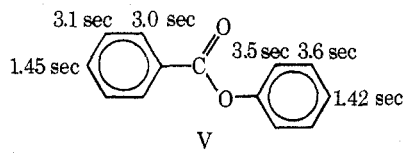

V

68° shows the following T_1's. The $T_1^{o,m}/T_1^{p}$ ratio is distinctly greater for the phenoxy ring than for the benzoyl group, presumable reflecting a lower barrier to rotation about the phenyl–oxygen bond than about the phenyl–carbonyl bond.

Other Applications. Some of the most important future applications for ^{13}C relaxation studies involve characterization of proteins, nucleic acids, and other biopolymer molecules.[25] Structure analysis of synthetic high polymers in solution from ^{13}C T_1 (and line shape T_2) measurements is also very promising.[26] Studies of solution effects such as hydrogen bonding and ion pairing may give new insight into these processes.[6,22,27,28] The ^{13}C T_1 experiment shown in Figures 3 and 4 illustrates the effects of strong solvation of ions. The tumbling of nonprotonated aniline is nearly isotropic, whereas the anilinium ion rotates approximately ten times faster around the C_2 axis. Other ^{13}C relaxation studies of rapid molecular motions will undoubtedly emerge soon, showing additional applications for this important new technique.

I wish to acknowledge the valuable contributions of Professor F. A. L. Anet and Joseph D. Cargioli. Professors D. M. Grant, A. Allerhand, and J. B. Grutzner and Doctors R. Freeman, H. M. Relles, and E. D. Becker provided helpful discussions. I also thank the management of General Electric Corporate Research and Development for their support.

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The S-Peptide–S-Protein System: a Model for Hormone–Receptor Interaction[1a]

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Structure–function studies with peptide hormones have provided considerable understanding of the contribution of individual amino acid residues within a sequence to overall biological activity. As a result of these investigations, it is now recognized that large structural modifications can be tolerated in many cases without loss of physiological activity. An adrenocorticotropic hormone ($ACTH_{1-20}$-amide[1b]) one-half the size of natural ACTH is fully active as concerns steroidogenesis and ascorbic acid depletion.[2] Recently, still shorter analogs with 5–10 times the activity of the natural molecule have been produced.[3,4]

The C-terminal tetrapeptide amide of gastrin is another example of a hormone fragment possessing biological activity. The natural heptadecapeptide amide is about fivefold more potent than the fragment, but the tetrapeptide amide still possesses the full spectrum of biological actions.[5] Furthermore, a number of amino acid substitutions can be made in peptide hormone fragments without decreasing their potency.

On the basis of these and other similar findings it was possible to formulate some generalizations regarding the role of amino acid residues within these structures.[6] Two major functional classes of amino acids seem to exist: those concerned with binding the

Dr. Klaus Hofmann's primary research interests are the chemistry and biochemistry of biologically important natural products. He received his graduate training under L. Ruzicka at the ETH in Zürich, where he received his Ph.D. in 1936. After 2 more years in Zürich and 2 years at the Rockefeller Institute for Medical Research with the late Max Bergmann, he joined the group of Vincent du Vigneaud at Cornell Medical College to work on the isolation and structure proof of biotin. After a 2-year residence as a scientific guest at the Laboratories of Ciba Pharmaceutical Products, Inc., he moved to the University of Pittsburgh in 1944. He is now Professor of Experimental Medicine and Director of the Protein Research Laboratory, University of Pittsburgh School of Medicine.

Dr. Frances Finn received her Ph.D. from the University of Pittsburgh in 1964 with Professor Hofmann and remains as Assistant Research Professor of Biochemistry. In 1964–1965 she was a U. S. Public Health Postdoctoral Fellow, working with Professor Westheimer at Harvard.

(1) (a) Work from the Protein Research Laboratory was generously supported by the National Institutes of Health, Grant No. AM01128, and by the Hoffmann-La Roche Foundation. (b) Three letter abbreviations of amino acids are those suggested by IUPAC (*J. Biol. Chem.*, **241**, 2491 (1966)). Synthetic peptides are abbreviated according to the following scheme, *e.g.*, Orn^{10}-S-$peptide_{1-14}$ denotes an analog corresponding to positions 1–14 in the sequence of natural S-peptide but containing an ornithine residue instead of arginine in position 10. The notation 3-$CMHis^{12}$-S-$peptide_{1-14}$ refers to a tetradecapeptide containing a carboxymethyl group on nitrogen 3 of the aromatic ring of histidine-12. F-Orn = N^{ϵ}-formylornithine; Met(→O) = methionine *d*-sulfoxide. Amino acids are of the L configuration.

(2) K. Hofmann, H. Yajima, T-Y. Liu, N. Yanaihara, C. Yanaihara, and J. Humes, *J. Amer. Chem. Soc.*, **84**, 4481 (1962).

(3) B. Riniker and W. Rittel, *Helv. Chim. Acta*, **53**, 513 (1970).

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hormone to its receptor (binding or attachment sites) and those intimately involved in the chemical event which is the key to physiological function (active sites). Candidates for active sites would be residues whose alteration or replacement eliminates completely biological function. Residues whose replacement diminishes but does not destroy activity are not likely to be involved in function at the molecular level.

Despite the many fundamental advances made in the peptide hormone field with regard to peptide structure and activity, the necessity for *in vivo* assays or measurements with highly organized biological systems to evaluate physiological activity imposes certain limitations on the kind of information that can be derived. When, for example, an amino acid substitution results in partial loss of biological activity, it is virtually impossible to assess whether this is due to greater susceptibility of the analog to attack by proteolytic enzymes, poor binding to a transport vehicle, or decreased affinity of the peptide for its receptor.

