A Study of Peptide–Peptide Interactions on an Insoluble Matrix*

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ABSTRACT: This study examines the possibility of obtaining quantitative binding data for peptide–peptide interactions when one of the binding components is attached to an insoluble matrix. The interaction of ribonuclease S protein with an Agarose–S peptide complex was studied by direct binding (analysis of free S protein in the absence of complex) as well as by activity assay (determination of bound S protein as active Agarose–ribonuclease S′). Good titration curves were obtained by either method at S-protein concentrations from 10^{-7} to 10^{-5} M, and the dissociation constant for Agarose–ribonuclease S′ was found to be $2.5 \pm 1.0 \times 10^{-6}$ M at 23° and pH 7.5. At lower pH anomalous titration curves were obtained. This can at least in part be explained on the basis of the pH-dependent aggregation of S protein. By sedimentation analysis the dissociation constant for the S-protein dimer was estimated to be of the order of 10^{-4} M at pH 7.5 and less than 10^{-5} M at pH 4.5. Thus at the lower pH a significant amount of S-protein dimer is present under the conditions of the titration experiments. In an attempt to eliminate S-protein dimer formation, S protein was attached to Agarose and the binding of S peptide to the Agarose–S-protein complex was studied. In this case good titration curves were obtained both at high and low pH and the dissociation constants ($K_d = 1 \times 10^{-6}$ M at pH 7.5 and $K_d = 2.5 \times 10^{-6}$ M at pH 4.5) were in good agreement with that determined from the reverse system at pH 7.5. For both the Agarose–S-peptide and Agarose–S-protein complexes only 60–70% of the bound component was available for interaction with its counterpart. The unavailable fraction of 30–40% appears to be completely inert in the system and does not complicate the analyses or the treatment of the data.

The study of complex interacting systems can often be greatly facilitated by immobilizing one of the interacting components on an insoluble matrix. This approach has so far been most fruitfully employed in preparative methods, such as the isolation of specific polynucleotide sequences by binding to immobilized complementary sequences (Nygard and Hall, 1964), the purification of specific antibodies by binding to immobilized antigen (Moudgal and Porter, 1963), the purification of enzymes by their binding to immobilized coenzymes (Arsenis and McCormick, 1964) or substrate analogs (Cuatrecasas *et al.*, 1968), and the purification of substrates such as tRNA by binding to immobilized enzyme (Denburg and DeLucca, 1970). The method should be applicable to any binding system, however, and should under the proper conditions allow quantitative evaluation of the binding parameters.

We have explored the possibility of studying quantitatively peptide–peptide interactions with a system in which one of the interacting components is fixed on an insoluble matrix, and have chosen the ribonuclease S-protein–S-peptide interaction for this study. The 124 amino acid peptide chain which makes up bovine pancreatic ribonuclease A can be cleaved by proteolytic enzymes into an S-peptide component, residues 1–20, and an S-protein component, residues 21–124. The two components can be separated at low pH. Neither component is active individually, but upon mixing at neutral pH they recombine to give active enzyme, designated ribonuclease S′ (Richards and Vithayathil, 1959).

The purpose of the present study was: (1) to establish whether quantitative binding data can be obtained when one of the binding partners is fixed on an insoluble matrix and (2) if the answer to the first question was affirmative, to explore the S-protein–S-peptide interaction with this system. Since this method measures binding directly and is limited only by the sensitivity of detection of free (unbound) component (in the solution phase) in equilibrium with the bound component (in the "insoluble" phase), such a study could be carried out with a wide range of derivatives, independent of activity and other physical and chemical secondary changes. With this plan and with the long-range goal of investigating the bonding regions of S protein, it was decided to make S protein the soluble component to be modified and derivatized in future experiments and to attach S peptide to the matrix as the constant, invariant component in the binding system. In the present paper we describe the properties of the products obtained when S peptide is attached to activated Agarose (Axen *et al.*, 1967) and the preliminary results of S-protein binding to the "insoluble" S-peptide–Agarose complex.

Materials and Methods

Bovine pancreatic ribonuclease (Sigma, type I-A) was purified according to the method of Hirs *et al.* (1953). In later preparations this procedure was eliminated and the commercial preparation was used directly. S protein and S peptide were prepared by nagarse digestion (Gordillo *et al.*, 1962) of the pure ribonuclease A or of the commercial ribonuclease A preparations and separated by gel filtration on Sephadex G-75 (Gross and Witkop, 1967) in 1 M formic acid. S protein was further purified by carboxymethylcellulose chromatography, and S peptide by gel filtration on Bio-Gel P-2. The final purity of S peptide, based on amino acid analysis, was 95%, and that of S protein, based on amino acid analysis and on activity in the absence and presence of S peptide was 94–98%.

