of improving the analytical precision and sensitivity of the method. To this end S protein was acetylated with high specific activity acetic anhydride, and the binding experiments were carried out with acetylated S-protein derivatives. With these derivatives, free S-protein derivative in equilibrium with Agarose–S-peptide–S-protein complex could be determined directly by radioactive counting and binding properties could be investigated independent of enzymatic activity.

While the present work was in progress a thorough study of the thermodynamics of the S-protein–S-peptide binding based on equilibrium and calorimetric measurements was reported (Hearn *et al.*, 1971). The thermodynamic parameters determined in that recent study are very different from those we had derived from our binding data; so different in fact, that while Hearn *et al.* (1971) conclude that the binding is completely energy driven, our results are consistent with a completely entropy-driven reaction. Although we do not understand the reason for the large discrepancies, some possible sources have been considered and are discussed.

Materials and Methods

The preparations of S protein, S peptide, and Agarose–S peptide are described in the companion paper (Gawronski and Wold, 1972) as are the procedures related to activity measurement with the cyclic cytidylate as substrate (Crook *et al.*, 1960) and methods for protein concentration measurement.

Preparation of [^{3}H]Acetyl–S Protein. The acetylated S protein derivatives were prepared by reacting S protein (5–10 mg/ml) with [^{3}H]acetic anhydride (50 mCi/mmole; New England Nuclear Corp.) in 1 M potassium acetate–0.5 M sodium borate buffer (pH 8.5) for 4–6 hr at 0°. The reacted protein was separated from salt and excess reagent by gel filtration on Sephadex G-25 with 0.01 M ammonium acetate buffer (pH 4.5) as solvent, and the pooled protein-containing fractions were lyophilized. The extent of incorporation of acetate was calculated from the specific radioactivity of each preparation, and was also evaluated by quantitative analysis of free and reacted amino groups, using the trinitrobenzenesulfonic acid method as described by Habeeb (1966).

S-Protein–S-Peptide Binding as a Function of Temperature. A special technique was developed for the thermodynamic studies reported here. Five sample vials, each containing exactly the same, known amount of Agarose–S peptide and five different levels of acetyl–S protein (corresponding to 5 points

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TABLE I: Properties of Acetylated Derivatives of S Protein.

S-Protein Derivative	Moles of [^{3}H]Acetic Anhydride/Mole of Protein (in Reaction Mixture)	Moles of [^{3}H]Acetate Incorporated/ Mole of Protein[a]	Moles of Amino Groups Reacted/Mole of Protein[b]	cpm/mg of S-Protein Derivative	Enzyme Act. (% of Unreacted)[c]
Lightly acetylated	2.0	0.76		1.45×10^6	95
Fully acetylated	17.0	8.9	7.74	17.2×10^6	<2

[a] Based on radioactivity incorporated. [b] Based on assay for free amino groups with trinitrobenzenesulfonate. The assay was run only for "fully acetylated" S protein. S protein contains 9 moles of amino groups/mole of protein. [c] Based on activity in the presence of an excess of S peptide.

on a titration curve) were incubated in a thermostatted aluminum block together with a blank vial, containing Agarose–glycylglycine (the same amount as Agarose–S peptide in the sample vials) and a quantity of acetyl–S protein corresponding to the median concentration of the sample vials. The vials were wetted to ensure good contact with the aluminum block, and trial runs showed that the temperature of the solutions in the vials matched that of the block within 5 min. The six vials were stirred continuously with magnetic stirrers and after temperature equilbration for 30 min, 2- to 5-μl samples were removed for analysis of free acetyl–S protein by determination of total radioactivity. Bound acetyl–S protein was obtained as the difference between the sample concentration and the appropriately adjusted blank concentration. Dissociation constants were then determined from each set of five sample concentrations (covering one decade of S-protein concentration in each experiment) as described before (Gawronski and Wold, 1972). After each analysis, the temperature of the block was changed, samples were reequilibrated and analyzed, and the entire temperature range desired was thus studied with a single set of samples. In the actual experiments 1 ml of settled Agarose–S peptide (or -glycylglycine) was suspended with 2 ml of 0.01 M potassium phosphate–0.09 M potassium chloride buffer (pH 7.5), containing the appropriate amount of acetyl–S protein. The range of S-protein concentration, covering all the binding studies reported here, was 2×10^{-7} to 1×10^{-5} M. The 2- to 5-μl samples removed for analysis at each temperature were too small to cause any need for volume corrections in routine experiments. The concentration of acetyl–S protein in the blank vial was not affected by temperature. Radioactivity analyses were performed using a Beckman Model LS-133 liquid scintillation counter. The counting medium was toluene, containing Beckman Fluoralloy, formula TLA as directed by the manufacturer. The counting medium also contained 10% Beckman Bio-Solv, formula BBS-3. Tritium-counting efficiency under these conditions was 40%.