The Model

Recognizing these inherent drawbacks, we searched instead for a model system that would permit a direct measurement of the effect of peptide modification on peptide binding and function. The S-peptide–S-protein system of Richards[7] presents an intriguing opportunity to study peptide–protein interaction and biological function.

Under controlled conditions, the bacterial protease subtilisin preferentially hydrolyzes the bond between amino acids 20 and 21 in bovine pancreatic ribonuclease A with formation of ribonuclease S. No loss of activity occurs as a result of this modification. However, separation of the eicosapeptide (S-peptide) from the rest of the molecule (S-protein) results in total inactivation. Neither fragment alone exhibits enzymic activity, but when the fragments are mixed in a molar ratio of 1:1 a fully active enzyme (ribonuclease S′ or S) is re-formed. The forces responsible for binding the peptide to the protein are noncovalent in nature. These events are depicted schematically in Figure 1.

Meaningful answers in structure–function studies are possible only when the peptides employed are of a high degree of purity; therefore, reliable methods had to be developed for the synthesis of S-peptide and its congeners.

Synthesis of S-Peptide and Activation Studies

The synthesis of S-peptide (Figure 2) was accomplished by two unequivocal routes both utilizing the protected tetrapeptide hydrazide (I) as the key intermediate.[8-12] The synthetic S-peptide obtained by both routes was indistinguishable from natural S-peptide by its ability to fully activate S-protein (Figure 3), optical rotation, amino acid ratios in aminopeptidase-M digests, and thin-layer chromatography. The same synthetic methods were used to prepare a great number of S-peptide analogs.

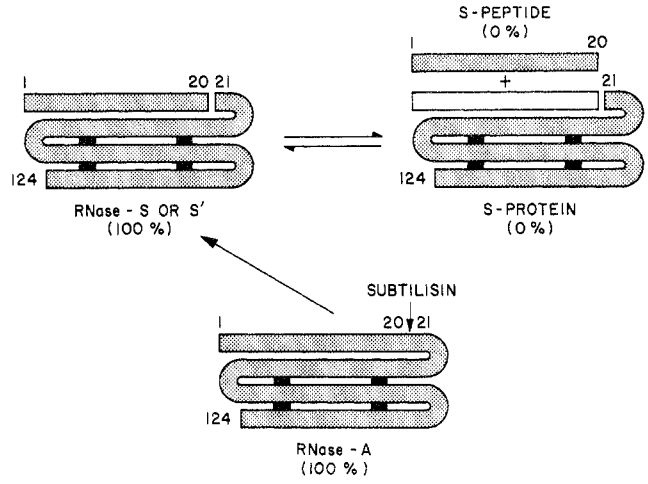

Figure 1. Schematic representation of subtilisin cleavage of RNase A. Black bars denote disulfide bridges.

During the course of the development of this synthetic scheme we had occasion to assay some of the intermediates for their ability to generate enzymatic activity in combination with native S-protein. S-Peptide$_{1-13}$ lacking seven of the carboxy-terminal amino acids of S-peptide$_{1-20}$ had the ability to restore 70–80% the activity of RNase S.[13] Activation of S-protein required larger amounts of S-peptide$_{1-13}$ than natural S-peptide, but the finding that an enzyme with a large portion of its primary structure missing still possessed enzymic activity was indeed surprising and prompted a systematic search for the minimum structural features necessary for full activity at a 1:1 ratio.

Digestion of natural S-peptide with carboxypeptidase A afforded a material (S-peptide$_{1-15}$) that restored 100% activity when mixed with an equimolar quantity of S-protein,[14] but it was eventually found that even the serine residue in position 15 is unnecessary and S-peptide$_{1-14}$ proved to be the smallest fragment capable of regenerating full activity at a 1:1 ratio[12] (Figure 4). Thus S-peptide$_{1-14}$ contains both the active site and all of the binding site residues of the natural molecule. Further shortening of the peptide chain to produce S-peptide$_{1-13}$ and S-peptide$_{1-12}$-amide necessitated larger peptide:protein ratios to achieve equivalent levels of activity, and so it was reasoned that the residues in position 14 and 13 (aspartic acid and methionine) represent binding sites. Elimination of histidine-12 resulted in a peptide (S-peptide$_{1-11}$) incapable of activating S-protein even at ratios as high as 8000:1.[15] These results are summarized in Figure 5.

Progressively shortening the chain of S-peptide$_{1-13}$ from the N-terminal end uncovered more attachment sites. Removal of lysine-1 does not seriously

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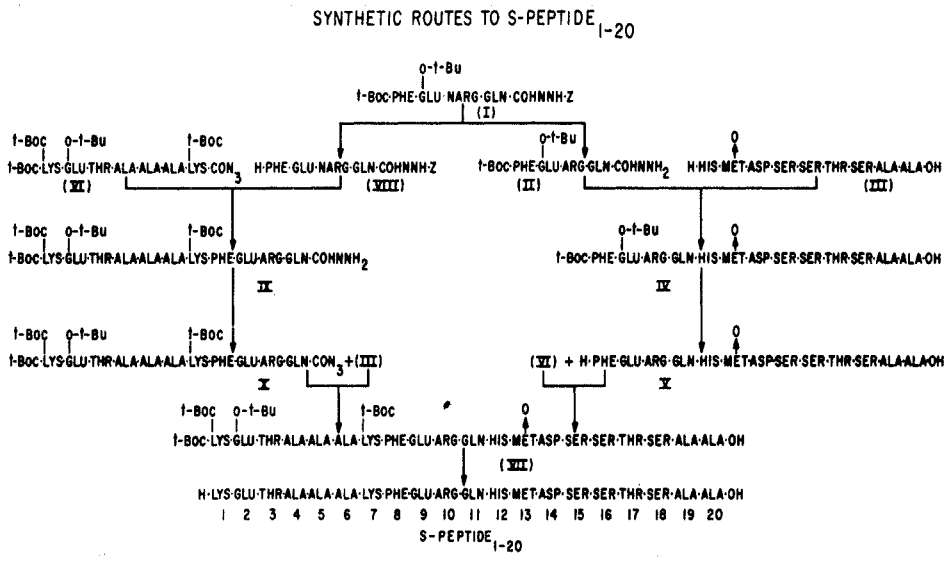

Figure 2.