Agarose (type A-15m, 50–100 mesh) was obtained from Bio-Rad Laboratories, cCMP and yeast RNA from Sigma Chemical Co., and cyanogen bromide from Eastman Organic

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Chemicals. All the reagents were reagent-grade products and were used without further purification.

Agarose was activated and reacted with ribonuclease A, S peptide, or S protein using a modification (Cuatrecasas *et al.*, 1968) of the method of Axen *et al.* (1967).

For quantitative S-peptide–S-protein interaction studies by direct titration, the following procedure was used throughout. S-peptide–Agarose complex (3 ml) was washed thoroughly with the appropriate buffer and finally suspended in 10 ml of the same buffer. A control vessel (blank) contained an identical quantity of Agarose which had been reacted with glycylglycine instead of with S peptide. Small aliquots (50–100 μl) of concentrated S-protein solution in the same buffer were added to both the experimental and blank titration vessels. The suspensions were stirred for 30 min and aliquots of the same size were removed for determination of free S protein (by activity assay in the presence of an excess of S peptide as described below). The amount of S protein bound was determined as the difference between the blank and the experimental values. Since the concentration of bound protein is equal to the concentration of protein–peptide complex, the following relation describes the interaction:

$$\frac{1}{[\text{S protein}]_b} = \frac{1}{[\text{S protein}]_f} \times \frac{K_d}{[\text{S peptide}]_t} + \frac{1}{[\text{S peptide}]_t}$$

Plots of (bound S protein)$^{-1}$ *vs.* (free S protein)$^{-1}$ should be linear and the intercepts at abscissa and ordinate should represent K_d^{-1} and (total S peptide)$^{-1}$, respectively.

Protein concentrations, including S peptide and S protein, were determined by measurements of ribonuclease activity. The activity was determined by the yeast RNA assay of Kalnitsky and Resnick (1959) or by the cyclic cytidylic acid assay (Crook *et al.*, 1960). In assaying S peptide or S protein, an excess of counterpart was added. This permitted reproducible and accurate determination of protein and peptide concentrations as low as 10^{-7} M. All optical densities were measured using a Zeiss PMQ II spectrophotometer.

To avoid the complications involved in working with insoluble components in the spectrophotometric assay, activity measurements involving Agarose–ribonuclease S′ were based on the rate of hydrolysis of cyclic cytidylic acid (1 mg/ml) as determined by the rate of base uptake in the pH-Stat. These titrimetric activity assays as described by Herries *et al.* (1962) were performed using a Radiometer type TTT 11 autotitrator equipped with a type ABU 1b autoburet. The reactions were carried out in 0.1 M KCl, maintained at pH 7.5 at 22°. The titrant was 0.005 M NaOH. The reaction was standardized using a known concentration of S protein assayed at several levels of S peptide to determine maximum attainable activity. Aliquots (1 ml) of Agarose–S peptide were preincubated either with or without S protein for 15 min, and the assay was initiated by addition of substrate to a final volume of 10 ml. The final substrate concentration was 3.1×10^{-4} M. Less than 1 ml of titrant was added. Bound S protein was determined by assuming that the specific activity of Agarose–ribonuclease S′ is the same as that of free ribonuclease S′. The data were analyzed by the method used in direct binding experiments.

Analytical centrifugation was performed using a Beckman Model E centrifuge equipped with schlieren optics. Sedimentation–diffusion measurements were made in a synthetic boundary cell. After the boundary was formed, the speed was maintained at 12,590 rpm and diffusion was observed for 20 min. The speed was then increased to 59,780 rpm and sedimentation was observed for 80 min. Molecular weight was calculated from the relation $M = (RT/1 - \bar{v}\rho)(s/D)$. The solvent was 0.01 M Tris-Cl–0.09 M KCl (pH 7.5). The solvent density was 1.005. $\bar{v}$ was taken as 0.695. The temperature was 20°.

Low-speed sedimentation equilibria were determined using the multichanneled cell described by Yphantis (1960). The rotor speed was 12,590 rpm. Sedimentation time was 20 h. At pH 7.5, the buffer was identical with that used in the sedimentation–diffusion studies. The pH 4.5 buffer was 0.01 M NH_4-Ac–0.09 M KCl (pH 4.5). The temperature was 20°. Weight-average molecular weights were calculated by the hinge point method.

Amino acid analyses were performed using a Beckman Model 120 C amino acid analyzer equipped with a Beckman Model 125 automatic digital integrator. Samples were hydrolyzed for 24 hr in 6 M HCl at 110°.

