Studies on Ribonuclease S

I. LIMITED CARBOXYPEPTIDASE DEGRADATION OF RIBONUCLEASE S-PROTEIN AND RIBONUCLEASE S-PEPTIDE: EFFECTS OF CHANGES IN PRIMARY STRUCTURE ON ENZYMIC ACTIVITY*

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Specific regions of the polypeptide sequence of pancreatic ribonuclease have been altered by chemical modification and by limited proteolytic digestion in attempts to implicate specific covalent portions of the molecule in the structure and stabilization of the active center. Digestion of the native molecule with trypsin at elevated temperatures has been shown to remove portions of the chain with only partial inactivation (1). Conversely, it has been possible to cause total inactivation of the enzyme through removal of the COOH-terminal tetrapeptide sequence, Asp-Ala-Ser-Val, by pepsin (2) or the 20-amino acid fragment at the NH₂-terminal end of the molecule by treatment with subtilisin (3).

The present studies were designed to analyze the specific roles of individual amino acid residues within the essential NH₂- and COOH-terminal regions of ribonuclease. In contrast to native RNase A (4), RNase S and its separated components, RNase S protein and RNase S peptide, are susceptible to extensive digestion by carboxypeptidase. The experiments described below show that changes in the conformation and the enzymic function of the RNase S derivatives produced can be related to the removal of individual amino acid residues.

EXPERIMENTAL PROCEDURE

Materials—RNase was obtained from Sigma Chemical Company (Lot R-60-B-204). This preparation has already been shown in a previous study to consist of 95% RNase A (5). RNase S, prepared by the method of Richards and Vilhayathill (3) with Nagarse subtilisin (Nagarse Company, Tokyo), was separated from unreacted RNase A by chromatography on Amberlite XE-64 (3), as were small peptides resulting from adsorption of the protein on a carboxymethyl cellulose column, (3), as were small peptides resulting from adsorption of the protein on a carboxymethyl cellulose column.

Enzymic procedures—All experiments were performed at pH 7.0 for the S-protein, which has 84% of the molecular weight of RNase A, by the measured shift in absorption maximum and extinction at 280 nm, which accompanies removal of S-peptide (J. T. Potts, Jr., and D. M. Young, unpublished observation).

With subsequent elution by 0.2 M ammonium bicarbonate. After lyophilization of the eluate, the essentially salt-free protein was dissolved in water to a concentration of 1% and then made 4% in trichloroacetic acid (3). The supernatant solution containing S-peptide was freed of trichloroacetic acid by continuous extraction with ether. The trichloroacetic acid precipitate (S-protein) (3) was desalted by gel filtration on Sephadex G-50.

Carboxypeptidase was obtained from the Worthington Biochemical Corporation (Commercial Grade). Special precautions were taken to minimize activity of any endopeptidases in the carboxypeptidase preparation. Freshly opened DFP (Aldrich Chemical Company, Inc.) was assessed for its ability to inactivate trypsin. The carboxypeptidase to be used throughout the experiments was then prepared in a single lot by dissolving the enzyme in 2.0 M NH₄HCO₃ to a concentration of 10 mg per ml. A 20-fold molar excess of the diisopropyl fluorophosphate was then added, and the mixture was incubated overnight at 4°. The final preparation was divided into 0.2-ml aliquots which were rapidly frozen with Dry Ice and ethanol and stored at -10°.

Methods—Quantitative amino acid analyses were performed with the Beckman/Spinco amino acid analyzer, model 120 (6). Preliminary analyses were performed by the one-dimensional electrophoretic method of Dreyer (7). For a more precise estimate of the completeness of removal of amino acids from S-protein, an internal standard of glycine was used. Prior to the addition of glycine, the concentration of the S-protein solutions used in each digestion was determined spectrophotometrically with a value of 0.781 as the extinction at 280 nm of a solution (1 mg per ml) of S-protein (1-cm light path). 3

* A preliminary report of some of the data in this paper was presented at the 47th Annual Meeting of the Federation of American Societies for Experimental Biology, April 1963.