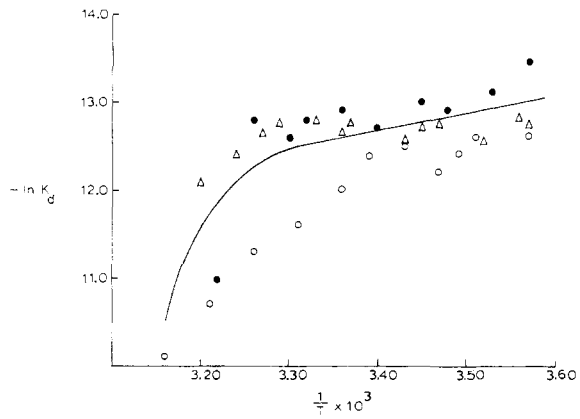

FIGURE 1: van't Hoff plot of the titration of Agarose–S peptide (0.09 mg of S peptide/ml of settled Agarose) with lightly acetylated S protein (0.76 mole of acetyl groups/mole of S protein). The 3-ml reaction mixtures contained 1 ml of Agarose–S peptide and 2 ml of S-protein solution in buffer as described in the text. The molar concentrations are calculated with the assumption that the volume occupied by the Agarose is unavailable and that the total reaction volume thus is 2 ml. This gives a molar concentration of S peptide of 2.3×10^{-5} M and the S-protein concentration ranges used in the three different experiments reported were 1.3×10^{-6} to 1.3×10^{-5} M (○), 6×10^{-7} to 6×10^{-6} M (●), and 1.1×10^{-6} to 1.1×10^{-5} M (△).

Measurement of Thermal Transitions in S Protein by Circular Dichroism. Circular dichroism measurements were made using a Durrum-Jasco circular dichorism spectrophotometer, Model CD-SP, with a thermostatted cell holder. Sample temperatures were determined using a thermistor probe immersed in the spectrophotometer cell. The solvent was 0.005 M sodium phosphate buffer (pH 7.5) and the cell path length was 0.1 cm. Protein concentrations were 0.1–0.2 mg/ml.

Results

Acetylation of S Protein. Two acetylated S-protein derivatives were prepared and their properties are given in Table I. Lightly acetylated S protein, containing slightly less than 1 mole of acetyl groups/mole of protein, retained essentially full enzymatic activity and was indistinguishable from unreacted S protein in other properties as well (see below). Fully acetylated S protein, on the other hand, containing about 9 moles of acetyl groups/mole of protein had no significant enzymatic activity and showed altered thermal stability (see below). Assuming that the presence of high concentration of acetate during the acetylation prevented the formation of *O*-acetyltyrosine derivatives (Riordan and Vallee, 1967), the incorporation of 9 moles of acetyl group corresponds to the total number of free amino groups in S protein (8 ϵ-amino groups and 1 α-amino group), and this is the basis for referring to this derivative as "fully" acetylated. However, direct determination of free amino groups (Habeeb, 1966) using unreacted S protein as standard showed that the derivative contained 1.26 moles of amino groups/mole of protein (Table

TABLE II: Typical Dissociation Constants for the Binding of S Protein and S-Protein Derivatives to Agarose–S Peptide at Different Temperatures.[a]

S-Protein Derivative	Expt No.	Temperature 10°	20°	30°	40°	50°
Unreacted S protein	1		2	3		
Lightly acetylated S protein	1	1.8	2.4	3.3	17	(No binding)
	2	3.5	3.6	6.3	28	
	3	3.0	3.2	2.9	5.6	
Fully acetylated S protein	1	2.3	2.4	5.1		
	2	1.1	2.0	6.0	>13	(No binding)
Lightly acetylated S protein in the presence of CMP	1	2.6	2.6	4.0	9.1	

[a] The dissociation constants are given in units of micromoles per liter.