Results

Preparation of Agarose–Ribonuclease A Complex. In preliminary control experiments, intact ribonuclease A was reacted with activated Agarose at pH 8.2. When 25 mg of ribonuclease was added per ml of settled activated Agarose, 8–10 mg of the enzyme was covalently bound per ml of Agarose. A comparison of the ribonuclease–Agarose reaction at pH 7.55, 8.2, and 9.0 showed that although the reaction was more rapid at the higher pH, the total amount of enzyme bound and the specific activity of the product were essentially identical. The bound enzyme retained 30% of its enzymatic activity toward cCMP. The ribonuclease–Agarose complex was subjected to several washes with 30% acetic acid and with 8 M urea at room temperature. After reequilibration with dilute phosphate buffer, the activity of bound enzyme was unaltered. The complex was also washed with gradually increasing concentrations of ethanol and was finally air-dried at room temperature after the last wash with absolute ethanol. After storage for several days, the complex could be rehydrated in about 72 hr to give back all the original activity.

Preparation and Characterization of Agarose–S-Peptide Complex. With the hope of limiting the reaction to the α-amino group, S peptide was reacted with activated Agarose at pH 7.5. In separate experiments, 5- or 10-ml samples of settled, activated Agarose were reacted with from 0.5 to 1 mg of S peptide per ml of Agarose by stirring at room temperature overnight. Based on the disappearance of free S peptide from the reaction mixture, essentially all of the added peptide became attached to the Agarose in all experiments. Subsequent studies, however, suggested that much smaller quantities of Agarose-bound, active S peptide were available for S-protein binding, and more careful analytical methods were employed to characterize the product.

In a typical experiment, a sample of Agarose–S peptide, which, based on S-peptide disappearance contained 0.7 mg of S peptide/ml of Agarose, was washed with large volumes of water, 10^{-3} M HCl, and with water again. A 1-ml sample of the settled Agarose was then lyophilized, subjected to acid hydrolysis and amino acid analysis (Cuatrecasas, 1969), using known quantities of norleucine and homoarginine as internal standards. Based on the normalized amino acid analysis the sample contained 0.25 mg of S peptide/ml of Agarose. This value, indicating that of the S peptide which disappeared from the supernatant in the initial reaction only 35–40% is actually bound in a stable complex, appears to be quite reproducible. (A summary of the data for several preparations is included in Table II.)

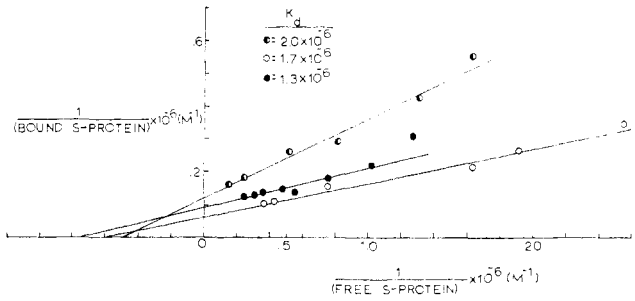

FIGURE 1: Typical plots of binding data derived from the direct titration of Agarose–S peptide with S protein at pH 7.5. The Agarose–S-peptide sample used was preparation I (Table II). Conditions are stated in the text.

Attempts were also made to determine which amino acid(s) of the S peptide reacts with the activated Agarose. Assuming that amino groups are the most reactive ones, there are three possible sites of reaction in S peptide, the α- and the ϵ-amino groups of Lys-1 and the ϵ-amino group of Lys-7. The rationale of the experiment thus was to digest the Agarose–S-peptide complex with trypsin, which cleaves the peptide bonds after Lys-7 and Arg-10, and to determine the amino acid composition of the peptides released after digestion. Any S peptide attached at Lys-1 should release peptides 7–10 and 11–20, whereas a complex involving Lys-7 should release only residues 11–20. The Agarose–S-peptide complex was digested for 4 hr at 37° and at pH 7.8 with a trypsin:peptide ratio of 1:3. After digestion, aliquots of the supernatant were withdrawn, hydrolyzed, and the amino acid composition was determined. Since trypsin contains several amino acids not found in S peptide, the trypsin contribution to the total amino acid content of the supernatant could be calculated and subtracted. Typical results, which are given in Table I, show that the yield of residues 11–20 was about three times that of residues 8–10 (Phe-Glu-Arg), and thus indicate that the reaction at Lys-1 was at least three times more frequent than that at Lys-7. The overall yield of peptide 11–20, however, was only 45% of the total peptide bound (based on amino acid analysis of the Agarose–S-peptide complex). This, as will be seen below, corresponds moderately well with the fraction of bound peptide which can be detected by S protein binding, and may suggest that binding sites other than Lys-1 and -7 may be involved in the insolubilization reaction (see Discussion). We have not pursued this point further. Nor have we attempted to determine the fate of the S peptide which disappeared from the reaction mixture and yet was not covalently bound. The characterization of the product simply shows that from 0.1 to 0.4 mg of S peptide can be attached per ml of settled Agarose through a stable bond and that 40–50% of this bound S peptide is susceptible to trypsin digestion in a pattern which suggests that Lys-1 and Lys-7 are the primary reaction sites, with a relative reaction frequency of about 3:1.