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3 The abbreviations used are: DFP, diisopropyl fluorophosphate; DNP-, dinitrophenyl-.

4 Trypsin (Worthington Biochemical Corporation, Commercial Grade) was dissolved in 0.01 M NH₄HCO₃ to a concentration of 8%, and sufficient DFP was added to make the molar ratio of DFP to trypsin 0.5:1.0. After 30 minutes of incubation at room temperature, the activity of the DFP-treated trypsin was compared with a solution of trypsin incubated similarly without DFP. A reduction of 50% in the rate of hydrolysis of benzoylarginine ethyl ester by the DFP-treated sample of trypsin indicated the potency of the diisopropyl fluorophosphate.

5 This value for S-protein was calculated by correcting the absorbance expected for S-protein, which has 84% of the molecular weight of RNase A, by the measured shift in absorption maximum and extinction at 280 nm, which accompanies removal of S-peptide (J. T. Potts, Jr., and D. M. Young, unpublished observation).
The incubations of RNase S-protein were performed at concentrations of 20 to 32 mg per ml in 0.2 M NH₄HCO₃ buffer. Incubation of S-peptide was likewise conducted in 0.2 M ammonium bicarbonate with the peptide present at a concentration of 10 mg per ml. Aliquots of the digestion mixture, removed for analysis during incubation, were acidified to pH 3 with acetic acid to stop the action of carboxypeptidase irreversibly. Assays of RNase activity were performed by the soluble oligonucleotide method (8) and by a modification of the spectrophotometric method of Kunitz (9). In the latter procedure, decrease in absorbance of a 0.1% RNase solution at 300 mμ was measured with a Cary recording spectrophotometer, model 14. Under these conditions, initial reaction rates are a linear function of RNase concentrations in the range of 0.1 to 1.5 μg per ml. At this concentration of enzyme and RNA, the rate of digestion follows zero order kinetics for several minutes. This permits comparison of RNase S derivatives and unmodified RNase S with respect to maximum velocity of enzymic activity.

Richards and Vithayathil (3) have shown that the recombination of S-peptide and S-protein behaves as a simple bimolecular reaction and that S-peptide is tightly bound to S-protein even in dilutions as high as 10⁻⁹ M. Consequently, addition of S-peptide to a solution containing S-protein and substrate (RNA) results in a linear increase in activity with a sharp break at molar equivalence of peptide to protein (Fig. 2). To assess the enzymic activity of samples of the separated RNase S-protein after digestion by carboxypeptidase, appropriate dilutions of the digestion mixture were combined with 1 molar equivalent of undegraded S-peptide. Molar equivalence was determined for each solution of RNase S-protein before digestion by titration of an aliquot with aliquots of a stock solution of S-peptide. A similar procedure was followed to assess the effects of digestion of S-peptide, i.e. by titrations of RNase S-peptide solutions with a solution of undegraded S-protein.

After digestion of S-protein with carboxypeptidase, the preparation was examined for peptide bond cleavage at sites other than the COOH terminus by dinitrophenylation (10, 11) and by two-dimensional peptide mapping (12) of performic acid-oxidized (13) samples of the digested S-protein. In all experiments, carboxypeptidase control incubations (carboxypeptidase incubated at a concentration of 1 mg per ml in 0.2 M ammonium bicarbonate without RNase) contained no detectable quantities of free amino acids. Similarly, control samples of S-protein or S-peptide were incubated in 0.2 M ammonium bicarbonate without carboxypeptidase and then were used as reference for the enzymic activity of the digested samples.

RESULTS

Before quantitative experiments were performed, the effects of carboxypeptidase incubation on S-protein were assessed with several small scale digestions. In these initial studies, amino acid release was followed by one-dimensional electrophoretic amino acid analysis. It was found that incubation of S-protein with carboxypeptidase at 25° caused the rapid release of serine and valine and resulted in 55% enzymic inactivation of the RNase S reconstituted from the altered S-protein. After longer digestion by carboxypeptidase, performed at 37°, more extensive amino acid release occurred: valine, serine, alanine, aspartic acid, phenylalanine, and histidine were detected. This more extensively degraded derivative of RNase S-protein was completely inactive when enzymic activity was assayed in the presence of 1 molar equivalent of control S-peptide.

