Studies on Polypeptides. XXXIII. Enzymic Properties of Partially Synthetic Ribonucleases[1-4]

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Synthetic peptides related to the N-terminal eicosapeptide portion of pancreatic ribonuclease A (S-peptide) were tested for ability to generate ribonuclease activity with S-protein. Of the S-peptide fragments studied lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylmethionine is the most active (50% activation ratio 3). The partially synthetic enzyme produced with this peptide hydrolyzes yeast RNA as well as uridine and cytidine 2',3'-phosphates. Lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutamine fails to generate active enzyme at molar peptide to S-protein ratios of 8000 in contrast to lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidine amide with a 50% activation ratio of 88. This observation provides direct evidence for a role of histidine 12 for enzymic activity and eliminates amino acid residues 13–20 of S-peptide as essential for S-protein activation. Glutamic acid 2 appears to be an important "binding site" for S-peptide–S-protein interaction since elimination of the N-terminal glutamic acid from glutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylmethionine (50% activation ratio 41) affords a peptide with a 50% activation ratio of 2000. The observation that phenylalanylglutamylarginylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine which corresponds to almost two-thirds of the S-peptide molecule possesses a 50% activation ratio higher than 8000 supports this concept. Phenylalanylglutamylarginylglutaminylhistidine amide constitutes the smallest fragment of S-peptide, studied to date, which activates S-protein with RNA as substrate. Replacement of glutamine 11 by glutamic acid in lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylmethionine markedly decreases its S-protein-activating ability. The S-protein-activating capacity of synthetic S-peptide d-sulfoxide is identical with that of the dl-sulfoxide derived from natural S-peptide. The apparent K_m values of ribonuclease S' and partially synthetic ribonucleases resulting from the combination of three peptides with S-protein are strikingly similar. The significance of these findings for ribonuclease function and their relation to certain observations in the peptide hormone field are discussed.

Introduction

Our demonstration[4a] that a derivative of S-peptide[5] from which a sizable portion of the peptide chain is missing has the ability, in combination with S-protein,[5] to regenerate ribonuclease activity prompted study of the S-protein-activating ability of synthetic peptides corresponding to sections of the S-peptide sequence (Table I). Delineation of the smallest fragment possessing this property may define those portions of the peptide chain which are essential for enzymic function. In addition, investigation of the affinity of such peptides for S-protein is likely to provide fundamental information pertaining to the structural elements of S-peptide which are responsible for its noncovalent, highly specific alignment on S-protein to form ribonuclease-S'.[5] This communication is concerned with the S-protein-activating ability of a series of peptides whose syntheses have been described.[3,6,7]

Experimental

Materials. Yeast RNA (Sigma Chemical Co., Lot. No. R52-B-074) was purified by precipitation from 0.1 *M* sodium acetate.[8] Uridine 2',3'-phosphate barium salt (Schwarz BioResearch Lot. No. 6202) and cytidine 2',3'-phosphate barium salt (Schwarz BioResearch Lot. No. 6302) were used without further purification. Ribonuclease A was prepared from bovine pancreatic ribonuclease (Sigma Chemical Company, five times crystallized, Lot. No. R42-B093, R23B-335, and 63B-1010) by the procedure of Crestfield, *et al.*[9] Ribonuclease S was prepared from ribonuclease A by the method of Richards.[10] S-Protein and S-peptide were separated by precipitation with trichloroacetic acid. The precipitate (S-protein trichloroacetate), dissolved in water (100 ml./g. of RNAase S), was chromatographed on acetate cycle Amberlite CG-400 (10 equiv. of resin per equiv. of

(1) The authors wish to express their appreciation to the U. S. Public Health Service, the National Science Foundation, and the American Cancer Society for generous support of this investigation. Frances M. Finn acknowledges financial support from U. S. P. H. Training Grant 2G-149.

(2) The peptides and peptide derivatives mentioned are of the L-configuration. In the interest of space conservation the customary L-designation for individual amino acid residues is omitted.

(3) See K. Hofmann, R. Schmiechen, M. J. Smithers, R. D. Wells, Y. Wolman, and G. Zanetti, *J. Am. Chem. Soc.*, **87**, 640 (1965), for paper XXXII in this series.

(4) Preliminary accounts of this work have appeared: (a) K. Hofmann, F. M. Finn, W. Haas, M. J. Smithers, Y. Wolman, and N. Yanaihara, *ibid.*, **85**, 833 (1963); (b) K. Hofmann, *Harvey Lecture Ser.*, **59**, 89 (1964); lecture delivered Dec. 19, 1963; (c) Presented in part at the Sixth International Congress of Biochemistry in New York, July 1964.

(5) F. M. Richards, *Proc. Natl. Acad. Sci. U. S.*, **44**, 162 (1958); the following abbreviations will be used: RNAase-S, subtilisin-modified beef ribonuclease RNAase-A; S-peptide, the eicosapeptide obtained from RNAase-S; S-protein, the protein component obtained from RNAase-S; RNAase-S', the reconstituted enzyme obtained by mixing equimolar proportions of S-peptide and S-protein; RNA, yeast ribonucleic acid.