Binding of S Protein to Agarose–S-peptide Complex. DIRECT BINDING STUDIES AT pH 7.5. The results of three separate titrations of Agarose–S peptide with S protein in 0.001 M Tris-Cl–0.1 M KCl buffer (pH 7.5) are given in Figure 1. Considering that these represent different experiments at different times the agreement between the dissociation constants (K_d = 1.3×10^{-6}, 1.7×10^{-6}, and 2.0×10^{-6} M) is quite good. The end points in the titrations in Figure 1 show rather wide variation. These titrations were run before gel-washing procedures were standardized, however. After thorough washing, ensuring complete removal of S protein, reproducible titration end points are obtained. The bound S protein can be removed from the Agarose–S-peptide complex simply by washing with large volumes of water or buffer, but washing with acid is both faster and more efficient. Thus two 30-min washes with 50 volumes of 0.01 M HCl, each followed by filtration, are sufficient to remove more than 90% of the S protein. After this washing routine, the Agarose–S-peptide complex can be used over again with completely reproducible results. The cycle has been repeated more than ten times over a 4-month period for one Agarose–S-peptide sample (preparation I) without any loss in S-protein binding capacity or change in K_d. Some mechanical breakdown of the Agarose beads may be observed after extended use, affecting the sedimentation time, but not the binding properties.

BINDING AT pH 7.5 IN THE PRESENCE OF SUBSTRATE. It was felt of interest to check these results by an independent method and also to seek an answer to the question: does S protein binding to the Agarose–S-peptide complex give enzymatically active Agarose–ribonuclease S′? To this end the Agarose–S peptide was titrated with S protein using enzymatic activity as a measure of bound S protein. The results of one such titration, using preparation III, are shown in Figure 2a. The calculated dissociation constant was 4×10^{-6}

TABLE I: Amino Acid Composition of Peptides Released from Agarose–S Peptide after Trypsin Digestion.[a]

	Amino Acid Content (nmoles)			
	A Total Hydrolysate	B Trypsin Contribution[b]	A − B S-Peptide Contribution	Calcd for 15 nmoles of 11–20 and 5 nmoles of 8–10
Aspartic acid	25	10	15	15
Threonine	20	4.5	15.5	15
Serine	60	15.3	44.7	45
Glutamic acid	27	6.3	20.7	20
Glycine	14	10	4.0	0
Alanine	37	6.3	30.3	30
Valine	8	7.7	0.3	0
Isoleucine	5.5	6.8	0	0
Leucine	4.8	6.3	0	0
Tyrosine	5.3	4.5	0.8	0
Phenylalanine	6.5	1.4	5.1	5
Lysine	8.2	6.3	1.9	0
Histidine	14.8	1.4	13.4	15
Arginine	6.6	0.9	5.7	5

[a] Agarose–S peptide (Agarose-[Lys$_1$-Glu-Thr-Ala-Ala-Ala$_5$-Lys-Phe-Glu-Arg$_{10}$-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala$_{15}$-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser$_{20}$-Ser-Thr-Ser-Ala-Ala] was treated with trypsin (see text for experimental detail), and the supernatant, containing trypsin plus liberated peptides from Agarose–S peptide was concentrated, hydrolyzed, and analyzed. [b] The trypsin contribution was estimated from an average factor calculated from the amino acids uniquely present in trypsin and not in S peptide (Gly,Val,Ile,Leu,Tyr,Lys).

TABLE II: Summary of Properties of Different Agarose–S-Peptide Preparations.

	Preparation No.			
	I	II	III	IV
1. S Peptide attached (mg/ml of Agarose)				
a. Based on disappearance from the reaction mixture[a]	0.37	0.34	1.0	0.7
b. Based on amino acid analysis of the Agarose–S-peptide complex[b]			0.38	0.25
2. Active S peptide attached (mg/ml of Agarose)				
a. Based on S-protein binding capacity	0.06–0.11	0.09	0.24	0.18
b. Based on maximum enzymatic activity in the presence of an excess of S protein			0.27 ± 0.03	0.20
3. Fraction of bound S peptide which is active (%)				
a. Based on 1.a.[a]	15–30	27	24	26
b. Based on 1.b.[b]			63	72
4. Fraction of bound S peptide available to trypsin digestion (% of 1.b)[b]				
a. Peptide sequence 11–20			45	
b. Peptide sequence 8–10			15	
c. Peptide sequence 1–7			0	
5. Dissociation constant for the Agarose–ribonuclease S′ complex at pH 7.5, 23° (μmoles/l.)				
a. Based on direct binding (in the absence of substrate)	1.7 + 0.3	2.5 ± 1.5	2.0	3.0
b. Based on activity assay (in the presence of substrate)			4.0	3.0

