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Spectrophotometric Measurement of Binding of S-Peptide Analogs to S-Protein†

Frances M. Finn

ABSTRACT: The binding to S-protein of S-peptide and eleven synthetic analogs was determined by a spectrophotometric method in the absence of substrate. Dissociation constants were in the range of 10^{-4}–10^{-5} M. Structural modifications of S-peptide which have a marked influence on S-protein activation or RNase S inhibition in the presence of substrate are not necessarily detectable by this technique. Modifications which disrupt the noncovalent interaction between Glu^2 and Arg^{10} in the S-peptide–S-protein complex and replacement of methionine by the sulfoxide are instances where a difference in the strength of the peptide–protein interaction is detectable spectrophotometrically.

The discovery of the S-peptide–S-protein system[1] (Richards, 1958) provided a unique opportunity to measure a number of parameters of peptide–protein interactions. The fortuitous location of part of the active site in the S-peptide portion of ribonuclease S permitted synthetic manipulations to be made leading eventually to the discovery of a number of potent inhibitors (Finn and Hofmann, 1967; Hofmann *et al.*, 1970, 1971). Furthermore a systematic replacement of residues along the peptide chain has allowed definition of amino acids contributing to the strong, highly specific, noncovalent binding between peptide and protein. Through this approach it has been possible to show that His^{12} is the only residue in the peptide essential for regeneration of enzymic activity in RNase S and that a number of hydrophilic (Glu^2,Arg^{10},Gln^{11},Asp^{14}) and hydrophobic (Phe^8,Met^{13}) residues are involved in binding. Such studies have led to the discovery that a peptide containing only the first 14 amino acid residues is equivalent to S-peptide as concerns activation of and binding to S-protein.

Initially the importance of amino acids in the S-peptide chain was assessed by comparing the ability of analogs to regenerate ribonuclease activity with S-protein using the potency of S-peptide as the 100% standard. However, the sharp break that occurs in the curve for activation of S-protein by S-peptide and a number of analogs indicated that the peptides bound quite firmly and that with such strong binding it seemed reasonable to expect that subtle differences in binding ability might not be discernible.

The finding that Pyr^{12}S-peptide$_{1-14}$ (Table I) is capable of competing as efficiently as S-peptide$_{1-20}$ for S-protein (50% inhibition at a ratio of 1:1, Pyr^{12}S-peptide$_{1-14}$:S-peptide$_{1-20}$) provided an alternate, more sensitive method for comparing peptide–protein interactions (Finn and Hofmann, 1967). Since both S-peptide and the various inhibitors were exposed simultaneously to the effects of substrate and since they were competing for the same binding sites, the ability to inhibit would be directly related to the relative binding efficiency and thus provide more information than activation studies. A number of such competitive inhibition studies were performed and the results of these will be discussed later.

Recently, we have become interested in determining directly the strength of peptide–protein interactions in general and the effect of amino acid substitutions on them. The S-peptide–S-protein system is obviously ideally suited to such an investigation owing to the availability of a large number of analogs. From the point of view of the techniques employed for the determination of binding constants, however, the system has several drawbacks. Equilibrium dialysis methods, as they are commonly used, are effective only when a large difference in molecular weight exists between the protein and the ligand. The combination of a ligand of molecular weight 2×10^3 and a protein of molecular weight 12×10^3 virtually precludes the use of conventional dialysis techniques. Even the Craig (Craig and Konigsberg, 1961) modification proved difficult insofar as quantitation was concerned. It should be noted that there are no tryptophan or tyrosine residues in S-peptide so that trials with dialysis techniques necessitated preparation of radioactively labeled peptides.

The generation of a difference spectrum upon addition of S-peptide to S-protein was first noted by Richards and Logue

† From the Protein Research Laboratory, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15213. *Received October 28, 1971.* Supported by Grant No. GB 13647 from the National Science Foundation.