(6) (a) K. Hofmann, R. Schmiechen, R. D. Wells, Y. Wolman, and N. Yanaihara, *J. Am. Chem. Soc.*, **87**, 611 (1965); (b) K. Hofmann, W. Haas, M. J. Smithers, R. D. Wells, Y. Wolman, N. Yanaihara, and G. Zanetti, *ibid.*, **87**, 620 (1965); (c) K. Hofmann, W. Haas, M. J. Smithers, and G. Zanetti, *ibid.*, **87**, 631 (1965).

(7) The syntheses of peptides I, VIII, XI, and S-peptide *d*-sulfoxide will be described in forthcoming publications.

(8) W. A. Klee and F. M. Richards, *J. Biol. Chem.*, **229**, 489 (1957).

(9) A. M. Crestfield, W. H. Stein, and S. Moore, *ibid.*, **238**, 618 (1963).

(10) F. M. Richards and P. J. Vithayathil, *ibid.*, **234**, 1459 (1959).

Table I. Amino Acid Sequence and S-Protein-Activating Capacity of S-Peptide and Related Synthetic Compounds; Substrate RNA.

	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20	% RNase S′ ACTIVITY AT MOLAR RATIO ×:1 100	200	300	700	2000	5000	8000
	H·LYS·GLU·THR·ALA·ALA·ALA·LYS·PHE·GLU·ARG·GLU(NH$_2$)·HIS·MET·ASP·SER·SER·THR·SER·ALA·ALA·OH	100						
	S PEPTIDE (NATURAL)							
I	H·PHE·GLU·ARG·GLU(NH$_2$)·HIS·MET·ASP·SER·SER·THR·SER·ALA·ALA·OH					8		
II	H·PHE·GLU·ARG·GLU(NH$_2$)·HIS·MET·OH					4	10	15
III	H·PHE·GLU·ARG·GLU(NH$_2$)·HIS·BUT·OH					3	7	13
IV	H·PHE·GLU·ARG·GLU(NH$_2$)·HIS·NH$_2$							11
V	H·LYS(F)·PHE·GLU·ARG·GLU(NH$_2$)·HIS·MET·OH		1	5	7	10	12	18
VI	H·ALA·ALA·ALA·LYS·PHE·GLU·ARG·GLU(NH$_2$)·HIS·MET·OH	15				39	61	70
VII	H·THR·ALA·ALA·ALA·LYS·PHE·GLU·ARG·GLU(NH$_2$)·HIS·MET·OH	24				48	71	74
VIII	H·GLU·THR·ALA·ALA·ALA·LYS·PHE·GLU·ARG·GLU(NH$_2$)·HIS·MET·OH	65	100					
IX	H·LYS·GLU·THR·ALA·ALA·ALA·LYS·PHE·GLU·ARG·GLU(NH$_2$)·HIS·MET·OH	80						
X	H·LYS·GLU·THR·ALA·ALA·ALA·LYS·PHE·GLU·ARG·GLU(NH$_2$)·HIS·BUT·OH	65			100			
XI	H·LYS·GLU·THR·ALA·ALA·ALA·LYS·PHE·GLU·ARG·GLU·HIS·MET·OH	10	16	21	26			
XII	H·LYS·GLU·THR·ALA·ALA·ALA·LYS·PHE·GLU·ARG·GLU(NH$_2$)·HIS·NH$_2$	52	100					
XIII	H·LYS·GLU·THR·ALA·ALA·ALA·LYS·PHE·GLU·ARG·GLU(NH$_2$)·OH	0		0			0	0

trichloroacetate). The column was developed with water, Pauly-positive eluates were collected, and one-tenth volume of glacial acetic acid was added. The solution was concentrated to a sirup at a bath temperature of 35°, dissolved in water (final protein concentration approximately 2%), and lyophilized. The combined supernatant solutions, from the trichloroacetate precipitation (S-peptide), were chromatographed on acetate cycle IRA-400 (2.5 equiv. of resin per equiv. of trichloroacetate) with water as the solvent. One-tenth volume of glacial acetic acid was added to the Pauly-positive effluent and the solution was concentrated to a sirup which was dissolved in water to yield a 1% solution and lyophilized (crude S-peptide).

Purification of S-Peptide. Crude S-peptide (308 mg.) dissolved in water (100 ml.) was applied to a carboxymethylcellulose column (3 × 25 cm.) which was eluted with the following pH 6.8 ammonium acetate buffers: 0.01 (100 ml.), 0.02 (500 ml.), and 0.04 *M* (300 ml.). The desired material was located in the 0.04 *M* eluates by the Lowry reaction.[11] Lowry-positive fractions were pooled, concentrated *in vacuo* at a bath temperature of 40°, and lyophilized to constant weight from water; yield 153 mg.; $[\alpha]^{28}D$ −42.0° (*c* 0.55, 10% acetic acid); R_f 0.48 × His in the pyridine system[12]; single Pauly- and ninhydrin-positive spot on paper electrophoresis at pH 1.9, 3.5, and 6.5 shows slight contamination with slower moving component at pH 8.0; amino acid ratios in acid hydrolysate $lys_{2.16}$-$his_{1.00}arg_{0.94}asp_{1.01}thr_{1.83}ser_{3.12}glu_{3.15}ala_{4.92}met_{0.89}phe_{0.98}$; amino acid ratios in LAP digest $(lys+orn+arg)_{2.60}$-$his_{0.99}asp_{1.06}thr_{2.00}(ser+gluta)_{3.44}glu_{2.56}ala_{5.24}met_{1.00}phe_{1.07}$. Natural S-peptide, natural S-peptide *dl*-sulfoxide, and synthetic S-peptide *d*-sulfoxide[7] exhibit the same paper electrophoretic behavior. Natural S-peptide *dl*-sulfoxide and synthetic S-peptide *d*-sulfoxide behave identically on paper chromatography; R_f 0.27 × His.[12]