[a] In some of the early preparations the amount of peptide bound to Agarose was determined only as the amount of peptide which was removed in the reaction with activated Agarose. Although subsequent experiments clearly established these estimates to be much too high, the values have been included here for comparative purposes. [b] Amino acid analysis of hydrolyzed Agarose–S-peptide complex which has been thoroughly washed free of noncovalent contaminations should give an accurate estimate of the amount of bound S peptide (Cuatrecasas, 1969). The numbers in the columns based on amino acid analysis thus represent the actual values for peptide binding.

M and the stoichiometry of binding indicated 0.27 mg of S peptide/ml of Agarose compared to 0.24 mg/ml of Agarose by the direct binding approach. Preparation IV gave comparable results as shown in Figure 2b. As a control for this experiment we also investigated the effect of the Agarose in the assay system, determining in parallel experiments the S protein binding to free S peptide in the absence and presence of glycylglycine–Agarose. The free ribonuclease S′ dissociation constant, estimated from a double-reciprocal plot of data like that shown in Figure 2 was 5×10^{-7} M in both systems.

Several conclusions can be drawn from this experiment. First, within the experimental errors substrate, at the concentration used (3.1×10^{-4} M), does not affect the Agarose–ribonuclease S′ dissociation. Secondly, since the end point of the titration of Agarose–S peptide with S protein using either the direct binding assay or the activity assay was the same (see Table II) it can be concluded that all S protein bound yields active Agarose–ribonuclease S′. Furthermore, since the maximal activity obtained at saturation of either Agarose–S peptide or free S peptide with S protein are similar, we also tentatively conclude that the specific activity is the same for free ribonuclease S′ and Agarose–ribonuclease S′. Finally, it appears that there may be an effect of Agarose on the S peptide–S protein binding, reflected in the fourfold decrease in the K_d for ribonuclease S′ (5×10^{-7} M) compared to Agarose–ribonuclease S′ (2×10^{-6} M). Since the presence of glycylglycine–Agarose in the reaction mixture did not affect the affinity between free S peptide and S protein the effect must be due to the covalent linking of S peptide to Agarose.

A summary of the properties of four different preparations of Agarose–S peptide is given in Table II, and illustrates the reproducible binding data obtained with this system. Even with quite different quantities of S peptide bound to Agarose, the properties of the Agarose–S peptide are remarkably constant. For all preparations the binding capacity of the immobilized peptide based on disappearance of peptide in the reaction with activated Agarose was 24.5 ± 4.4%. Based on actual content of S peptide determined by amino acid analysis, this corresponds to a binding capacity of 60–70%. The average dissociation constant for all titrations was $2.5 \pm 1.0 \times 10^{-6}$ M.

BINDING STUDIES AT LOW pH. In an attempt to reproduce the conditions of other studies on S-peptide–S-protein interaction (Richards and Logue, 1962; Woodfin and Massey, 1968), we have also investigated S protein binding to Agarose–S peptide at lower pH (4.5, 5.5, and 6.5). When treated

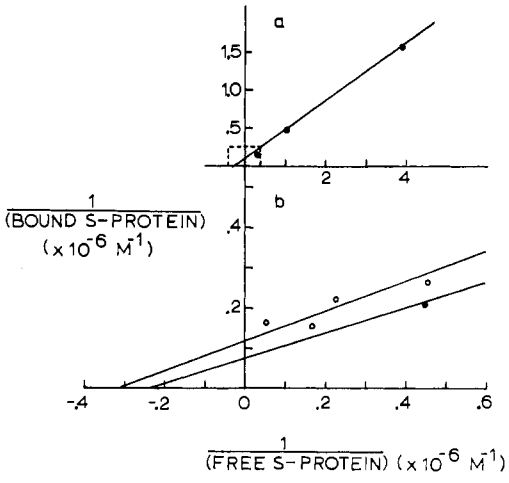

FIGURE 2: Plots of binding data derived from the titrimetric assay of Agarose–S peptide in the presence of added S protein. Part a shows results obtained using a wide range of S-protein concentrations and Agarose–S-peptide preparation III (Table II). The box indicates the portion shown in part b. Part b shows the high S protein point (●) and the line derived from part a as well as a titration of preparation IV (○) at high protein concentration. Conditions are described in the text. The dissociation constants derived from the data are 4×10^{-6} and 3×10^{-6} M for preparations III and IV, respectively.