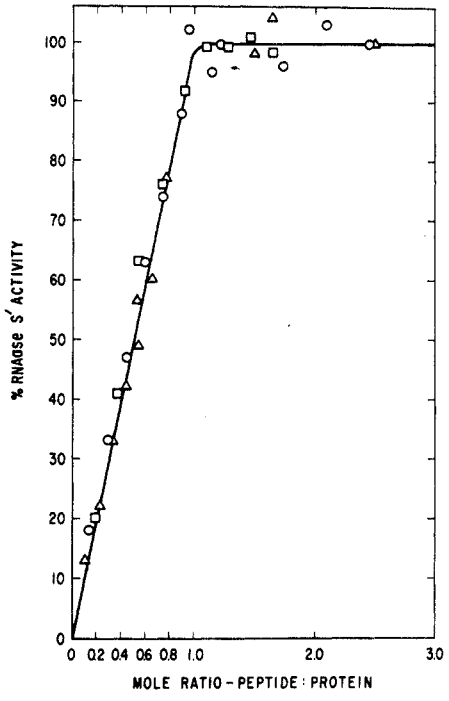

Figure 3. Activity of partially synthetic and natural ribonucleases S′ using yeast RNA as substrate: ○, natural enzyme; △, partially synthetic enzyme, first method; □, partially synthetic enzyme, second method.

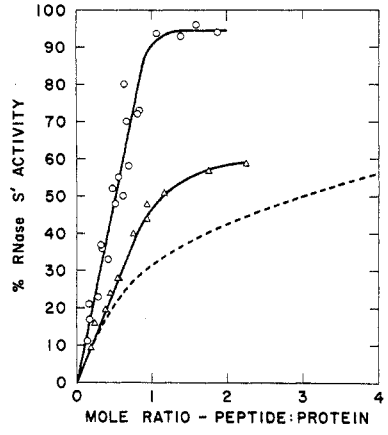

Figure 4. Ability of S-peptide$_{1-14}$ (○), Met(→O)^{13}-S-peptide$_{1-14}$ (△), and S-peptide$_{1-13}$ (- - -) to regenerate ribonuclease activity with S-protein using yeast RNA as substrate.

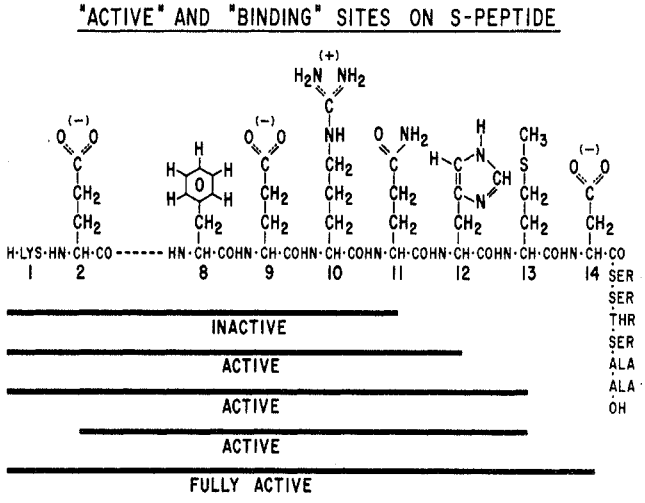

Figure 5. Summary of results of S-protein activation studies. Residues 15 through 20 are unnecessary for binding or function. Binding sites are Glu^{2}, Phe^{8}, Gln^{11}, Met^{13}, and Asp^{14}. Histidine-12 is the only active site in S-peptide.

alter the molar ratios, but deletion of both lysine-1 and glutamic acid-2 markedly weakens peptide–protein binding. In order to regain 50% activity an S-peptide$_{3-13}$:S-protein ratio of 2000:1 is required.[15]

Comparison of the activation potentials became more and more difficult as the list of synthetic analogs grew; hence, the term 50% activation ratios was introduced. The 50% activation ratio defined as the molar ratio of peptide to protein necessary to restore 50% activity allows comparisons to be made among various peptides in a fashion analogous to comparisons of substrate affinities for enzymes through their K_m values, the only difference being that no absolute values are assigned in the case of 50% activation ratios.

Replacement of the glutamine residue (position 11) with glutamic acid in S-peptide$_{1-13}$ had a dramatic effect on the activity. Although the complex was still enzymatically active, the 50% ratio increased from 3:1 to 8000:1. In a separate study

conducted with analogs of S-peptide$_{1-20}$ all containing ornithine instead of arginine in position 10, Scoffone and coworkers[16] also found that introduction of the negatively charged glutamic acid residue weakened binding between the resulting peptide and S-protein.