On the basis of this preliminary information, three digestions of S-protein were performed at 35° and the products were subjected to quantitative amino acid analysis. The data in Table 1 permit the comparison of amino acid release and enzymic activity. Fig. 1 has been constructed from representative data in Table 1. As illustrated, incubation of RNase S-protein, at concentrations of 2 to 3 mM, with carboxypeptidase at 25° was accompanied by a rapid release of COOH-terminal valine, Val-124, within 5 to 10 minutes, without change in enzymic activity. However, after 4 hours of incubation there occurred, in addition, a stoichiometric release of the penultimate serine residue, Ser-123. This was accompanied by a 55% reduction in the maximum rate of enzymic activity after recombination with 1 molar equivalent of S-peptide.

The homogeneity of the modified S-protein was examined by several methods to rule out possible endopeptidase activity of the carboxypeptidase preparation and to ensure that the serine and valine released actually represented molar equivalents of the two COOH-terminal residues, Val-124 and Ser-123. Dinitrophenylation was performed on an aliquot of an S-protein sample, taken after 4 hours of digestion, from which all of Val-124 and Ser-123 had been removed. After hydrolysis of the precipitated DNP-protein, the ether extract was chromatographed with DNP-amino acid standards (10, 11). Only DNP-serine was seen, as expected for S-protein, and the estimated recovery of the DNP-peptide bond (by elution of the DNP-serine spot and measurement of absorbance at 360 mμ) indicated 70% of theoretical recovery.

As illustrated in Fig. 1, incubation of ribonuclease S-protein samples at room temperature for periods of up to 24 hours did not liberate amino acids other than serine and valine or cause
The results of three separate digestions of S-protein indicate the progressive release of valine and serine and the decline in enzymic activity to a final value of 45% (when the samples of degraded S-protein are reconstituted to RNase S by addition of S-peptide). All incubations of S-protein were conducted in 0.2 M NH₄HCO₃ with carboxypeptidase added to a final concentration of 1 mg per ml. In the third digestion, glycine was added as an internal standard. Predicted values for amino acid release indicate the volume of digest mixture (in which the S-protein content was initially determined spectrophotometrically) applied to the amino acid analyzer. The predicted value for the enzymic activity of each sample of S-protein (after addition of S-peptide) reflects the extent of inactivation expected for the amount of valine and serine removed (with a value of 45% activity for RNase S lacking all of Val-124 and Ser-123).

Further change in enzymic activity. In order to evaluate the effect of the removal of amino acid residues from the COOH-terminal region of S-protein beyond residues 124 and 123, an incubation was performed at 38° as follows. An S-protein solution (0.75 mM) containing 1 mg of carboxypeptidase in a total volume of 1 ml was digested for 2 hours. Quantitative analysis of an aliquot representing one-tenth of the digestion mixture revealed the presence of the following quantities of amino acids (in micromoles): valine, 0.07; serine, 0.08; alanine, 0.04; aspartic acid, 0.05; phenylalanine, 0.03; and histidine, 0.02. After 2 hours of further digestion at room temperature, analysis of one-twentieth of the digested S-protein solution gave the following values: valine, 0.04; serine, 0.06; alanine, 0.04; aspartic acid, 0.04; phenylalanine, 0.04; and histidine, 0.02. After 2 hours of further digestion at room temperature, analysis of one-twentieth of the digested S-protein solution gave the following values: valine, 0.04; serine, 0.06; alanine, 0.04; aspartic acid, 0.04; phenylalanine, 0.04; and histidine, 0.02. These results indicated essentially complete and equal removal of residues 124 through 120, with only partial removal of the histidine residue at position 119. After such treatment, the resulting S-protein derivative, when combined with 1 molar equivalent of S-peptide, showed no enzymic activity against RNA. Analysis of this more extensively degraded derivative by dinitrophenylation and by two-dimensional peptide mapping of a performic acid-degraded derivative by dinitrophenylation revealed the presence of the following amino acids: valine, 0.07; serine, 0.08; alanine, 0.04; aspartic acid, 0.05; phenylalanine, 0.03; and histidine, 0.02. After 2 hours of further digestion at room temperature, analysis of one-twentieth of the digested S-protein solution gave the following values: valine, 0.04; serine, 0.06; alanine, 0.04; aspartic acid, 0.04; phenylalanine, 0.04; and histidine, 0.02. These results indicated essentially complete and equal removal of residues 124 through 120, with only partial removal of the histidine residue at position 119. After such treatment, the resulting S-protein derivative, when combined with 1 molar equivalent of S-peptide, showed no enzymic activity against RNA. Analysis of this more extensively degraded derivative by dinitrophenylation and by two-dimensional peptide mapping of a performic acid-degraded sample revealed no evidence of internal peptide bond cleavage.