I) and thus that some acetylation of other residues had taken place.

Effect of Temperature on the Dissociation Constant for Agarose–Ribonuclease S'. The results of the titration experiments are given in Figures 1, 2, and 3 in the form of van't Hoff plots and typical K_d values are summarized in Table II. The data for lightly acetylated S protein are given in Figure 1 and represent three separate experiments covering a protein concentration range from 6×10^{-7} to 1×10^{-5} M. The data for fully acetylated S protein given in Figure 2 represent two separate experiments and cover a protein concentration range from 6×10^{-7} to 6×10^{-6} M. Most other studies of S-protein–S-peptide binding have used enzymatic activity to measure the equilibrium concentration of complex (ribonuclease S') and consequently most data on the interaction parameters have been obtained in the presence of substrate. For the sake of comparison to other work and for the sake of evaluating the effect of substrate on the ribonuclease S' dissociation in our system, we carried out one additional titration of Agarose–S peptide with lightly acetylated S protein (concentration range 2×10^{-7} to 2×10^{-6} M) in the presence of 1.5×10^{-2} M substrate analog, in the form of the mixture of 2'- and 3'-cytidylate. We did not determine the actual concentration ratio of the two isomers, but established by kinetic assay that the K_I for the mixture used was 64 μg/ml (2×10^{-4} M), and the concentration of total cytidylate in the titration mixture was thus about $100 \times K_I$. The results of this last titration are shown in Figure 3 together with data from the studies of Hearn *et al.* (1971) who also carried out their S-protein–S-peptide binding studies in the presence of substrate.

Two features of the data in the three figures should be noted. First the relative values of individual dissociation constants at any given temperature (typical values have been compiled in Table II for easy access) and secondly, the nonuniform change in the dissociation constant at increasing temperature, also illustrated in Table II, but equally apparent from the curvatures of the van't Hoff plots. The conclusion from the first point is that at the low temperatures where the van't Hoff plots are linear, the dissociation constants for the Agarose–S-peptide–acetylated S-protein complexes are es-

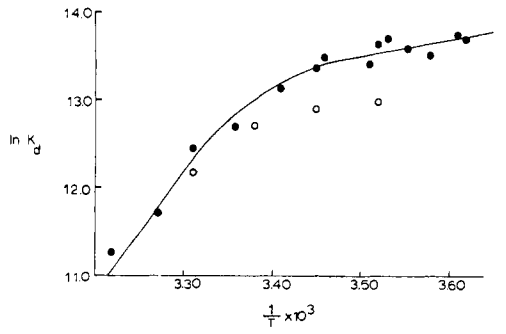

FIGURE 2: van't Hoff plot of the titration of Agarose–S peptide (0.09 mg of S peptide/ml of settled Agarose) with fully acetylated S protein (8.9 moles of acetyl groups/mole of S protein). The conditions and calculations are the same as those given in Figure 1; an S-peptide concentration of 2.3×10^{-5} M and S-protein concentration ranges of 6.5×10^{-7} to 5.4×10^{-6} M (○) and 6.5×10^{-7} to 6.5×10^{-6} M (●).