The importance for binding of Phe^{8} was demonstrated by Scoffone with a series of Orn^{10} analogs. The conservative substitution of this phenylalanine by tyrosine has no noticeable effect on activity or binding.[17] Tyr^{8},Orn^{10}-S-peptide$_{1-20}$ and Orn^{10}-S-peptide$_{1-20}$ are equally capable of activating S-protein; however, introduction of an aliphatic residue at this site weakens the peptide–protein interaction. Larger ratios of Ile^{8}- or Ala^{8},Orn^{10}-S-peptides are necessary for activation,[18] and at a ratio of 100:1 Gly^{8},Orn^{10}-S-peptide$_{1-20}$ is inactive.[19]

Using S-peptide$_{1-14}$ as the prototype a number of analogs were prepared to test the effect of amino acid substitutions on the strength of binding. Particular attention was focused on methionine-13. Substitution of the *d*-sulfoxide of this methionine increases the 50% activation ratio approximately tenfold, although a highly active RNase ensues when sufficient amounts of the peptide are used (Figure 4). Replacement of methionine with norleucine improves the binding.[20] The 50% activation ratio for Nle^{13}-S-peptide$_{1-14}$ is approximately 0.5. Curiously, though, full S-protein activation is never attained with either the sulfoxide or the norleucine analog. Possibly the active site formed when these peptides combine with S-protein is deformed, and this change results in a decreased catalytic efficiency.

Development of Competitive Inhibitors

From the results of these activation studies several conclusions can be drawn, namely, that the amino acids occupying positions 15–20 are not necessary for binding or activation; that glutamic acid-2, phenylalanine-8, glutamine-11, methionine-13, and aspartic acid-14 are binding sites, and that histidine-12 is very likely the only active-site residue in S-peptide. A role for this histidine in the catalytic activity of ribonuclease A was suggested by results of carboxymethylation studies.[21] Reaction of ribonuclease A with iodoacetic acid at pH 5.5 inactivates the enzyme. Two products, 1-CMHis^{119}-RNase and 3-CMHis^{12}-RNase, formed during this reaction are both enzymatically inactive. If, as postulated, the function of the binding sites is to bring about the correct alignment of the peptide with S-protein so that histidine^{12} is positioned properly to participate in catalysis, then it was reasoned that replacement of this histidine in a peptide containing the binding sites should result in an inactive analog capable of

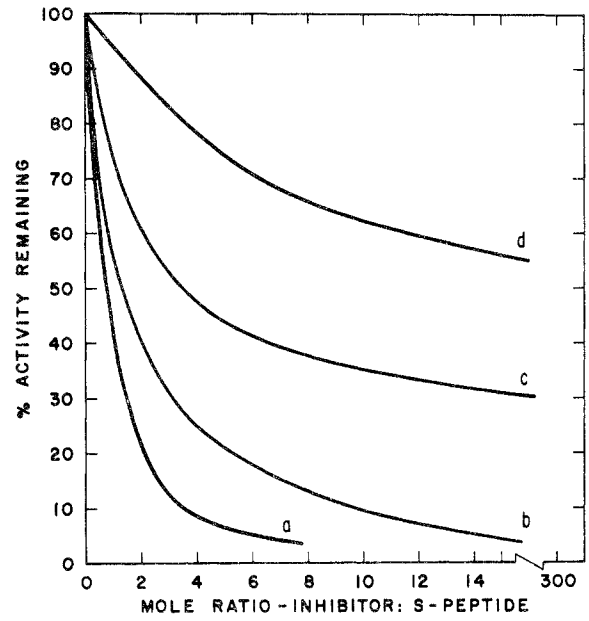

Figure 6. Competition of analogs of S-peptide$_{1-14}$ with natural S-peptide for S-protein. Curve a: 3-CMHis^{12}-S-peptide$_{1-14}$; curve b: Pyr(3)ala^{12}-S-peptide$_{1-14}$; curve c: Orn^{10}-3-CMHis^{12}-S-peptide$_{1-14}$ and F-Orn^{10}-3-CMHis^{12}-S-peptide$_{1-14}$; curve d: Orn^{10}-Pyr(3)ala^{12}-S-peptide$_{1-14}$ and F-Orn^{10}-Pyr(3)ala^{12}-S-peptide$_{1-14}$. Substrate in all cases was yeast RNA.

competing with S-peptide for binding to S-protein. β-(Pyrazolyl-3)-L-alanine (Pyr(3)Ala) was chosen as a substitute for histidine. This amino acid is isosteric with histidine, a fact which was demonstrated by X-ray diffraction analysis,[22] but exhibits a markedly different acid–base behavior (pK of 2.5) and, therefore, is unable to reversibly donate protons at the pH optimum for ribonuclease catalysis. Pyr(3)Ala^{12}-S-peptide$_{1-14}$[23] is, as predicted, incapable of activating S-protein at peptide–protein ratios up to 1000:1, but when the peptide is added to an equimolar mixture of S-peptide$_{1-14}$ and S-protein it displaces the histidine-containing analog. Even more importantly, Pyr(3)Ala^{12}-S-peptide$_{1-14}$ competes with S-peptide$_{1-20}$ for binding to S-protein.

When natural S-peptide and the pyrazole analog of S-peptide$_{1-14}$ are mixed with S-protein in a molar ratio of 1:1:1, approximately 50% inhibition is observed (Figure 6, curve b). Complete inhibition ensues when the Pyr(3)Ala peptide is present in about a 20-fold excess over S-peptide$_{1-20}$. Inhibition can be reversed by adding S-peptide$_{1-20}$ to the inactive complex.[24] The inhibition curve generated by addition of increasing amounts of Pyr(3)Ala^{12}-S-peptide$_{1-14}$ to RNase S follows closely one that can be calculated for equal binding of the two peptides, a result which again demonstrates that, at least in the presence of RNA, a peptide containing only 14 amino acids binds as firmly as the natural eicosapeptide. The *d*-sulfoxide of this peptide (Pyr(3)Ala^{12}-Met(→O)^{13}-S-peptide$_{1-14}$) is a far less potent antagonist. Approximately a 500-fold molar excess of this peptide is required to inhibit a 1:1 S-peptide$_{1-20}$–S-protein complex 50%.