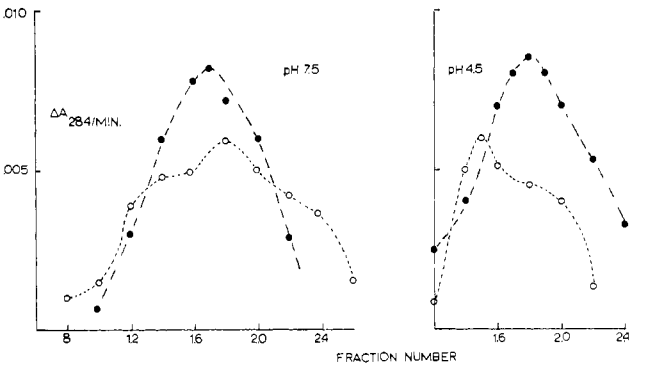

FIGURE 4: Gel filtration of ribonuclease A and S protein on Sephadex G-50 at pH 7.5 and 4.5. The solvent was 0.1 M KCl in both cases with 0.005 M NH_4Ac added as buffer at pH 4.5 and 0.005 M Tris-Cl added as buffer at pH 7.5. The column volume was 56 ml. The elution volume was 43 ml. The protein was applied at a concentration of approximately 5 mg/ml. In all cases the sample was applied in a volume of 400 μl. Ribonuclease A and S protein were determined by enzymatic activity in the presence and absence of excess S peptide. (○) S protein and (●) RNase A.

in the same way as binding data obtained at pH 7.5, the lower pH binding data gave anomalous titration curves, however. Typical results are shown in Figure 3. We have established (by going to very high S-protein concentrations) that the end points of these titrations are identical with those observed in pH 7.5 titrations, but it is not possible to determine K_d from these data.

Since it is known that S protein tends to aggregate, especially at lower pH (Allende and Richards, 1961), the aggregation was considered a possible explanation of the anomalous binding data, and the molecular weight of S protein was investigated at both pH 4.5 and 7.5. The preliminary results obtained by gel filtration with ribonuclease A as marker are given in Figure 4 and illustrate a rather complicated pattern of molecular size. At pH 7.5, most of the material elutes after ribonuclease A as expected for the lower molecular weight

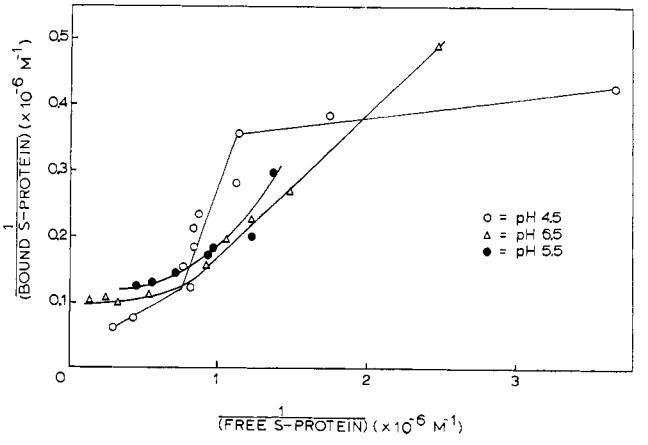

FIGURE 3: Plots of binding data derived from the direct titration of Agarose–S peptide with S protein at pH values below 7.5. The Agarose–S-peptide sample used was preparation I. Conditions are stated in the text.

monomeric form. At pH 4.5, however, most of the S protein elutes ahead of ribonuclease A, indicating a higher molecular weight.

The gel filtration results were confirmed in the analytical ultracentrifuge. According to the results shown in Figure 5, S protein has a molecular weight of 22,000 at pH 4.5 and at protein concentrations from 2 to 6 mg per ml ($1.7–5.2 \times 10^{-4}$ M). There appears to be no effect of concentration on the molecular weight in this concentration range, and the dissociation constant for the S protein dimer at pH 4.5 must, therefore, be less than 10^{-5} M. At pH 7.5, however, the molecular weight is strongly dependent on concentration. Calculations based on the $M_{s,D}$ data indicate an S-protein–dimer dissociation constant of approximately 10^{-4} M at pH 7.5. All of our titrations were done at S-protein concentrations about two orders of magnitude below this level.

If S protein aggregation is a major cause of the anomalous binding data at low pH, it should be possible to obtain normal titration curves by preventing aggregation through covalent binding of S protein to activated Agarose. One such experiment was carried out. An Agarose–S-protein sample was prepared containing 0.68 mg of S protein/ml of settled Agarose based on amino acid analysis of the hydrolyzed complex, and titrations were carried out as in the S-protein titrations except that the titrant now was free S peptide. The results of titrations at pH 7.5 and 4.5 are shown in Figure 6. The protein–peptide dissociation constant of 10^{-6} M at pH 7.5 is in excellent agreement with that determined in the reversed titration system (Table II). The dissociation constant calculated for the pH 4.5 case is slightly higher at 2.5×10^{-6} M, but the most significant part of this experiment is that in the absence of S-protein aggregation, the direct titration with S peptide at pH 4.5 gives a normal titration curve. The titration end points indicated 0.36–0.43 mg of S protein/ml of settled Agarose or 60% binding capacity for S peptide.