Assay Procedures. With RNA as substrate, ribonuclease assays were performed essentially as described by Kunitz.[13] For preparation of enzyme solutions S-protein ($3.5–5.0 \times 10^{-6}$ *M*) and varying amounts of S-peptide or the synthetic peptides were incubated at 25° for 30 min. before assay. These enzyme solutions (0.2 ml.) were added to a 0.1% solution of RNA in 0.1 *M* pH 5.0 sodium acetate buffer (2.0 ml.) and water (1.8 ml.). Optical density changes at 300 mμ were recorded within 15 sec. after addition of enzyme. A solution of uridine 2′,3′-phosphate (96.5 mg.) in 0.3 *M* sodium chloride (50 ml.) was cooled at 0°. S-Protein ($3.5–7.0 \times 10^{-6}$ *M*) in 0.3 *M* sodium chloride (0.2 ml.) was added to a 3-ml. aliquot of the substrate solution. The mixture was incubated for 5 min. at 30°, the pH was adjusted to 7.0 with 0.01 *N* sodium hydroxide, and the rate of 0.01 *N* sodium hydroxide consumption was measured with a Radiometer pH-stat for 5 min. to establish a blank. Varying amounts of S-peptide or of the synthetic peptides in 0.3 *M* sodium chloride were then added. Cytidine 2′,3′-phosphate (5 mg.) was dissolved in 25 ml. of pH 7.09 sodium phosphate buffer (NaH_2PO_4, 3.23 g.; $Na_2HPO_4 \cdot H_2O$, 7.52 g./l.). To 2.8 ml. of this solution was added 0.2 ml. of a solution containing S-protein (3.5×10^{-6} *M*) and varying amounts of S-peptide or synthetic peptides preincubated for 30 min. in the same buffer at 25°. Buffer (0.2 ml.) was substituted for enzyme in the blank. Increase in absorbancy *vs.* time was measured at 284 mμ with a slit width of 0.59 mm.

Activities are expressed as per cent of initial rate obtained using the same concentration of ribonuclease S′. Spectrophotometric measurements were made with a Cary 14 recording spectrophotometer equipped with a scale expander (0–0.1 optical density).

Oxidation–Reduction Experiments. For preparation of sulfones, S-peptide or peptide IX (1 μmole) was dissolved in 98% formic acid (0.30 ml.) containing methanol (0.05 ml.) and the solution was cooled at

(11) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(12) S. G. Waley and J. Watson, *Biochem. J.*, **55**, 328 (1953).

(13) M. Kunitz, *J. Biol. Chem.*, **164**, 563, (1946).

0°. Performic acid freshly prepared according to Hirs[14] (25 μmoles) precooled to 0° was added and the mixture was kept for 2 hr. at 0°. Water (2.5 ml.) was added and the solution was lyophilized. Lyophilization from water was repeated three times to yield a fluffy white material, which was used for assay without further purification. For preparation of sulfoxides, S-peptide or IX (1 μmole) was dissolved in water (1.0 ml.) and 3% hydrogen peroxide (100 μmoles) was added. The solution was kept at room temperature for 45 min. at which time excess hydrogen peroxide was destroyed by addition of 200 units of catalase (Worthington Biochemicals). The resulting solution, negative to starch–potassium iodide paper, was diluted to 2 ml. with water. An aliquot (0.5 ml.) was used for enzyme assay. To the remainder aqueous 4% thioglycolic acid (0.5 ml.) was added and the solution was incubated under nitrogen at 48° for 24 hr. This solution was used for enzyme assays with a solution identical in composition but containing no peptide serving as blank.

Determination of Apparent K_m Values. Apparent K_m values were determined for the ribonucleases formed by combining S-peptide, peptide IX, peptide X, or peptide XII, respectively, with S-protein. Peptide to protein ratios large enough to ensure a measurable rate of hydrolysis throughout the range of substrate concentrations were selected (Table II). A 0.2-ml. aliquot of a solution of RNAase S′ or the partially synthetic ribonucleases ($3.5–4.0 \times 10^{-6}$ *M* with respect to S-protein) in pH 6.69 Tris buffer[15] was added to 2 ml. of a solution containing cytidine 2′,3′-phosphate ($10^{-3}–10^{-4}$ *M*) in the same buffer. Increase in absorbancy with time was monitored at 284 mμ.

Table II. Apparent K_m Values with Cytidine 2′,3′-Phosphate for Various Ribonucleases

Enzyme	Activating peptide[a]	$K_m \times 10^3$	Rel. V_{max}	pH	Temp., °C.
RNAase S′	S-peptide (1.43)	1.5	100	6.9	25
	IX (29.1)	3.3	66	6.9	25
	X (57.8)	2.7	38	6.9	25
	XII (147)	1.5	20	6.9	25

[a] Roman numerals refer to peptides listed in Table I; numbers in parentheses represent peptide to S-protein ratios.