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Discovery of a ribonuclease S inhibitor provided a more sensitive method for evaluating the effect of peptide modifications upon the strength of the peptide–protein interaction than did measurement of S-protein activation since the sharp break occurring in the activation curve with many peptides indicates that the binding is strong and hence small differences might not be discernible. The term 50% inhibition ratio, in analogy to 50% activation ratio, is used to compare the relative inhibition capacities of various peptides.

The binding contribution of the aromatic ring of histidine or Pyr(3)Ala was investigated by substituting serine at this site.[20] Interestingly, the enzyme formed by mixing Ser^{12}-S-$peptide_{1-14}$ and S-protein has some of the elements, namely serine and histidine in close proximity, of the active site of chymotrypsin. The finding that the Ser^{12}-peptide proved to be a weaker inhibitor (50% inhibition ratio of 7) than the Pyr(3)Ala analog indicates that the aromatic side chain has a binding function.

As mentioned previously, carboxymethylation of ribonuclease A at either histidine-119 or histidine-12 destroys enzymic activity,[21] a finding consistent with the hypothesis that both residues are in the catalytic site of the enzyme. It was, therefore, difficult to rationalize a report that carboxymethylation of S-peptide sulfone did not alter the capacity of this peptide to activate S-protein.[25] In order to resolve this discrepancy, we synthesized 1-$CMHis^{12}$,3-$CMHis^{12}$- and 1,3-$(CMHis^{12})_2$-S-$peptides_{1-14}$ by unequivocal routes and explored their interaction with S-protein and RNase S.[24]

3-$CMHis^{12}$-S-$peptide_{1-14}$ failed to activate S-protein; in fact, this peptide proved to be a most effective inhibitor of RNase S. Based on the inhibition curve for this peptide (Figure 6, curve a) and a 50% inhibition ratio of 0.8, it can be concluded that 3-$CMHis^{12}$-S-$peptide_{1-14}$ binds to S-protein more firmly than does S-$peptide_{1-20}$. The 1-CMHis derivative activates S-protein, but large proportions of the peptide are necessary, while the 1,3-$(CMHis)_2$ analog neither activates nor inhibits. The difference in behavior of these various carboxymethylated peptides is remarkable, indeed. Both the 1- and 3-carboxymethyl isomers exhibit pK values similar to that of histidine (6.13). The pK of the imidazole ring of 1-CMHis is 6.33, and that of 3-CMHis is 5.74.[26] Thus, ability to donate and accept protons is not drastically altered, yet these carboxymethylated peptides behave quite differently. In the case of 1-$CMHis^{12}$-S-$peptide_{1-14}$, the presence of the carboxymethyl group does not destroy enzymatic activity, but this bulky substituent probably interferes with binding and may deform the active site as well. The situation with the 3-$CMHis^{12}$-tetradecapeptide is quite different. In this case, the bulky group is directed toward the substrate cavity in RNase S and, therefore, exerts no steric hindrance; on the contrary, the CM carboxyl group may form a salt bridge with the imidazole ring of histidine-119 of S-protein (Figure 7). Such an interaction would account for the increased

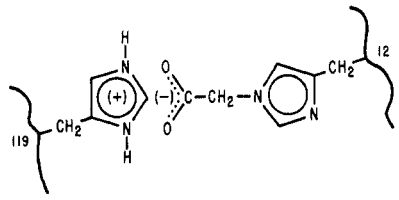

Figure 7.

binding strength of this peptide. Furthermore, it would prevent His^{119} from undergoing deprotonation and interfere with substrate binding. Yang and Hummel[27] have reported that 3-$CMHis^{12}$-Rnase A fails to bind 2′-cytidine monophosphate and that both 3-$CMHis^{12}$- and 1-$CMHis^{119}$-Rnase A exhibit greater stability toward urea below pH 6.5 than does RNase A. They attribute this behavior to an interaction between the carboxymethyl group and a cationic site on the enzyme. The model proposed in Figure 7 is consistent with all of these results.

The presence of methionine in the form of its sulfoxide has a pronounced effect on the binding of 3-$CMHis^{12}$-S-$peptide_{1-14}$. Approximately 3000 times more of the *d*-sulfoxide are needed to achieve a level of inhibition equivalent to that shown by the corresponding reduced derivative.

In view of the sensitivity of the inhibition method to differences in peptide–protein binding strength we decided to use this technique to try to resolve a controversy which had arisen over the importance of the glutamic acid residue in position 2 as a binding site. Scoffone and collaborators[28] synthesized a number of analogs of Orn^{10}-S-$peptide_{1-20}$ to measure the effect of chain shortening from the N-terminus on the ability of peptides to activate S-protein. These authors concluded that removal of both lysine-1 and glutamic acid-2 from the N-terminus of Orn^{10}-S-$peptide_{1-20}$ has little effect on S-protein-activating ability of the resulting analogs. They suggested that the behavior of glutamic acid-2 as a binding site might be discernible only in short fragments of S-peptide and that particularly the absence of aspartic acid-14 might have been responsible for our earlier results with fragments of S-$peptide_{1-13}$. Another important difference between the two groups of peptides is the substitution of ornithinine for arginine in position 10. Although active enzyme is formed when S-protein is mixed with Orn^{10}-S-$peptide_{1-20}$, full activity is never realized, and the possibility existed that the presence of Orn in position 10 was responsible for the conflicting results. To clarify this point, we synthesized a number of analogs of S-$peptide_{1-14}$ and measured their affinity for S-protein *via* competitive inhibition experiments.