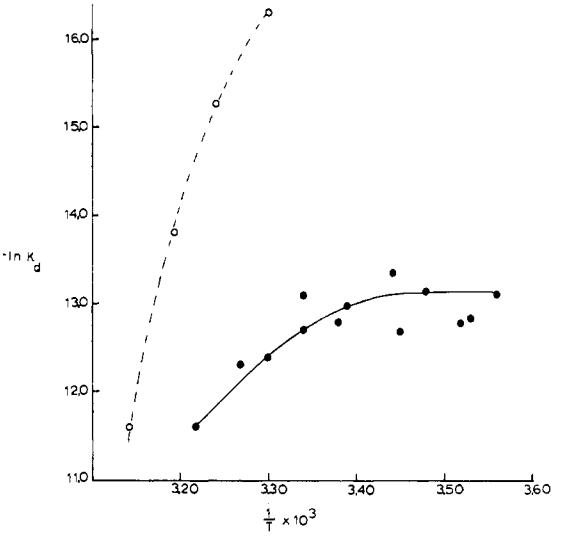

FIGURE 3: The solid line and closed circles represent the van't Hoff plot for the titration of Agarose–S peptide (0.09 mg of S peptide/1 ml of settled Agarose) with lightly acetylated S protein (0.76 mole of acetyl groups/mole of S protein) in the presence of 1.5×10^{-2} M cytidylate (a mixture of the 2'- and 3'-monophosphates). The conditions and calculations are the same as those given in Figure 1; an S-peptide concentration of 2.3×10^{-5} M and an S-protein concentration range of 2×10^{-7} to 2×10^{-6} M. The dotted line and open circles represent comparable data from Hearn *et al.* (1971) who determined ribonuclease S' concentrations by assaying for enzymatic activity. Their binding experiments were conducted at pH 7 using 4.7×10^{-7} M S protein, 6×10^{-6} to 5×10^{-5} M S peptide, and 3×10^{-4} M cyclic cytidylate.

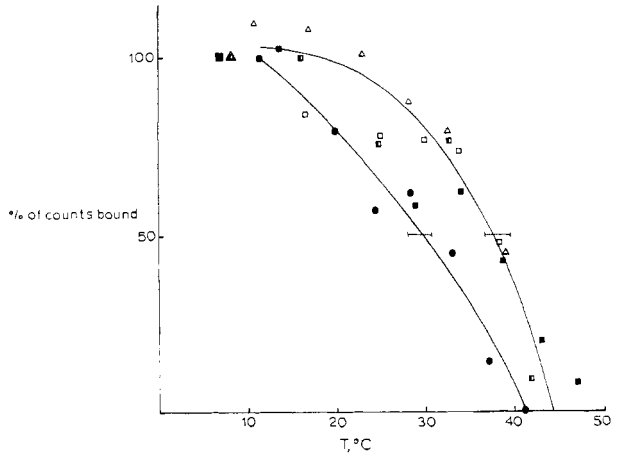

FIGURE 4: An estimate of the transition temperature based on the data in Figures 1 (□, ◧, ■), 2 (●) and 3 (△). (The data shown as open circles in Figure 2 were not included.) Each point represents the mean percent of radioactive counts bound for five samples at the given temperature relative to the mean counts bound at the lowest temperature used for that set of five samples.

sentially identical for lightly acetylated S protein both in the absence and in the presence of cytidylate and for fully acetylated (and enzymatically inactive) S protein. The average value of these dissociation constants $2.5 \pm 1 \times 10^{-6}$ M is in excellent agreement with the value 2.5×10^{-6} M determined for unreacted S protein (Gawronski and Wold, 1972). Our values for K_d, however, are uniformly one order of magnitude larger than those determined by Hearn *et al.* (1971) and illustrated by the dotted line in Figure 3. The conclusion from the second point is that at least one component in the reaction mixture (either S protein, S peptide, or ribonuclease S′) undergoes a conformational transition in the temperature range studied and that the system involving fully acetylated

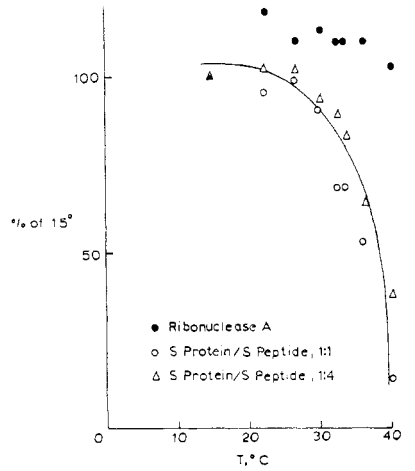

FIGURE 5: An estimate of the transition temperature from enzymatic activity. The activity of ribonuclease A was determined as a function of temperature as a control (●). (It is interesting to note that under the conditions of the experiment, opposing effects of temperature on V_{max} and K_m essentially cancel each other to give an apparent temperature coefficient very close to 1.) S protein (7×10^{-7} M) was incubated at varying temperatures together with equimolar (○) or 4× molar excess (△) of S peptide for 5 min. The initial reaction rate was determined at that temperature by addition of substrate. The transition temperature was taken as the temperature at which one-half of the activity remained.