Apparent K_m values were obtained graphically from a Lineweaver–Burk plot. Lines were fitted to the experimentally determined points by the method of least squares.

Results

The ability of a series of homogeneous synthetic peptides related to S-peptide to generate ribonuclease activity when added to S-protein is summarized in Table I. The peptides were tested at molar ratios which varied from 1 to 8000 with yeast RNA as substrate. Based on the molar ratios necessary to bring about activation these peptides may be grouped into four categories, *i.e.*, those which generate high RNAase activity at ratios of 100 and lower (peptides VIII, IX, X, and XII), those whose activating potential is low at ratios of 100 but approaches high levels at ratios of 8000 (peptides VI, X, and XI), those exhibiting a low level of potency at ratios between 2000 and 8000 (peptides I–V), and finally those possessing no activity at ratios as high as 8000 (peptide XIII), and others (see below).

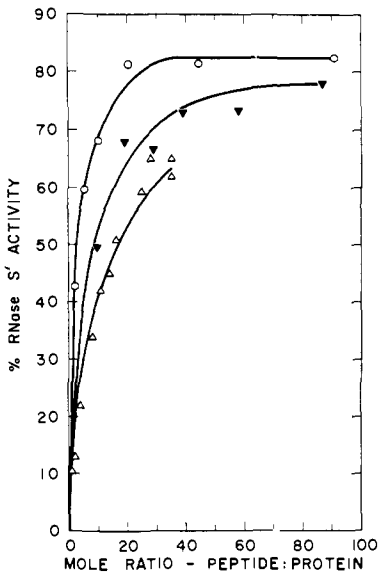


Figure 1. Ability of peptide IX to activate S-protein toward RNA and 2′,3′-phosphates: ○, RNA; ▼, cytidine 2′,3′-phosphate; △, uridine 2′,3′-phosphate.

A ribonuclease exhibiting 80% the activity of ribonuclease S′ ensues when the tridecapeptide IX is added to S-protein at a molar ratio of 30 (Figure 1). Not only does this peptide activate S-protein for yeast RNA as the substrate but the partially synthetic ribonuclease which results from its combination with S-protein has the ability to hydrolyze cytidine and uridine 2′,3′-phosphates as well (Figure 1). Thus the partially synthetic enzyme produced with this peptide is a true ribonuclease. However, higher molar peptide–S-protein ratios are required to bring about hydrolysis of the cyclic phosphates at rates comparable to those observed with RNA.

To gain information regarding the role of the methionine 13 residue for ribonuclease activity, peptides in which methionine is modified, exchanged, or eliminated were tested for their capacity to activate S-protein. Richards[16] showed that conversion of S-peptide into the sulfone lowers the S-protein-activating ability. Since the possibility existed that other amino acid residues, in addition to methionine, may have undergone change during performic acid oxidation of S-peptide, we synthesized the *d*-sulfoxide of S-peptide and compared its ability to activate S-protein with that of the *dl*-sulfoxide of natural S-peptide. The results (Figure 2) demonstrate that the activities of the two preparations are identical within the error of assay. Three moles of the sulfoxide are required to activate fully 1 mole of S-protein. As has been stated previously[4a] larger proportions of the sulfone, obtained from peptide IX by performic acid oxidation, are required to activate S-protein. This effect is illustrated in Figure 3. Similarly, oxidation of peptide IX to

(14) C. H. W. Hirs, *J. Biol. Chem.*, **219**, 611 (1956).
(15) E. M. Crook, A. P. Mathias, and B. R. Rabin, *Biochem. J.*, **74**, 234 (1960).
(16) P. J. Vithayathil and F. M. Richards, *J. Biol. Chem.*, **235**, 2343, (1960).

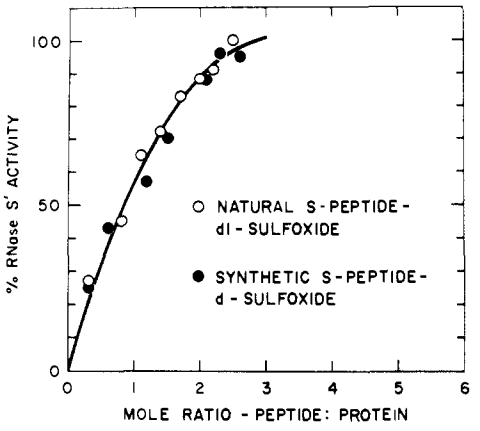


Figure 2. Activation of S-protein by natural and synthetic S-peptide sulfoxides: substrate RNA.