[1] The following abbreviations will be used: RNase S, Subtilisin-modified beef pancreatic ribonuclease A; S-peptide, the peptide obtained from RNase S; S-protein, the protein component obtained from RNase S; RNase complex, a general term denoting the reconstituted enzyme obtained by mixing S-peptide or S-peptide analogs with S-protein; Pyr, β-(pyrazolyl-3)-alanine; 3-CM-His, 3-carboxymethylhistidine; RNA refers to yeast ribonucleic acid.

TABLE I: Dissociation Constants for S-Protein–Peptide Complexes (Reaction 2).

Peptides	H-Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala-OH 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20	Dissocn Constant, Moles/l. × 10^5	50% Ratio[a]
S-Peptide$_{1-20}$	H–[1–20]–OH	0.96 ± 0.15	1.0[b]
S-Peptide$_{1-20}$ sulfoxide	H–[1–20, O↑ Met]–OH	20.7 ± 7.6	
S-Peptide$_{1-14}$	H–[1–14]–OH	1.33 ± 0.58	1.0[b]
Pyr^{12}S-peptide$_{1-14}$	H–[1–11, Pyr]–OH	1.95 ± 0.56	1.0[c]
Nle^{13}S-peptide$_{1-14}$	H–[1–12, Nle]–OH	1.42 ± 0.53	1.0[d]
Ser^{12}S-peptide$_{1-14}$	H–[1–11, Ser]–OH	1.70 ± 0.40	7[c]
3-CM-His^{12}S-peptide$_{1-14}$	H–[1–11, 3CM-His]–OH	1.06 ± 0.11	0.8[c]
3-CM-His^{12}S-peptide$_{1-14}$ sulfoxide	H–[1–11, 3CM-His, O↑ Met]–OH	14.3 ± 3.1	3000[c]
Orn^{10}Pyr^{12}S-peptide$_{1-14}$	H–[1–9, Orn, 11, Pyr]–OH	4.22 ± 0.33	20[c]
Orn^{10} 3-CM-His^{12}S-peptide$_{1-14}$	H–[1–9, Orn, 11, 3CM-His]–OH	1.46 ± 0.44	4[c]
Pyr^{12}S-peptide$_{2-14}$	H–[2–11, Pyr]–OH	1.71 ± 0.37	1.2[c]
Pyr^{12}S-peptide$_{3-14}$	H–[3–11, Pyr]–OH	6.82 ± 0.33	30[c]

H-Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp Ser-Ser-Thr-Ser-Ala-Ala-OH

[a] The 50% ratio refers to enzyme activity measurements using RNA as substrate (Finn and Hofmann, 1965). [b] Mole ratio of peptide to inhibitor (Pyr^{12}S-peptide$_{1-14}$) required to regenerate 50% of the activity of RNase S. [c] Moles of peptide required to inhibit 50% the activity of 1 mole of RNase S. [d] Mole ratio of peptide to inhibitor (Pyr^{12}S-peptide$_{1-14}$) required to regenerate 50% of the activity of the complex Nle^{13}S-peptide$_{1-14}$–S-protein; this complex is not as active as RNase S (see Hofmann *et al.*, 1971).