Comparison of the RNase S inhibition produced by Pyr(3)Ala^{12}-S-$peptide_{2-14}$ with that by Pyr(3)Ala^{12}-S-$peptide_{3-14}$ (Figure 8) shows that, while the peptide missing $lysine^1$ is approximately as effective an inhibitor as the corresponding tetradecapeptide, the des-Lys^1,Glu^2-peptide is a considerably poorer antagonist.[20] Thus, although the presence of aspartic acid in position 14 unquestionably strength-

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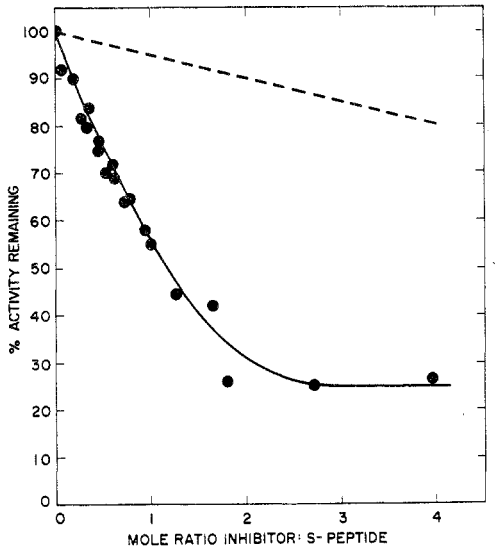

Figure 8. Inhibition of RNase S by Pyr(3)ala^{12}-S-peptide$_{2-14}$ (●) and Pyr(3)ala^{12}-S-peptide$_{3-14}$ (- - -) (substrate: yeast RNA). This figure is taken from ref 20 and is reproduced by courtesy of Academic Press, Inc.

ens binding, glutamic acid-2 still acts as a binding site.

The effect of the Orn–Arg exchange was also investigated by contrasting the inhibitory activity of Pyr(3)Ala^{12}-peptides containing either ornithine or arginine in position 10 (Figure 6, curve d) as well as the 3-CMHis^{12} derivatives (Figure 6, curve c) of both.[24] The results in both series were comparable. Peptides containing ornithine had less affinity for S-protein than their arginine counterparts. Even more interesting was the observation that the presence or absence of a charged side chain on ornithine made no difference in the 50% inhibition ratio. The inhibition curves produced by formyl-Orn^{10},Pyr(3)Ala^{12}-S-peptide$_{1-14}$ and formyl-Orn^{10}-3-CMHis^{12}-S-peptide$_{1-14}$ were superimposable on those of corresponding peptides containing a free δ-amino group. Apparently the shorter, less basic side chain of ornithine has lost its functional importance, and further alterations at this site are of little consequence. In order to account for inability to demonstrate a binding contribution of glutamic acid-2 in Orn^{10}-peptides, we have postulated that glutamic acid-2 and arginine-10 interact in S-peptide in a manner that favors a particular conformation required for optimal binding to S-protein. Many lines of evidence support this hypothesis. The functional groups of both Glu^2 and Arg^{10} in RNase A are relatively unreactive,[29,30] even though models derived from X-ray crystallographic studies of RNases A and S[31,32] indicate that these amino acids are exposed to the solvent. Eaker[33] has shown that <Glu^2-RNase$_{2-124}$ exhibits markedly lower enzymic activity than either RNase A, or RNase$_{2-124}$. Finally the X-ray structure of RNase A shows that the γ-carboxyl of Glu^2 and the guanidinium group of Arg^{10} are within hydrogen bonding distance of each other.[34] The distance between Orn^{10} and Glu^2 may be too great, owing to the shorter ornithine side chain, to permit such an interaction.

Peptide–Protein Interaction without Substrate

Thus far, comparisons of the effect of various structure alterations on peptide–protein affinity relative to S-peptide$_{1-20}$ have been made in the presence of substrate. In the inhibition studies both S-peptide and the various inhibitors are exposed simultaneously to the effects of substrate and the peptides compete for the same binding sites. Unquestionably though the presence of substrate, especially RNA, exerts an effect on binding. Regeneration of active enzyme by addition of weakly binding peptides, as, for example, S-peptide$_{1-12}$-amide, requires considerably higher peptide:protein ratios when pyrimidine 2′,3′ cyclic phosphates are used as substrates than when RNA is hydrolyzed.[15] Furthermore, since measurements are made in terms of enzymic activity, the formation of an enzyme–substrate complex and other activated states further complicates the situation.

Binding of peptides to S-protein is accompanied by an ultraviolet spectral change[35] whose magnitude is proportional to the concentration of re-formed enzyme.[36] This difference spectrum has been used to measure the dissociation constant for the reaction

$$\text{RNase S} \rightleftharpoons \text{S-protein} + \text{S-peptide}$$

Application of this technique to the determination of relative binding strengths of a number of analogs of S-peptide$_{1-20}$ and S-peptide$_{1-14}$ produced results qualitatively similar to those obtained by the inhibition approach; however, the magnitude of the differences determined by the spectral technique was considerably smaller.[37] For example, the dissociation constant for the *d*-sulfoxide of 3-CMHis^{12}-S-peptide$_{1-14}$ is only approximately tenfold greater than the value for the corresponding thioether peptide, whereas the 50% inhibition ratios of the two analogs differ by a factor of over 3000 (Table I). The binding function of Glu^2 and Arg^{10} is evidenced by the elevated dissociation constants for Pyr(3)Ala^{12}-S-peptide$_{3-14}$ and Orn^{10},Pyr(3)Ala^{12}-S-peptide$_{1-14}$, but here again the differences are not as large as those detected by inhibition measurements.