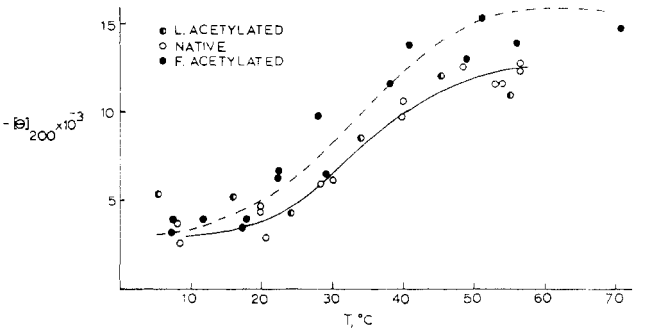

FIGURE 6: An estimate of the transition temperatures of S protein and acetyl-S protein by circular dichroism.

S protein apparently has a lower transition temperature than does the system involving the lightly acetylated derivative. Approximate values for the transition temperature were obtained from the binding data in Figures 1–3 by estimating the temperature at which the dissociation constant was twice that determined in the low temperature, linear region of the van't Hoff plots. We also re-plotted the binding data to give S protein bound as a function of temperature and estimated the transition temperature as that at which one-half of the protein bound at low temperature was released (Figure 4). These treatments yielded transition temperatures of 36–38 and 29° for the reactions involving lightly acetylated and fully acetylated S protein, respectively.

It was felt important to establish that this temperature dependent change was not merely an artifact of the Agarose–ribonuclease S′ system. To this end the effect of temperature on the activity of free ribonuclease S′ was determined (Figure 5) and the transition temperature obtained was 37–39°, in good agreement with that estimated from the binding data. These values of 36–39° are also in good agreement with those determined by Tsong *et al.* (1970) for S protein, but not with their value for ribonuclease S′ (47°). There is, however, a major difference in the experimental conditions in that Tsong *et al.* (1970) worked at protein concentrations about 500 times higher than the ones used in our work.

Because of the obvious importance of defining as precisely as possible all the conformation isomers of the acetylated S-protein derivatives that may exist in solution and thus affect the overall thermodynamics of the binding process, we sought to determine the transition temperature by an independent method. This was done by determining the effect of temperature on the circular dichroism of both lightly acetylated and fully acetylated S protein in comparison to unreacted S protein. The circular dichroism spectra of all three species are very similar at low temperature. The most prominent feature is a trough at approximately 208 nm. At higher temperatures, a new trough appears at approximately 198 nm. The thermal transition temperature is most clearly visualized in plots of mean residue ellipticity at 200 nm *vs.* temperature, as illustrated in Figure 6. The transition temperatures obtained from these plots are 37° for lightly acetylated and unreacted S protein and 29° for fully acetylated S protein. The transition temperatures derived from all of these techniques are summarized in Table III, and it appears safe to conclude that the thermal transitions observed in direct binding experiments are due entirely to changes in the structure of the S-protein derivatives.

One final point must be mentioned regarding the effect of high temperature on the S-protein derivatives. It was apparent

TABLE III: Transition Temperatures for S Protein and Acetylated S Protein as Determined by Different Experimental Approaches.[a]

S-Protein Derivative	From Direct Binding Studies	From CD Measurements	From Act. Assays	From Calorimetry[b]
Free S protein		36–38	36–38	37.6 (45°)[b,c]
Lightly acetylated	36–39	36–38		
Fully acetylated	28–31	28–30		

[a] Transition temperatures are given in degrees (centrigrade); for experimental detail, see text. [b] From Tsong *et al.* (1970). [c] The transition temperature for ribonuclease S′. This should be comparable to the data under "from activity assays," except for the concentration used (see text).

in both the binding experiments and in the circular dichroism studies that prolonged exposure to the highest temperatures led to some irreversible loss of acetylated S protein. The thermal transition of S protein can therefore be treated as a simple reversible process only in short time experiments at moderate temperatures.

It was the purpose of this work to evaluate the thermodynamic parameters for the interaction of Agarose–S peptide with the S-protein derivatives. With the complex van't Hoff plots, this evaluation must be restricted to the lower temperature range where the plots appear linear. The values estimated for the Agarose–acetylated ribonuclease S′ dissociation at 10° are given in Table IV and show that the driving force for the binding of acetylated S protein to Agarose–S peptide is essentially all entropic.