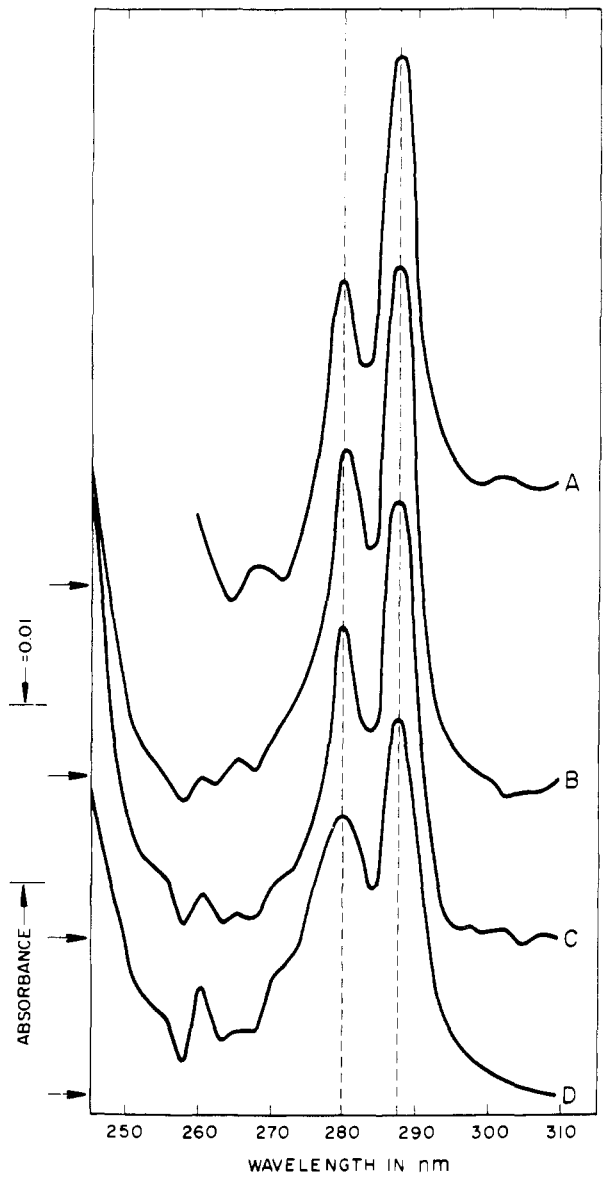

FIGURE 1: Ultraviolet difference spectra for the reassociation of S-protein and synthetic S-peptide and three analogs. See Experimental Section for conditions. Arrows on the ordinate refer to zero absorbance. Curve A, S-peptide$_{1-20}$ 4.72×10^{-5} M, S-protein 4.62×10^{-5} M; curve B, 3-CM-His^{12}S-peptide$_{1-14}$ sulfoxide 22.73×10^{-5} M, S-protein 5.36×10^{-5} M; curve C, S-peptide$_{1-14}$ 2.74×10^{-5} M, S-protein 4.87×10^{-5} M; curve D, Orn^{10}Pyr^{12}S-peptide$_{1-14}$ 3.65×10^{-5} M, S-protein 4.20×10^{-5} M.

(1962) who then used this spectrum to compare the velocity of the reaction of S-peptide with S-protein to that of tetramethyl S-peptide. Later Woodfin and Massey (1968) applied the difference spectrum technique to the measurement, in the absence of substrate, of the dissociation constant for the reaction.

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

This paper is concerned with the use of the difference spectrum technique for the determination of dissociation constants for S-peptide and a number of S-peptide analogs (Table I) in the absence of substrate according to reaction 2.

$$\text{RNase complex} \rightleftharpoons \text{S-protein} + \text{analog} \qquad (2)$$

Experimental Section

Materials

The peptides were prepared synthetically in this laboratory as previously described (Hofmann *et al.*, 1966, 1969–1971; Finn and Hofmann, 1967). S-Protein was obtained from purified ribonuclease S (Richards and Vithayathil, 1959) which had been desalted according to the Dixon (1959) procedure, by precipitation with 20% trichloroacetic acid one-fifth volume). The trichloroacetate ions were exchanged for acetate ions on IRA-400 (20–50 mesh). S-Protein had <2 Kunitz units/mg (Kunitz, 1940) in the absence of S-peptide and 130 Kunitz units/mg when fully activated. Yeast RNA (Sigma commercial grade) was purified as described (Hofmann *et al.*, 1969).