It was impossible to obtain S-protein derivatives from which carboxypeptidase had removed only Ala-122 in addition to Val-124 and Ser-123. Even in several other incubations with carboxypeptidase at 38°, alanine and aspartic acid were liberated from S-protein at nearly equal rates.

Binding of S-Protein to Carboxypeptidase Derivatives of S-Protein. Richards and Vithayathil (14) have studied derivatives of S-peptide (e.g. methionine sulfone S-peptide) with altered affinity of binding to S-protein. With such derivatives, enzymic activity is a nonlinear function of the ratio of S-peptide to S-protein, and a hyperbolic plot is obtained (14). It was essential, therefore, to establish normal affinity of binding of the carboxypeptidase derivatives of S-protein to S-peptide before assuming that the reduced enzymic activity of the S-protein derivative after recombination with 1 molar equivalent of S-peptide reflected structural deficiency in the reconstituted RNase S. Since the combination of S-peptide and S-protein behaves as a bimolecular reaction, the concentration of either component may be increased in order to examine enzymic activity at high molar ratios of peptide to protein (14). For this reason, enzymic assays with the S-protein derivative, lacking Val-124 and Ser-123, were conducted in the presence of a large excess of S-peptide. Under these conditions, however, the S-protein derivative showed no increase in enzymic activity above the level (45% of the activity of control RNase S) observed with only 1 molar equivalent of S-peptide. This is illustrated in Fig. 2, which presents the results of titrations of equal amounts of degraded and

### Table I

<table>
<thead>
<tr>
<th>Digestion</th>
<th>Valine</th>
<th>Serine</th>
<th>Glycine</th>
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<tr>
<td></td>
<td>Predicted</td>
<td>Observed</td>
<td>Per cent</td>
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<td>1 (32 mg of S-protein per ml)</td>
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<td>0.12</td>
<td>80</td>
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![Fig. 1. The effects of carboxypeptidase digestion of S-protein.]
enzymic activity. In all assays, 1 µg of RNase S-protein was added to 1 ml of 0.1% RNA in sodium acetate buffer. These samples were titrated with increasing quantities of S-peptide. The upper curve represents addition to unmodified S-protein of the unmodified S-peptide (Δ) and modified S-peptide consisting of residues 1 to 15 (∗). The lower curve shows the results of additions of the same S-peptide solutions (O, unmodified S-peptide; ●, modified S-peptide) to S-protein lacking Val-124 and Ser-123.

FIG. 2. Assays of enzymic activity after reconstitution of RNase by addition of either unmodified or degraded S-peptide to samples of S-protein. In all assays, 1 µg of RNase S-protein was added to 1 ml of 0.1% RNA in sodium acetate buffer. These samples were titrated with increasing quantities of S-peptide. The upper curve represents addition to unmodified S-protein of the unmodified S-peptide (Δ) and modified S-peptide consisting of residues 1 to 15 (∗). The lower curve shows the results of additions of the same S-peptide solutions (O, unmodified S-peptide; ●, modified S-peptide) to S-protein lacking Val-124 and Ser-123.