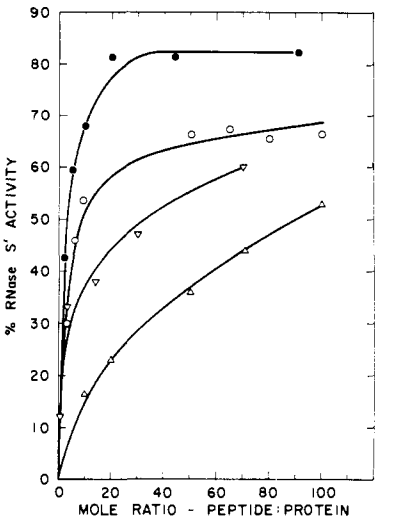


Figure 3. Effect of alteration and elimination of methionine 13 on ability of synthetic peptides to activate S-protein toward RNA. ●, peptide IX; O, peptide X; ▽, peptide XV; △, peptide XII.

the sulfoxide lowers its activating potential, but full activity is regained when the sulfoxide is incubated with thioglycolic acid.[4a]

To establish the methionine sulfur as the only site for reversible oxidation–reduction of peptide IX the ability of its α-amino-*n*-butyric acid analog (X) to restore active enzyme before and after hydrogen peroxide oxidation was evaluated. As may be seen in Figure 3 peptide X is highly active. Its ability to form active enzyme with S-protein remains unchanged after oxidation by either performic acid or hydrogen peroxide. Finally the activity of peptide XII, which C-terminates in histidine amide (Figure 3), unequivocally eliminates methionine 13 as essential for ribonuclease activity.

The importance of histidine 12 for S-protein activation is most strikingly demonstrated in the peptide pair XII and XIII where addition of the histidine amide moiety to the *totally* inactive peptide XIII results in formation of the peptide amide XII which fully activates S-protein at molar ratios lower than 200. Elimination of the N-terminal lysine residue from peptide IX appears to exert little effect; however, further shortening of the chain affords a compound (peptide VII) whose activating potential is low at a ratio of 100 but approaches high levels at ratios of 8000. Removal of threonine from peptide VII to give peptide VI does not change significantly the ability to regenerate enzyme (Table I). Peptide V, which differs from peptide VI by the three N-terminal alanine residues and contains an N^{ϵ}-acylated amino group on lysine 7, is still less effective.

The smallest S-peptide fragment studied thus far which possesses S-protein-activating properties, but only at high peptide-to-protein ratios, is peptide IV. The potency of the related peptides II and III is of the same order of magnitude. It is particularly significant to note that the activity of the tridecapeptide I, which corresponds to over 60% of the S-peptide sequence, does not differ markedly from that of these small fragments.

Replacement of the carboxamide function of the glutamine in position 11 by a carboxyl group (peptide XI) lowers drastically the S-protein-activating capacity. In addition to the peptides listed in Table I we have tested histidylmethionine, phenylalanylglutamylarginylglutamine, lysylglutamylthreonylalanylalanylalanyllysine, aspartylserylserylthreonylserylalanylalanine, methionylaspartylserylserylthreonylserylalanylalanine, and histidylmethionylaspartylserylserylthreonylserylalanylalanine at peptide-to-protein ratios of 8000 and found them *totally* inactive.

In an effort to determine whether partially synthetic ribonucleases formed from combination of certain of the synthetic peptides with S-protein differ in some of their kinetic parameters from RNAase-S′, V_{max} and apparent K_m values were determined using cytidine 2′,3′-phosphate as the substrate. No significant differences were observed in the apparent K_m values when S-protein was activated by S-peptide or by peptides IX, X, and XII, despite large variations in V_{max} which reflect the different levels of activation employed (Table II).

Discussion

Two possible answers come to mind when one inquires into the molecular events that bring about regeneration of ribonuclease activity from S-peptide and S-protein: either their combination results in formation of an active site constituted from amino acid residues belonging to both parts, or their interaction elicits a conformational change in the protein to create an active site whose structural features are located exclusively within the protein portion.

Discussion of our experimental results is based on the premise that any peptide which generates ribonuclease activity with S-protein, irrespective of the peptide–protein ratios necessary, embodies within its structure those elements which are essential for catalytic function. The assumption that peptides which bring about activation only at high peptide–protein ratios bind less firmly to S-protein is also implied. On this basis the pentapeptide IV which generates 11% activity at 8000:1 contains the structural features necessary for activation but exhibits low affinity for S-protein.

In a preliminary communication[4a] we reported that peptide IX had the ability to generate 68–72% of the biological activity of RNAase-S′ when approximately

10 moles of the peptide was added per mole of S-protein. It was also stated that peptide XIII, which differs from peptide IX by the C-terminal histidylmethionine moiety, was inactive at a molar ratio of 100. We have since repeatedly tested different preparations of peptide XIII at ratios as high as 8000 and find it *totally* inactive. These observations implicate histidylmethionine in the catalytic function of ribonuclease.

Performic acid oxidation of the methionine in S-peptide lowers somewhat its S-protein-activating ability.[16] In the case of peptide IX, which appears to bind less firmly to S-protein, oxidation to the sulfoxide or sulfone brings about a more pronounced decrease in activity. However, the observation that oxidation only decreases but does not destroy the S-protein-activating properties of peptide IX eliminates methionine 13 as functionally essential. Thus, the histidine residue must be responsible for the difference in activity between peptides IX and XIII.