Discussion

The primary purpose of the work reported in this paper was to assess whether quantitative binding data can be obtained for peptide–peptide interactions when one of the binding partners is fixed on an insoluble matrix. It appears from the

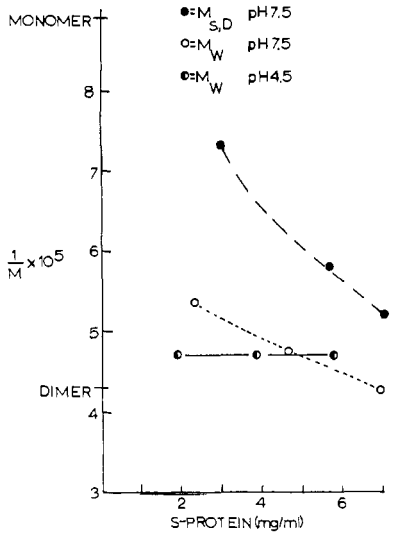

FIGURE 5: Molecular weight of S protein derived from equilibrium and sedimentation–diffusion measurements in the analytical ultracentrifuge. Experimental details are given in Methods.

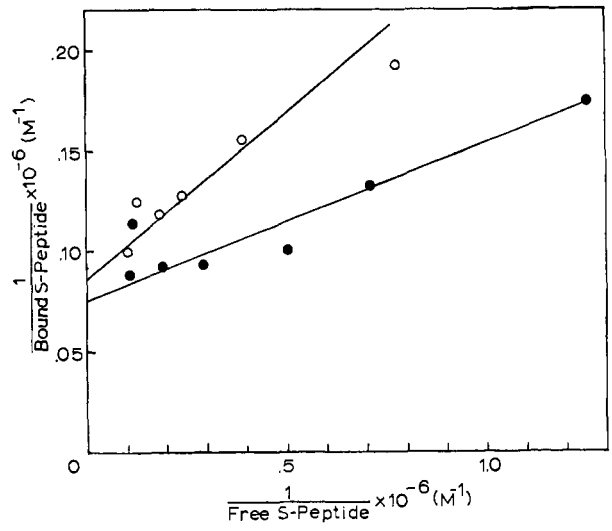

FIGURE 6: Plots of binding data derived from the direct titration of Agarose–S protein with S peptide at pH 7.5 (●) and 4.5 (○). Conditions are as stated in the text for S protein titrations except that the buffer composition at pH 7.5 was 0.01 M KPO_4–0.09 M KCl (pH 7.5) while at pH 4.5 the buffer composition was 0.01 M KAc–0.09 M KCl (pH 4.5).

data presented here that this approach is perfectly valid, and that the method next can be used to explore the S-protein–S-peptide interaction in greater detail.

Some questions are left unanswered, however. The most serious of these is the elucidation of the nature of all the bonds responsible for the firm binding of S peptide to Agarose. These bonds are presumably all covalent bonds, and some evidence was presented that Lys-1 and Lys-7 are involved. However, trypsin only hydrolyzed about 45% of the bound S peptide, and similarly only 60–70% of the bound S peptide can be titrated with S protein. Thus, more than 30% of the covalently attached S peptide is unavailable to either reaction. We have been unable to conceive of an experimentally testable explanation for this binding. It has been shown that derivatization of Lys-7 has no effect on the S-protein–S-peptide interaction or on ribonuclease S′ activity (Vithayathil and Richards, 1960). However, while free S peptide in solution exists primarily in a random coil structure, it assumes a partial α-helical structure when it combines with S protein (Brown and Klee, 1971). Thus, if the attachment of free S peptide to Agarose prevents the coil-to-helix transition, the lack of S-protein binding would be explained. Since the tryptic digestibility agrees reasonably well with the S-protein binding capacity, one would probably have to postulate that an amino acid residue other than Lys-1 and -7 is involved in giving the inactive Agarose–S-peptide derivative. We have no information on this point. Another possible explanation for the inactive fraction of attached S peptide is that it is simply structurally unavailable in the Agarose matrix. Considering that extensive cross-linking may take place in the activated Agarose (Axen *et al.*, 1967), it is quite reasonable that some interior regions of the Agarose could attain small enough pore size to effectively block access of both trypsin and S protein to S peptide in these regions.