Neither the weaker binding of Ser^{12}-S-peptide$_{1-14}$ nor the somewhat greater affinity of 3-CMHis^{12}-S-peptide$_{1-14}$ are discernible by this method, although the added stabilization of the complex afforded by the postulated 3-CMHis salt bridge can be seen by comparing the constants for Orn^{10},Pyr(3)Ala^{12}-S-peptide$_{1-14}$ and Orn^{10},3-CMHis^{12}-S-peptide$_{1-14}$. Clearly, substitutions at or near the active site of the enzyme which might alter the substrate binding site can be more easily detected by techniques based on enzymic activity; however, dissociation measurements in the absence of substrate provide a better indication of the effect of amino acid substitutions

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Table I
Dissociation Constants for S-Protein–Peptide Complexes

PEPTIDES	DISSOCIATION CONSTANT M/L x 10^5	50% RATIO
S-PEPTIDE$_{1-20}$	0.96 ± 0.15	1.0[a]
S-PEPTIDE$_{1-20}$ SULFOXIDE	20.7 ± 7.6	—
S-PEPTIDE$_{1-14}$	1.33 ± 0.58	1.0[a]
PYR^{12}S-PEPTIDE$_{1-14}$	1.95 ± 0.56	1.0[b]
NLE^{13}S-PEPTIDE$_{1-14}$	1.42 ± 0.53	1.0[c]
SER^{12} S-PEPTIDE$_{1-14}$	1.70 ± 0.40	7[b]
3-CM HIS^{12} S-PEPTIDE$_{1-14}$	1.06 ± 0.11	0.8[b]
3-CM HIS^{12} S-PEPTIDE$_{1-14}$ SULFOXIDE	14.3 ± 3.1	3000[b]
ORN^{10} PYR^{12} S-PEPTIDE$_{1-14}$	4.22 ± 0.33	20[b]
ORN^{10} 3-CM HIS^{12} S-PEPTIDE$_{1-14}$	1.46 ± 0.44	4[b]
PYR^{12} S-PEPTIDE$_{2-14}$	1.71 ± 0.37	1.2[b]
PYR^{12} S-PEPTIDE$_{3-14}$	6.82 ± 0.33	30[b]

H–LYS·GLU·THR·ALA·ALA·ALA·LYS·PHE·GLU·ARG·GLN·HIS·MET·ASP·SER·SER·THR·SER·ALA·ALA–OH

[a] Mole ratio of peptide to inhibitor (Pyr(3)ala^{12}-S-peptide$_{1-14}$) required to regenerate 50% of the activity of RNase S. [b] Moles of peptide required to inhibit 50% of the activity of 1 mol of RNase S. [c] Mole ratio of peptide to inhibitor (Pyr(3)ala^{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.[20]

on the simple interaction of the peptide with S-protein.

Binding and Conformation

The results of X-ray diffraction studies of ribonuclease clearly indicate that the S-peptide portion of the enzyme contains approximately 2–3 turns of helix involving residues 2 or 3 through 12 or 13.[31,32] The arginine-10 side chain is bent back toward the amino terminus of the peptide although, unfortunately, in the RNase S picture glutamic acid-2 is poorly resolved. Existence of helix in S-peptide in the absence of S-protein as measured by solution methods is questionable. Klee[38] estimates that there is 10–15% helicity. This is not to suggest that a single structure with this per cent of helical character is found, but rather that during the course of conformational fluctuations helical sequences occur with this frequency. Scoffone and coworkers[39] contend that S-peptide is essentially in a randomly coiled conformation and that in the process of binding the peptide undergoes a coil-to-helix transition. In an attempt to obtain further information about the details of the binding process, Finn, *et al.*,[40] examined the 250-MHz proton nuclear magnetic resonance (nmr) spectra of S-peptide and some of its congeners and compared these with spectra obtained in the presence of 0.05–0.40 equiv of S-protein.

Titration of S-peptide$_{1-20}$ or S-peptide$_{1-14}$ with S-protein is accompanied by a broadening and decrease in intensity of the nmr peaks arising from the δ protons of arginine and the S-methyl protons of methionine (Figure 9). A gradual upfield shift in the histidine proton signal is also noted (not shown).

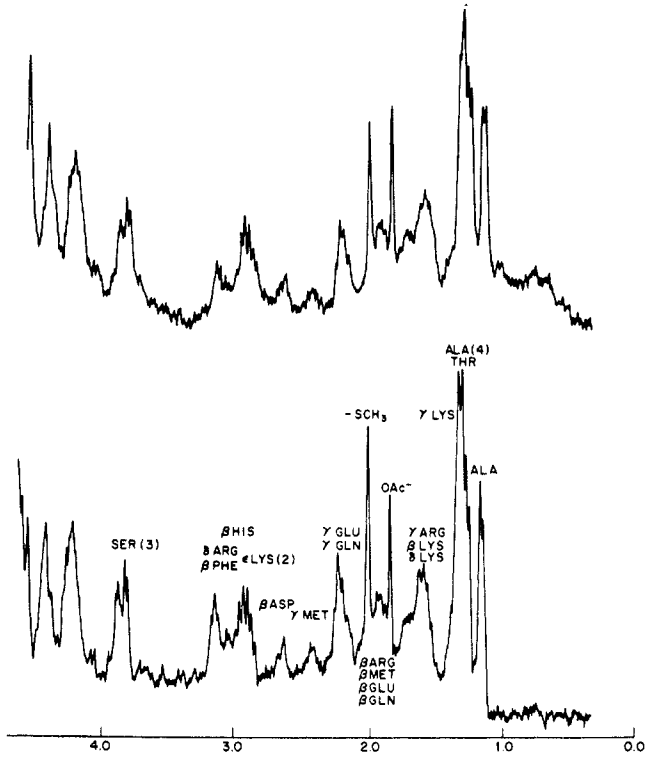

Figure 9. The proton nuclear magnetic resonance spectra (portion upfield from water) of S-peptide$_{1-20}$ 2% in D_2O in the absence of S-protein (lower trace) and upon addition of 0.1 equiv of S-protein (upper trace).