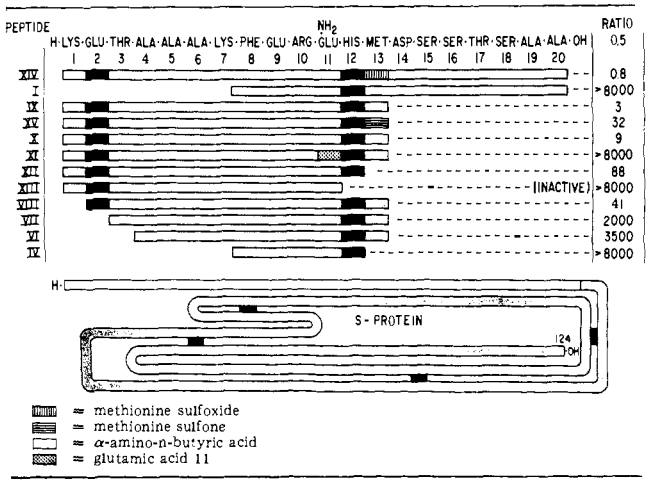
Prior to our studies Richards[5] observed that photooxidation destroyed the S-protein-activating properties of S-peptide, but that carboxymethylation of the histidine in S-peptide sulfone exerted "no marked effect on the binding of the peptide to S-protein or on the enzymic activity of the resulting complex."[16] On the basis of the behavior of RNAase toward iodoacetate at various pH values Stark, *et al.*,[17] postulated the presence, close to the active site, of a second histidine whose position was not identified. Barnard and Stein[18] had previously associated histidine 119 with the active site of the enzyme. Crestfield, *et al.*,[19] suggested a role for both histidine 12 and 119 in the catalytic site of ribonuclease based on dimerization experiments with ribonucleases carboxymethylated at either histidine. The pronounced ability of peptide IX to bring about activation of S-protein eliminates amino acid residues 14–20 of S-peptide as essential for catalytic activity. The observation that peptide XII is also endowed with S-protein-activating properties adds methionine to the list of nonessential amino acid residues. Thus, the section of S-peptide corresponding to positions 13–20 can be eliminated as functionally important for ribonuclease catalysis. The finding of Parks, *et al.*,[20] that Witkop's C-peptide[21] (lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylhomoserine or its lactone) has the ability to activate S-protein supports our conclusions.

Aspartylserine, implicated in the function of several proteolytic and esteratic enzymes,[22] can play *no* role with respect to ribonuclease catalysis since it is located in the nonessential portion of S-peptide. However, it would appear from studies with carboxypeptidase digests of S-peptide that aspartylserine or possibly the aspartic acid residue alone exerts a function as a binding site.

Conflicting reports have appeared concerning carboxypeptidase inactivation of S-peptide. Richards[5] observed that the biological properties of S-peptide are unchanged after incubation with this enzyme and Anfinsen[23] concluded from similar experiments that threonine 17 was probably essential for S-protein activation. More recently Potts, *et al.*,[24] regenerated ribonuclease activity on addition to S-protein of a crude carboxypeptidase digest of S-peptide, believed to contain the pentadecapeptide fragment positions 1 to 15. Apparently full activation of S-protein resulted when an aliquot of the digest corresponding to 1 mole of S-peptide was added per mole of S-protein. Peptide IX which differs from the pentadecapeptide by the C-terminal aspartylserine moiety brings about 30–35% activation of S-protein at the same molar ratio. In this connection it must be noted that elongation of the peptide chain of peptide II to give peptide I is accompanied by only an insignificant rise in S-protein-activating capacity.

Inspection of the shapes of activity *vs.* molar ratio curves (Figures 1–3) suggests that comparison of the peptide-to-protein molar ratio at 50% activation provides a meaningful measure of the relative activation potentials for the various peptides. Such values are compiled in Table III.

Table III. Fifty Per Cent Activation Ratios of S-Peptide and Related Compounds; Substrate RNA[a]



[a] Black rectangles serve to designate glutamic acid 2 and histidine 12.

Of the N-terminal amino acid residues in peptide IX glutamic acid 2 appears to be particularly effective as a binding site. This point is well illustrated in Table III. Peptide VIII which N-terminates in glutamic acid is approximately 500 times more effective than peptide VII which lacks this residue.

Eaker[25] isolated a naturally occurring isomer of RNAase which differs from ribonuclease A in that it lacks the N-terminal lysine. The observation that this "delysyl enzyme" possesses full ribonuclease activity eliminates lysine 1 as essential for activity of the intact enzyme. The present observation that peptide VIII forms enzyme only slightly less efficiently than peptide IX indicates that lysine 1 is not critical for the binding

(17) G. R. Stark, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **236**, 436 (1961).
(18) (a) E. A. Barnard and W. D. Stein, *Biochem. J.*, 19P (1959); (b) W. D. Stein and E. A. Barnard, *J. Mol. Biol.*, **1**, 350 (1959).
(19) A. M. Crestfield, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **238**, 2413 (1963).
(20) J. M. Parks, M. B. Barancik, and F. Wold, *J. Am. Chem. Soc.*, **85**, 3519 (1963).
(21) E. Gross and B. Witkop, *J. Biol. Chem.*, **237**, 1856 (1962).
(22) J. A. Cohen, R. A. Oosterbaan, H. S. Jansz, and F. Berends, *J. Cell. Comp. Physiol.*, **54** (Suppl. 1), 231 (1959).
(23) C. B. Anfinsen, *Brookhaven Symp. Biol.*, **15**, 184 (1962).
(24) J. T. Potts, Jr., M. Young, and C. B. Anfinsen, *J. Biol. Chem.*, **238**, PC 2593 (1963).
(25) D. Eaker, *J. Polymer Sci.*, **49**, 45 (1961).

of S-peptide to S-protein as well. Removal of the threonyl residue from peptide VII to give peptide VI increases the 50% activation ratio only by a factor of 1.75. Likewise the contributions of the individual alanine residues 4, 5, and 6 to peptide–protein binding are not significant. The methionine residue, although not contributing to catalytic function, appears to represent another binding site. This conclusion follows from results presented in Table III and Figure 3. Whereas removal of the C-terminal methionine from peptide IX results in a significant increase in the 50% activation ratio (from 3 to 88), its replacement by α-amino-*n*-butyric acid elicits only a change from 3 to 9. Conversion of the hydrophobic thioether sulfur to the hydrophilic sulfone is also accompanied by a significant increase in the 50% activation ratio from 3 to 32. The difference in the affinity for S-protein between peptides IX and X appears to be a reflection of the weaker hydrophobic binding ability of the ethyl side chain of α-amino-*n*-butyric acid.