An important part of the question about the inactive fraction of S peptide is to clearly establish that it is completely inert in the system, and thus does not bias the binding data. The fact that the titration end points obtained by extrapolation from a low S-protein concentration agree well with titration end points obtained at concentrations approaching the actual saturation level suggests that the inactive fraction of S peptide is indeed inactive and completely inert in the concentration ranges to be used in future work.

How does the dissociation constant determined in this work agree with those determined earlier in other laboratories? Richards and Vithayathil (1959) using recovery of activity as a measure of reassociation, titrated S protein with S peptide at pH 4.5 in the presence of either yeast RNA or uridine 2′, 3′-cyclic phosphate as substrate. This method of measuring the reassociation restricts the study to low concentrations of enzyme (below 10^{-8} M), but good stoichiometry was obtained and the calculated dissociation constant for the interaction was less than 5×10^{-9} M. In a more recent study using the same method, but at pH 7, Hearn *et al.* (1971) obtained a K_d of 10^{-7} M. Based on the small but significant spectral change associated with formation of ribonuclease S′ from S peptide and S protein (Richards and Logue, 1959), it is also possible to study the process by spectrophotometry, but because of the small spectral changes, such studies are limited to concentrations approaching 10^{-4} M. Woodfin and Massey (1968) carried out such a study of the S-protein–S-peptide interaction by spectrophotometric titration and reported a dissociation constant of 7×10^{-5} M at pH 5.4. They also used the spectrophotometric titration data reported by Richards and Logue (1962) to calculate a dissociation constant of 2×10^{-5} M at pH 4.5. Our values for the dissociation constant at pH 7.5 both by direct binding of S protein to Agarose–S peptide and of S peptide to Agarose–S protein and by activity titration fall in the range from 1×10^{-6} to 4×10^{-6} M, and for the binding of S peptide to Agarose–S protein at pH 4.5 a value of 2.5×10^{-6} M was obtained. Our value for K_d is thus in conflict with those determined in the earlier work.

Perhaps, however, some of the discrepancies are only apparent ones. If we consider that the high concentration spectrophotometric titration at pH 4.5 and 5.4 might be affected by S-protein dimerization, the total equilibrium would be

$$(\text{S protein})_2 \overset{K_a}{\rightleftharpoons} 2\ \text{S protein}$$

$$\text{S protein} + \text{S peptide} \overset{K_d}{\rightleftharpoons} \text{ribonuclease S}'$$

with the experimentally observed constant

$$K_d' = \frac{[\text{S protein}]_t[\text{S peptide}]}{[\text{ribonuclease S}']} = K_d\left(1 + \frac{[\text{S protein}]}{K_a}\right)$$

$$K_d = \frac{K_d'}{1 + \frac{[\text{S protein}]}{K_a}}$$

If K_a has a value around 10^{-6} M, K_d' for the spectrophotometric titration would be two orders of magnitude too high. The K_a step according to our data is pH dependent and the dimerization could then also explain the apparent effect of pH on S-protein–S-peptide binding observed in the study of Woodfin and Massey (1968).

This very qualitative treatment of the system is based on necessity since all our attempts at more quantitative evaluations of the dimerization effect have failed. It is, therefore, likely that the total system at low pH must be considerably more complicated than the simple monomer–dimer model suggests.

We would like to argue that the apparent dissociation constants derived from the direct binding measurements in this work are true estimates of the protein–peptide dissociation constant. The findings that the apparent constants derived for S-protein binding to immobilized S peptide essentially are identical with those observed for S-peptide binding to immobilized S protein, and that the associated species are fully active in the hydrolysis of cCMP are in favor of the arguments. However, in considering all the available binding data for low concentration S-protein–S-peptide interaction (where no S-protein dimer exists), the Agarose system does appear to give somewhat higher dissociation constants than do the systems involving the free S protein and S peptide. The difference may be more than an order of magnitude although the results obtained in our studies ($K_d = 2 \times 10^{-6}$ M for Agarose–ribonuclease S′ and $K_d = 5 \times 10^{-7}$ M for free ribonuclease S′) show a smaller disparity than that. At present there is no obvious basis for selecting one set of values as being more reliable than the other.

Even if the affinities measured with the Agarose–S-peptide titration should be established to be consistently too low, however, they should still serve as good quantitative measures of the relative affinities of different S-protein derivatives. This type of binding system has very significant advantages, due to the ease of sampling and rapid attainment of equilibrium. Experimental parameters can be easily varied without preparation of new samples and because the method measures binding directly, it can be applied to enzymatically inactive derivatives as well as to active ones. Based on the well-defined titration data obtained at pH 7.5, it is our intention to proceed with the second part of the plan: to use the Agarose–S-peptide complex to determine the thermodynamic parameters for the S-protein–S-peptide interaction, and to investigate the interaction of systematically modified S-protein derivatives with S peptide.

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