Methods

An aliquot (2.5 ml) of 0.1 M sodium acetate buffer (pH 5.0) containing S-protein ($5–6 \times 10^{-5}$ M) was pipetted into each of two 1-cm cuvets. The cells were placed in the reference and measuring chambers of a Cary 15 recording spectrophotometer equipped with a 0–0.1 OD scale. The absorbance of the solutions in the two cells was balanced over a spectral range of 310–265 nm. Solutions of S-peptide or its analogs (0.5×10^{-5} to 3×10^{-3} M) in the same buffer were added in 0.1-ml increments. Corresponding additions of acetate buffer were made to the reference cuvet. After 5 min, absorbance was measured throughout the spectral range or when the exact location of absorption peaks had been confirmed measurements were made at this wavelength only. A second set of measurements was made for similar additions of the peptide solutions to buffer using a buffer blank. True difference spectra were then obtained for the combinations of various peptides with S-protein by subtracting the peptide curve from the peptide plus protein curve. Spectrophotometric titrations were carried out at 25°. Concentrations of S-protein solutions were established by using the extinction coefficient of Potts *et al.* (1964). Peptide concentrations were based on the average recovery of amino acids from an acid hydrolysate.

Results

The difference spectra generated by addition of some peptide analogs (Table I) to S-protein are shown in Figure 1. Little variation is noted in the general shapes of the curves regardless of structure of the peptide or the value for the dissociation constant. In all cases absorbance maxima were observed at 280 and 287 nm. The 287-nm peak was used in the calculation of the dissociation constant. The molar $\Delta\epsilon_{287}$ for eq 2 could be estimated by a modification of the method of Nakamura *et al.* (1965). This modification which involves normalizing values for absorbance and peptide concentration to a single protein concentration was necessitated by the experimental design, since the concentrations of the solutions decreased with addition of peptide. Within the limit of experimental error $\Delta\epsilon_{287} = 1 \times 10^3$ M^{-1} cm^{-1} for all peptides tested. In several cases this value was confirmed by independent measurements under conditions where volume was kept constant. Using this value it was possible to determine the concentration of RNase S or RNase complex and hence the concentration of unbound peptide and protein for each spectral curve.

Based on the results of competitive inhibition experiments with this same series of peptides it was concluded that S-pep-

tide$_{1-14}$, Pyr^{12}S-peptide$_{1-14}$, Nle^{13}S-peptide$_{1-14}$, and S-peptide$_{1-20}$, bind equally firmly to S-protein. 3-CM-His^{12}S-peptide$_{1-14}$ exhibited a slightly enhanced binding which was attributed to a postulated electrostatic interaction between this substituted histidine and histidine^{119} (Hofmann *et al.*, 1970). These results are confirmed in the absence of substrate by the dissociation constants in Table I, although, the difference detected with the 3-CM-His^{12} peptide is not discernible. Substitution of a hydroxyl side chain for the aromatic five-membered ring of histidine as in Ser^{12}S-peptide$_{1-14}$ was found to decrease the binding between seven- and eightfold (Finn *et al.*, 1968). No difference is observed in the simple dissociation constants as determined here. The sulfoxides of S-peptide$_{1-20}$ and 3-CM-His^{12}S-peptide$_{1-14}$ do show a definite increase in dissociation constants. Although, the S-protein activation curves for the sulfoxides of S-peptide$_{1-14}$ or S-peptide$_{1-20}$ are not remarkably different from those of the corresponding thioether peptides, with competitive inhibition studies, it has been shown that peptides containing methionine sulfoxide bind generally from 500 to 3000 times less firmly than their methionine counterparts (Hofmann *et al.*, 1970; Finn and Hofmann, 1967).

Removal of lysine from position 1 of S-peptide$_{1-13}$ has been shown to have little effect on the ability of the resulting peptide to reform active enzyme (Finn and Hofmann, 1965). These results have been confirmed with the competitive inhibitor Pyr^{12}S-peptide$_{2-14}$ which binds approximately as firmly as S-peptide$_{1-14}$ in the presence (Hofmann *et al.*, 1971) or absence of substrate. However, deletion of the glutamic acid from position 2 of S-peptide$_{2-13}$ or Pyr^{12}S-peptide$_{2-14}$ seriously impairs the ability of the resulting peptide to bind to S-protein. Similar results are obtained with the dissociation constants.