Discussion

With reference to the objectives stated earlier, the results presented in this paper lead to some firm conclusions, but the main objective, the characterization of the S-peptide–S-protein interaction in terms of thermodynamic parameters, remains in a rather unsatisfactory state of ambiguity.

To start with the positive aspects, we feel that the reproducibility and the internal consistency of the binding data strengthen the proposition that the use of an insolubilized peptide derivative in the quantitative study of peptide–peptide interactions is an acceptable experimental device, justified by the great ease of sampling and analysis that it permits. By tagging the second binding partner with a radioactive label, the binding studies can be carried out with precision at low enough concentrations to allow collection of binding data down to the lowest part of the binding curve for most peptide pairs. With the radioactive assay available for quantitation of S protein in this work, it was furthermore possible to study binding of an enzymatically inactive derivative. From the results presented here, we conclude that different extent of acetylation of S protein has no effect on its affinity for S peptide. Although our data are not complete on this point, it is reasonable to propose that the loss of activity in the fully acetylated derivative (7.5–8 of the 9 available amino groups reacted) is due to the acetylation of the essential, active-site component, Lys-41 (Hirs, 1962). In S-protein–S-peptide binding, however, neither Lys-41 nor the other amino groups in S protein appear to be involved. Finally, we conclude from our results that even high concentrations of substrate analog (the mixed 2′ and 3′ isomers of CMP) have no effect on S-protein–S-peptide affinity. This issue has been an important one in trying to resolve the large discrepancies in affinities determined by different methods in different laboratories (see, for example, the discussion by Woodfin and Massey (1968)). Our findings are consistent with those of Richards and Vithayathil (1960), who from kinetic experiments concluded that S protein binds substrate, and that no obligatory binding order appears to be preferred in forming the ternary ribonuclease S′–substrate complex from S protein, S peptide, and substrate. The binding of substrate analogs to S protein has also been demonstrated by Wilchek and Gorecki (1969) who found that an affinity chromatography system developed for ribonuclease A purification strongly retarded S protein.

With regard to the thermodynamic parameters obtained in this work, the first problem encountered in trying to interpret the data is the very large difference in the results reported here and those in the literature (Hearn *et al.*, 1971). At present, it is not possible to explain the discrepancies. It seems safe, however, to conclude that the association of S peptide and S protein to form ribonuclease S′ is a very complex chemical reaction, and it may be useful to review some of the known aspects of the reaction.

Scheme I illustrates the individual reactions that have been demonstrated experimentally, using P as the symbol for S protein, p for S peptide, S′ for ribonuclease S′, and s for substrate (or substrate analogs), to simplify the scheme.

The following is a brief review of what is known about each of the reaction steps. *Reaction 1* ($2P \rightleftharpoons P_2$). Aggregation of S

TABLE IV: Thermodynamic Parameters at 10° for the Dissociation of Agarose–(acetyl–) Ribonuclease S′.

	Lightly Acetylated	Fully Acetylated
ΔG (kcal mole^{-1})	+7.5 ± 0.4	+7.7 ± 0.4
ΔH (kcal mole^{-1})	+1.3 ± 1	+1.3 ± 1
ΔS (cal deg^{-1} mole^{-1})	−22 ± 5	−22 ± 5

SCHEME I

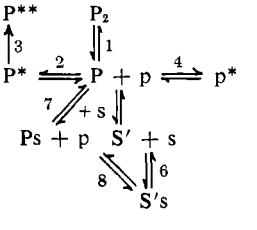

TABLE V: The Contribution of Different Known Processes to the Overall Thermodynamics of S-Protein–S-Peptide Interaction.