In his extensive studies[16,26] on ribonuclease, Richards investigated the S-protein-activating potential of S-peptide derivatives produced by chemically altering the peptide from natural sources. In those instances where the enzymic characteristics of the modified peptides differ only slightly from those of the starting material, valid conclusions may be drawn pertaining to the importance of the functional group thus modified. Deamination of the N-terminal amino group and guanidation of the N^{α}- and N^{ϵ}-amino groups are modifications of this type. However, our experience indicates that meaningful evaluation of the S-protein-activating potential of S-peptide variants exhibiting low activity must involve their testing over a wide range of concentrations. As has been pointed out in a previous communication[6a] contamination of peptides possessing a low level of activity with trace amounts of unreacted S-peptide may lead to erroneous conclusions. The testing of synthetic S-peptide fragments or analogs eliminates this difficulty.

Studies pertaining to the importance of the carboxamide group of glutamine 12 for ribonuclease function illustrate this point. Richards[26b] exposed S-peptide to sodium hydroxide under rather drastic conditions which may have caused racemization or α,β-rearrangement of the aspartic acid residue, and evaluated the ability of the product to activate S-protein at molar ratios up to 8:1. From the results he "tentatively concluded that hydrolysis of the amide group in the peptide has no demonstrable effect on the binding of the peptide and protein components, and that the resulting complex has no enzymatic activity." Our preliminary results with peptide XI do not support this conclusion. This peptide which differs from peptide IX solely by substitution of glutamic acid for glutamine activates S-protein but to a markedly lesser degree (Table I). This decreased activity may be the result of lower affinity of the peptide for the protein, or the negative charge in proximity to the functionally critical histidine 12 may interfere with substrate binding.

In order to explore the possibility that peptide modifications which appear to alter the enzyme's efficiency effect substrate binding, the relationship between the activities of three partially synthetic ribonucleases and their apparent K_m's (with cytidine 2′,3′-phosphate as substrate) was studied. The apparent K_m values of ribonuclease A, ribonuclease S′, and three partially synthetic ribonucleases resulting from the combination of peptides IX, X, and XII with S-protein (Table II) are strikingly similar.

Based on kinetic measurements Richards inferred that the protein portion of ribonuclease S′ is the site for substrate binding. Similar conclusions were reached by Barnard and Ramel[27] from a comparison of the binding of cytidine 2′-phosphate to ribonuclease A and its histidine 119 carboxymethyl derivative. Our results can also be interpreted in this manner. It has been shown[4a] that mixtures of peptides whose combined sequences correspond to that of an active peptide fail to exhibit the biological properties of the parent structure. For example, addition of 100 moles of an equimolar mixture of lysylglutamylthreonylalanylalanylalanyllysine and peptide II to 1 mole of S-protein does not result in restoration of enzymic function at a level comparable to that observed with peptide IX. Similarly, histidylmethionine plus peptide XIII fails to act synergistically. Apparently the conformational requirements which ensure proper alignment between peptide and S-protein cannot be fulfilled in the absence of an intact peptide backbone.

Although the studies with partially synthetic ribonucleases are only at their beginning one notes a striking parallelism between the results of structure–function studies in the peptide hormone field and those with these enzymes.

The biological testing of fragments of the melanocyte-expanding hormone α-MSH and of the adrenocorticotropic hormone ACTH has resulted in the recognition that certain sections of these peptide hormones are the carriers of physiological function.[28] These fragments appear to bring about all of the physiological manifestations of the intact hormone but much higher amounts are required to elicit comparable effects. Based on these observations it was postulated[29] that these peptides contain "active sites" and "attachment" or preferably "binding" sites. The "active sites" were defined as "comprising those parts of the peptide molecule which can function as such although usually much less efficiently than the intact hormone"; the "binding sites" are inactive biologically, but in combination with the "active site" enhance biological potency. We have pointed to the S-peptide–S-protein system as an attractive model for peptide hormone function at the molecular level.[29] Our hypothesis pictures the cell receptors as incomplete enzymes, comparable to S-protein, and visualizes the hormone as the missing part, analogous to S-peptide, whose combination with the receptor brings about formation of an active enzyme. The "active site" in the peptide may thus become an integral part of the active site of the newly created enzyme. The observation by McKerns[30] that corticotropin increases by 30% the initial velocity of adrenal glucose 6-phosphate dehydrogenase preparations may be highly significant in this connection.

The results of this study have revealed a remarkable

(26) (a) P. J. Vithayathil and F. M. Richards, *J. Biol. Chem.*, **235**, 1029 (1960); (b) P. J. Vithayathil and F. M. Richards, *ibid.*, **236**, 1380 (1961).