Replacement of arginine^{10} in S-peptide$_{1-20}$ by ornithine was shown to lower the enzymatic activity of the peptide–S-protein complex to approximately 45% that of RNase S (Moroder *et al.*, 1972). Hofmann *et al.* (1970, 1971) replaced arginine^{10} by ornithine in peptides containing either Pyr or 3-CM-His in place of histidine to evaluate, *via* competitive inhibition experiments, the effect of the Arg–Orn interchange on the binding capabilities of the peptides. Neither ornithine peptide was as good a competitive inhibitor as its arginine counterpart, however, the added stabilization afforded by the carboxymethyl group is apparent in this instance. The dissociation constants for these two analogs similarly indicate that the Orn^{10}Pyr^{12} peptide binds less firmly to S-protein; the constant for the Orn^{10}-3-CM-His^{12} peptide does not differ, within the limit of error of the method, from that of S-peptide$_{1-20}$.

Discussion

Binding constants for the reaction shown in eq 1 have recently been determined in a number of laboratories (Woodfin and Massey, 1968; Moroder *et al.*, 1972; Hearn *et al.*, 1971; Kenkare and Richards, 1966; Berger and Levit, 1972). In general, binding measurements based on regeneration of enzymic activity upon addition of S-peptide to S-protein in the presence of substrate have resulted in values ranging from 10^8 to 10^9 moles per l. whereas measurements based on a spectrophotometric change occurring at 287 nm upon addition of peptide to protein in the absence of substrate would indicate that the strength of the peptide–protein association is several orders of magnitude weaker. Association constants from 10^4 to 10^5 moles per l. have been obtained in this manner.[2] Comparison of binding results from different laboratories is still further complicated since these measurements have been performed under a variety of conditions of temperature, pH, and ionic strength. For this reason the conditions chosen for the spectrophotometric measurements reported here are the same (with the exception of the absence of substrate) as those employed for the determination of 50% inhibition ratios.

Constants obtained in the presence of substrate actually measure more than simple peptide–protein binding since measurement of enzymic activity implies formation of an ES complex which disturbs the simple equilibrium shown in eq 1 and 2. In addition, Erman and Hammes (1966) have detected, by stop-flow techniques, an isomerization of the enzyme–substrate complex which still further complicates the interpretation of such binding constants. For these reasons the 50% ratio (Table I) has been used to compare the relative activation or inhibition capacities of the various peptides under conditions where substrate is present.[3] It is hoped that some useful information may be derived by comparing the data obtained through both approaches.

One striking difference in the data collected with and without substrate is the magnitude of the effect created by peptide alteration. From the results in Table I, it would seem that dissociation constants are relatively less affected by amino acid substitutions than the 50% ratios. This is especially evident for the sulfoxide of 3-CM-His^{12}S-peptide$_{1-14}$.

Neither the somewhat weaker binding of the serine^{12} peptide nor the slightly greater affinity of the 3-CM-His^{12} peptide are demonstrable in the dissociation constants. In fact the only instances where the differences in dissociation constants approximate the size of those found with substrate are for Orn^{10}Pyr^{12}S-peptide$_{1-14}$ and Pyr^{12}S-peptide$_{3-14}$. In both of these cases weaker binding has been attributed to the inability of the peptide to form a noncovalent bridge along the axis of the helical portion of the peptide in the RNase complex[4] between Glu^2 and Arg^{10}. In the first peptide, substitution of the shorter side chain of ornithine for arginine results in too great a distance between the side chains of the two participating residues. In the second peptide, of course, one of the residues is absent.

Using nuclear magnetic resonance techniques it was possible to show that S-peptide and a number of its analogs and fragments undergo a conformational change upon binding to S-protein and that the side chains of glutamic acid^2 and arginine^{10} residues are involved in this change. Furthermore, when either residue is missing or substituted the protons of its compliment cease to be affected by binding (Finn *et al.*, 1972). Based on these experiments it would seem that a better correlation between 50% inhibition ratios and dissociation constants for a series of peptides is achieved in those cases where the peptide modifications involve residues not in the immediate vicinity of the active site of the RNase complex, *i.e.*, His^{12} and Met^{13}. In those cases where amino acid substitutions occur at or near the active site, comparison of 50% inhibition ratios is a far more "sensitive" barometer of change.