Source of Study	Conditions and Results: pH	S Protein Concn (M)	Substrate Present	Method	K_d (M)	Processes Involved (See Scheme I)
Richards and Vithayathil (1959)	4.5	5×10^{-7}	+	Activity assay	$<5 \times 10^{-9}$	4, 5, 6, 7, 8,
Woodfin and Massey (1968)	5.4	1×10^{-4}	–	Spectrophotometry	7×10^{-5}	1, 2, 3, 4, 5
	4.5[a]	8×10^{-5}	–		2×10^{-5}	1, 2, 3, 4, 5
Hearn *et al.* (1971)	7.0	$2\text{–}90 \times 10^{-5}$	–	Calorimetry		1, 2, 4, 5
	7.0	4.7×10^{-7}	+	Activity assay	1×10^{-7}	2, 4, 5, 6, 7, 8
This work (Gawronski and Wold, 1972)	7.5	$2\text{–}500 \times 10^{-7}$	–	Direct binding	2.5×10^{-6}	2, 3, 4, 5
	7.5		+	Activity assay	4×10^{-6}	2, 4, 5, 6, 7, 8
	4.5		–	Direct binding	2.5×10^{-6}	(1[b]), 2, 3, 4, 5

[a] Calculated values (by Woodfin and Massey (1968)) from the data of Richards and Logue (1962). [b] By binding S protein to Agarose it was proposed that S-protein dimerization was eliminated, and thus that reaction 1 (Scheme I) did not influence this system.

protein has been shown to occur at all pH values where the enzyme is active (Allende and Richards, 1962), but according to our data (Gawronski and Wold, 1972), there is no evidence that the aggregation exceeds the dimer stage at protein concentrations up to 5×10^{-4} M. We have estimated that the dimer dissociation constant must be about 10^{-4} M at pH 7.5 and $<10^{-5}$ at pH 4.5. In terms of the contributions of this reaction to the thermodynamics of the system, Hearn *et al.* (1971) have shown that ΔH in their reaction system is independent of protein concentration in the range from 2×10^{-5} to 2×10^{-3} M, and have concluded that S-protein dimer dissociation makes no contribution to the interaction enthalpy. We have measured the heat of dilution of S protein over the concentration range from 7×10^{-4} to 4×10^{-6} M (unpublished data) by flow calorimetry and find essentially no enthalpy of dissociation, in agreement with the conclusions of Hearn *et al.* (1971). However, at pH 7 (using the dissociation constant of 10^{-4} M and the corresponding $\Delta G°$ of 5.5 kcal/mole), this reaction must then have an entropy of dissociation of about -18.5 cal deg^{-1} mole^{-1} at 25°. This entropy contribution should appear in any ribonuclease S′ dissociation involving protein concentrations at which an appreciable amount of dimer exists.

Reaction 2 ($P \rightleftharpoons P^*$) may be taken to represent the thermal transition of S protein. In our work, the S-protein transition is clearly defined. Apparently, one sees the ribonuclease S′ transition only at high protein concentrations or at high peptide:protein ratios. From our results, we concluded that the variation in K_d with temperature above 30° reflected only the S-protein thermal transition, and that to use data from this region to determine ΔG for the binding process as Hearn *et al.* (1971) have done could be misleading. The thermal transition of S protein should not influence either the calorimetric or direct binding experiments performed at temperatures below 30°.

Reaction 3 ($P^* \longrightarrow P^{**}$). In our hands, S protein lost approximately 20% of its activatable enzymatic activity on preincubation for 30 min at temperatures above 18° at the assay concentration of about 7×10^{-7} M. When S protein was activated immediately on dilution into the assay medium, this loss in activity was not observed. In our direct-binding system, where protein is incubated uncombined and in the monomeric state for extended periods, this reaction is likely to occur. If the reaction is irreversible, however, its major effect on the ribonuclease S′ dissociation would be to obscure the actual S-protein concentration.