(27) E. A. Barnard and A. Ramel, *Nature*, **195**, 243 (1962).
(28) K. Hofmann, *Ann. Rev. Biochem.*, **31**, 213 (1962).
(29) K. Hofmann, *Brookhaven Symp. Biol.*, **13**, 184 (1960).
(30) K. W. McKerns, *Biochim. Biophys. Acta*, **90**, 357 (1964).

similarity between the findings with the hormones and observations with S-peptide. Large portions of S-peptide can be removed without destroying its function and small subunits (peptide IV), when employed in high enough concentrations, generate enzymic activity. The "active site" of S-peptide appears to be located in the peptide phenylalanylglutamylarginylglutaminylhistidine. The rest of the molecule, particularly the glutamic acid residue in the N-terminal end and the sequence methionylaspartylserine, provides important "binding sites." Histidine must play a key role in catalysis; the involvement in catalytic function of the other amino acid residues located within the "active site" of S-peptide remains to be established.

Communications to the Editor

On the Optical Absorptions of Phthalocyanines

Sir:

In the course of our investigations of dyes as saturable absorbers for use in laser Q-switching, we have remeasured the optical absorption and fluorescence spectra of phthalocyanine solutions. In this note, we wish to compare the spectra of both the metal-free (H_2Pc) and copper (CuPc) derivatives and report on extra absorption and emission bands observed for these complexes. Pure phthalocyanine single crystals were ground into powder and dissolved in 1-chloronaphthalene to yield 10^{-5} *M* solutions. Each solution was examined visually by passing an intense beam of light through it. Colloidal particles were discovered in the solutions, indicating that we do not have a true solution but rather a mixture of a true solution and a small amount of solid phase. Consequently, the given amplitudes of the extinction coefficient are lower limits.

The spectral absorption curves were measured at room temperature with a Cary Model 14R recording spectrometer. Beckman absorption cells of 5-cm. and 10-cm. path length were used. An absorption spectrum for the 1-chloronaphthalene solvent recorded in the spectral region of interest revealed one strong band at 3450 Å. and therefore limited our measurements in the ultraviolet region. The absorption spectra of H_2Pc and CuPc are shown in Figure 1, and the pertinent

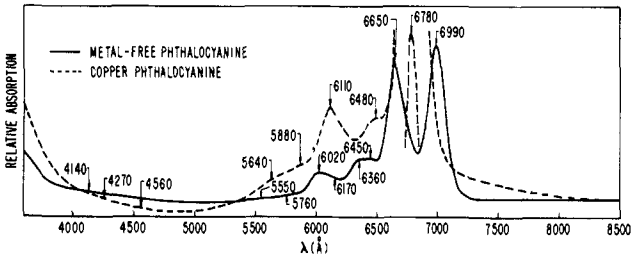


Figure 1. Absorption spectra of phthalocyanines in 1-chloronaphthalene.

data are listed in Table I. The fluorescence emission spectrum (Figure 2) for H_2Pc dissolved in 1-chloronaphthalene was recorded at 77°K. by passing the spectral output through a Bausch and Lomb grating monochromator to a photomultiplier detector (RCA 7102-Sl) and electrometer, the output of which was automatically recorded. The fluorescence bands were observed at 6990, 7350, and 7775 Å., respectively.

Table I. Wave Length and Extinction Coefficient of Absorption Bands

	H_2Pc		CuPc	
	λ_{max}, Å.	log ε	λ_{max}, Å.	log ε
a	6990	4.23	6780	5.1
b	6650	4.19	6480	4.36
c	6450	3.71	6110	4.39
d	6360	3.71	5880	3.57
e	6170	3.44	5640	3.37
f	6020	3.54	4560	2.38
g	5760	2.89	4270	3.0
h	5550	2.59	4140	3.69

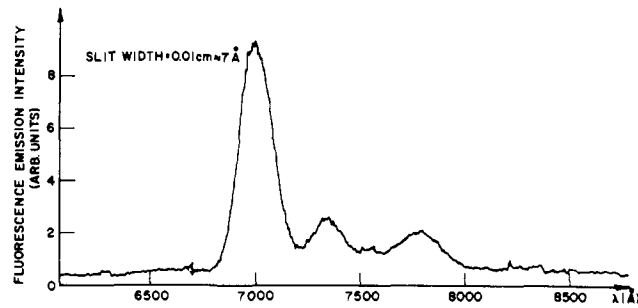


Figure 2. Fluorescence emission spectrum of metal-free phthalocyanine.

In the absorption spectra, no peaks in the ultraviolet due to the absorption of the 1-chloronaphthalene are listed here. However, ultraviolet measurements of thin films of H_2Pc gave peaks at 3334 and 2892 Å., while CuPc has peaks at 3292 and 2613 Å. The absorptions a through e in CuPc have been observed in the spectra recorded recently by Whalley,[1] which are considered to be more accurate than those reported previously. The H_2Pc spectrum is shown here to have eight distinct absorption maxima in the region 5500–7000 Å., while that of CuPc has five. Previously unrecorded absorptions at 6360 and 6170 Å. were observed. Although in Figure 1 these additional bands and others appear to be weak and therefore doubtful,

(1) M. Whalley, *J. Chem. Soc.*, 866 (1961).