[2] Hearn *et al.* (1971) and Moroder *et al.* (1972) have recently reported values of 10^7 and 10^5 moles per l., respectively, for association constants obtained by microcalorimetry in the absence of substrate. The reason for this large discrepancy is unclear.

[3] It has been shown (Visser, 1969) that this quantity is directly proportional to the relative dissociation constants calculated by the method of Kenkare and Richards (1966).

[4] Kartha (personal communication) has indicated that the R groups of Glu^2 and Arg^{10} are within hydrogen bonding distance in the RNase A crystal.

However, it is likely that in these situations the effect of substrate is most evident.

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Studies on Heart Phosphofructokinase. Binding of Cyclic Adenosine 3′,5′-Monophosphate, Adenosine Monophosphate, and of Hexose Phosphates to the Enzyme†

Barbara Setlow and Tag E. Mansour*

ABSTRACT: The binding of adenosine 3′,5′-monophosphate (cyclic 3′,5′-AMP), adenosine 5′-monophosphate (5′-AMP), and hexose phosphates to purified sheep heart phosphofructokinase were studied using the technique of equilibrium dialysis. The native enzyme binds, at saturation, 1.0 mole each of cyclic 3′,5′-AMP and AMP per 100,000 g of enzyme. Both nucleotides bind to the same site on the enzyme. The total number of sites as well as the dissociation constant for cyclic 3′,5′-AMP is the same for native enzyme and for enzyme which has been desensitized to allosteric control. Binding of the cyclic nucleotide is relatively insensitive to pH. Several effectors of enzyme activity alter the binding of cyclic 3′,5′-AMP by changing its dissociation constant. The enzyme inhibitors, citrate and tripolyphosphate, slightly increase the K_D for cyclic 3′,5′-AMP whereas the activator, fructose 1,6-diphosphate (fructose 1,6-di-P), decreases the K_D about twofold. Studies of the binding of fructose 6-phosphate (fructose-6-P) to the enzyme revealed the presence of two classes of binding sites; high-affinity sites and low-affinity sites. K_D's for the high-affinity sites varied from 0.08 μM in the presence of 5′-AMP to 1.7 μM in the presence of citrate. K_D's for the low-affinity sites ranged from 9.0 to 43 μM under the same conditions. Both the high- and low-affinity sites can also bind fructose-1,6-di-P.

Purified phosphofructokinase (ATP:D-fructose-6-P 1-phosphotransferase, EC 2.7.1.11) from sheep heart displays many of the kinetic properties associated with a typical allosteric enzyme (Mansour, 1963; Mansour and Ahlfors, 1968). At an acidic pH there is a sigmoidal response to one of its substrates, fructose-6-P. At a pH less than 7, a low ionic strength, and a suboptimal concentration of fructose-6-P, phosphofructokinase from the heart as well as from other sources is inhibited by ATP (Lardy and Parks, 1956; Mansour and Mansour, 1962, Mansour, 1963; Passonneau and Lowry, 1962), citrate (Garland *et al.*, 1963; Parmeggiani and Bowman, 1963; Passonneau and Lowry, 1963), and creatine-P[1,2]

† From the Department of Pharmacology, Stanford University School of Medicine, Stanford, Californoa 94305. *Received November 29, 1971.* Supported by Public Health Service Research Grant (AI04214) from the National Institute of Allergy and Infectious Diseases and grant-in-aid from the American Heart Association.

[1] Abbreviations used in this work are: creatine-P, creatine phosphate; β-glycero-P, β-glycerophosphate.

[2] A. S. Otani and T. E. Mansour, unpublished observation.