Reaction 4 ($p \rightleftharpoons p^*$). It is known that S peptide, when combined with S protein, has a large helical segment. It has been assumed that the peptide is an essentially random coil conformation in free solution. However, Brown and Klee (1971), working with a shorter segment of S peptide, residues 1–13 (the C peptide), have shown that this peptide possesses considerable helicity in the free state. The helix content of the free peptide is increased by increasing the ionic strength or decreasing temperature. At 0.1 ionic strength, one can use the values of mean residue ellipticity reported by Brown and Klee for C peptide at 224 nm to construct a van't Hoff plot for the coil–helix conversion. Various choices for the limiting values of mean residue ellipticity for helix and coil yield different values for the thermodynamic parameters, but the following estimates can nevertheless be made: $\Delta G°$ from +1.4 to 0 kcal per mole, $\Delta H°$ from -9 to -16 kcal per mole, and $\Delta S°$ from -34 to -50 cal deg^{-1} mole^{-1}. Furthermore, the helix content of the free peptide can be estimated to be from 20 to 50% at 0.1 ionic strength, pH 6–7, and at 1°. It seems clear that all measurements of the S-peptide–S-protein interaction include the contribution from the coil–helix transition of S peptide, and that the actual magnitude of this contribution must be quite sensitive to the experimental conditions. Reaction 4 could, therefore, be a major cause of the discrepancies in results from different laboratories. An important question regarding our work and related to the S peptide transition is: does the agarose affect the conformation of S peptide? In our initial titration experiments (Gawronski and Wold 1972) we obtained identical dissociation constants for S protein binding to Agarose–S peptide and for S peptide binding to Agarose–S protein. Based on this observation we tentatively conclude that Agarose–S peptide and free S peptide are identical with respect to S protein binding, and therefore also with respect to any S-peptide conformational change that could affect the S protein binding.

Reactions 5–8. The influence of substrate on the binding reaction has been briefly discussed above. Since it appears that the two paths of ribonuclease S′–substrate complex formation (reactions 5 + 6 or 7 + 8) are equivalent, the presence of substrate should not influence the apparent dissociation

constant for ribonuclease S′ under conditions where P_2 and P* are insignificant. Assuming, however, that neither P* nor P_2 binds substrate, any equilibrium involving P, P*, and P_2 should be affected by the presence of substrate and the effect should appear as an apparent effect on S-peptide–S-protein binding.

Using the limited data given above, it is possible to estimate to what extent the different reactions in Scheme I influenced the different binding studies reported in the literature, and such a survey has been carried out in Table V. Clearly this qualitative comparison is only approximate, but it may nevertheless serve as a first step toward a definition of all the variables that ultimately will have to be described in order to understand the S-protein–S-peptide interaction.

At present it is clear that the experimental conditions under which meaningful data on reaction 5 can be obtained will have to be restricted until more quantitative data are available for all the remaining seven reactions. It appears, however, that low-temperature, low S-protein concentration, and absence of substrate contribute a good deal toward simplifying the system, and these are the conditions under which our results were obtained.

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Proton Nuclear Magnetic Resonance Studies of the Association of Ribonuclease S-Peptide and Analogs with Ribonuclease S-Protein*

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ABSTRACT: The 250-MHz proton nmr spectra of D_2O solutions of S-peptide and a number of its subfragments and analogs have been recorded. The binding of S-peptide and its analogs to S-protein is accompanied by an upfield shift of the histidine-12 ring proton signals and a broadening and diminution in intensity of the signals from the arginine-10 δ protons and the methionine SCH_3 protons. These observations are in accord with a model of binding which postulates a random-coil conformation for the free S-peptide and the formation of a salt-bridge link between glutamate-2 and arginine-10 on binding. The findings are consistent with the structures deduced for ribonuclease S by X-ray diffraction.

Bovine pancreatic ribonuclease A has been extensively investigated from the point of view of structure and function. The primary amino acid sequence was determined by Smyth *et al.* (1963). X-Ray diffraction studies of crystalline ribonuclease A have been conducted by Kartha *et al.* (1967), who have refined the three-dimensional structure to 2 Å. Cleavage of a single peptide bond between alanine-20 and serine-21 may be brought about by hydrolysis with subtilisin under mild conditions (Richards, 1958). The products, S-peptide and S-protein, combine in solution to form a 1:1 complex, ribonuclease S, with the full enzymatic activity of the original ribonuclease, as demonstrated by Richards (1958). Crystalline ribonuclease S has been studied by X-ray diffraction by Wyckoff *et al.* (1970), who have established its structure to 2-Å resolution. The dissociation constant of ribonuclease S has been measured using various methods and has been assigned rather widely varying values, from 7×10^{-5} M (Woodfin and Massey, 1968; Moroder *et al.*, 1971) to 1×10^{-8} M (Kershaw and Richards, 1966; Hearn *et al.*, 1971; Berger and Levit, 1971). The latter value was measured in the pres-

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