The binding function of methionine has been amply documented. In the X-ray model of RNase S this methionine side chain fits in a hydrophobic pocket formed by valine-47, leucine-51, and valine-54. Presumably, van der Waals forces immobilize the residue sufficiently that dipolar broadening by the protons of the valine and leucine methyl groups accounts for the spectral changes. Transition from a random-coil structure for S-peptide alone to the helical conformation it assumes when binding to S-pro-

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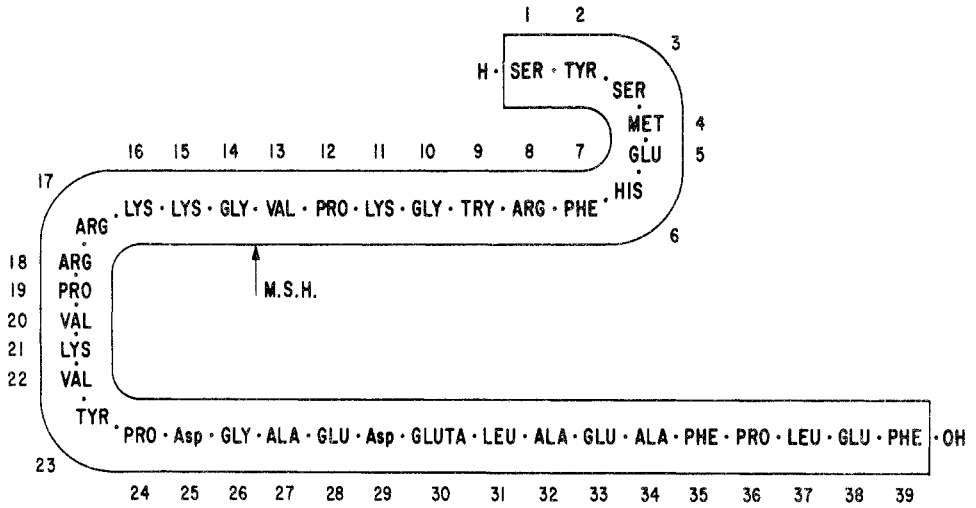

Figure 10. The structure of porcine ACTH.

tein with formation of the proposed salt bridge between Glu^2 and Arg^{10} would result in an immobilization of the arginine side chain and a broadening of the δ-proton signals. To test the latter conclusion, the spectral behavior of 3-$CMHis^{12}$-S-$peptide_{1-14}$ and Orn^{10},3-$CMHis^{12}$-S-$peptide_{1-14}$ upon titration with S-protein was investigated. With the 3-$CMHis^{12}$ analog the arginine and methionine signals undergo changes similar to those encountered with S-$peptide_{1-14}$ or S-$peptide_{1-20}$. However, when arginine-10 is replaced by ornithine the signals from this side chain remain unchanged on addition of S-protein. Finally, the progressive shift in the histidine protons can also be ascribed to the formation of helix on binding. In the helical conformation the histidine-12 protons are brought into position 5–6 Å in front of the plane of the benzene ring of phenylalanine-8. An upfield shift of about 0.25–0.5 ppm would be predicted for such an arrangement.

The correlation between results derived from this chemical approach and those obtained by X-ray crystallography is remarkable. Residues 15 through 20 which have been eliminated as binding or active sites extend far out into the solvent in the RNase S crystal and apparently move about freely so that this region of the chain is poorly defined. Hydrophobic interactions involving Phe^8, Met^{13}, His^{12}, and Ala^4 and a charge–charge interaction between Asp^{14} and Arg^{33} help to hold the peptide in position. As previously mentioned, the glutamic acid residue in position 2 is not readily visualized in the RNase S structure, but its proximity to arginine-10 is clearly apparent in the ribonuclease A model.[34] The hydrophobic environment in which methionine-13 is buried cannot accommodate the more bulky sulfoxide.[32] The side chain of methionine sulfoxide is, therefore, displaced from the normal position, and this apparently occurs with loss of binding capacity.

Is the Model Applicable to Hormones?

The structure–function studies with S-peptide were undertaken with the aim of discovering general principles involved in binding between peptides and proteins, principles that would be applicable to the study of interactions between antigens and antibodies and between peptide hormones and their receptors. From these investigations it can be concluded that both hydrophobic and charge–charge interactions are important for binding.

Recently an *in vitro* system for measuring hormone–receptor binding, using purified bovine adrenal cortical plasma membranes, has been described.[41] Addition of increasing amounts of the biologically highly active corticotropin analog Gln^5,[^{14}C]Phe^7-$ACTH_{1-20}$-amide to a given amount (based on protein measurements) of membrane produces a binding curve similar to the S-protein activation curve. The results of competitive inhibition experiments performed by exposing the membranes to mixtures of the radioactive eicosapeptide amide and nonradioactive fragments and analogs have delineated binding and active sites in this molecule. The cluster of positive charges contributed by the side chains of the residues -Lys-Lys-Arg-Arg- comprising positions 15 to 18 (Figure 10) constitute a major binding site for ACTH. The single residue qualifying for the role of an active site seems to be tryptophan-9. Replacement of this residue by phenylalanine does not alter the strength of hormone–receptor binding, but the Phe^9 peptide is virtually inactive.[42,43]

It has been postulated that the combination of inactive S-peptide with inactive S-protein which leads to the creation of active ribonuclease may provide a profitable hypothesis pertaining to the working of polypeptide hormones. It has been speculated that the mode of action of these hormones may involve their combination in a highly selective manner with a receptor protein to create an active enzyme.[6] Evidence has accumulated to the effect that many hormones activate specific adenylyl cyclase systems and that such activation may provide the key to their physiological function.[44] Details pertaining to the mechanisms involved are missing, but it appears likely that hormones do indeed function as enzyme activators.

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