FIG. 3. Studies of the competitive binding affinity of unmodified and carboxypeptidase-degraded S-protein for S-peptide. Each assay solution contained 0.1% RNA in sodium acetate buffer and a quantity of S-peptide representing 1 molar equivalent for 1 µg of unmodified S-protein. On the abscissa is indicated the molar ratio of S-protein derivative added simultaneously with the 1 µg of unmodified S-protein. △—△ represents the percentage of inhibition of enzymic activity resulting from competition for the limiting quantity of S-peptide by unmodified S-protein and S-protein lacking Val-124 and Ser-123. △—○ represents the inhibition produced by competition between unmodified S-protein and the extensively degraded S-protein derivative lacking Val-124 through Phe-129. The dotted line represents the restoration of enzymic activity to 100% by addition of a second molar equivalent of S-peptide.

degraded S-protein in 0.1% RNA with equal quantities of S-peptide. A linear increase in activity with a sharp break at molar equivalence of peptide to protein occurred in both instances.

With the more extensively degraded S-protein derivative, lacking residues Val-124 through Phe-129, a molar excess of S-peptide as high as 100:1 did not restore any measurable enzymic activity.

Relative Binding Affinities to S-Peptide of S-Protein and Carboxypeptidase Derivatives of S-Protein—Although S-protein lacking Val-124 and Ser-123 was able to bind S-peptide with sufficient affinity to exhibit its full enzymic activity (45%) at a 1:1 molar equivalence with S-peptide, this finding did not establish that the binding affinity was as strong as that displayed by undegraded S-protein and S-peptide. Furthermore, although S-protein lacking Val-124 through Phe-129 had no demonstrable enzymic activity in the presence of a 100-fold molar excess of S-peptide, this lack of enzymic activity might indicate complete inability of the more extensively degraded derivative to bind S-peptide. Therefore, the relative affinities of binding to S-peptide of S-protein and the carboxypeptidase derivatives of S-protein were compared quantitatively in a series of assays. The combination of molar equivalent quantities of S-protein with S-peptide provided an estimate of the maximal activity possible for the given quantity of S-peptide. Enzymic activity was then studied with the same quantity of S-protein and S-peptide but with the simultaneous addition of increasing molar quantities of the carboxypeptidase derivatives of S-protein (Fig. 3). Addition of an equal quantity and of a 2-, 3-, and 4-fold molar excess of S-protein lacking Val-124 and Ser-123 markedly decreased the observed enzymic activity below the control value (unmodified S-protein alone). Since the product formed by recombination of S-peptide with S-protein Val-124 and Ser-123 has only 45% of the activity obtained with undegraded S-protein, the quantity of S-peptide bound by the carboxypeptidase derivative in competition with unmodified S-protein could be calculated. This was done by comparing the enzymic activity observed with the activity expected if the native and degraded S-protein had equivalent binding affinity for S-peptide (in which the peptide would be distributed in direct proportion to the relative concentration of control and derivative S-protein). For example, at a 2-fold molar excess of S-protein lacking Val-124 and Ser-123, the enzymic activity predicted on this basis would be 63% of the control value. The observed enzymic activity was 79%. Hence, the S-peptide bound by the S-protein derivative was only 80% of that expected on a random basis. At a 3-fold molar excess of the S-protein derivative, the binding of S-peptide by derivative was 84% of that expected for equivalent distribution, and at a 4-fold molar excess, 93%. It is clear that the S-protein derivative, lacking Val-124 and Ser-123, can bind S-peptide strongly but with only 80 to 90% of the affinity of binding displayed by unmodified S-protein.

This approach also permitted us to evaluate the binding capacity for S-peptide of the more extensively degraded carboxypeptidase derivative of S-protein (S-protein lacking Val-124 through Phe-129). With this derivative of S-protein present at 20 times the molar concentration of the native S-protein, 40% inhibition of enzymic activity occurred. This indicates that this more extensively degraded S-protein is also capable of binding S-peptide, but that its binding affinity is much weaker than the binding by control S-protein or the S-protein derivative lacking only Val-124 and Ser-123.

That the above procedure accurately reflected competition between undegraded and an excess of degraded S-protein molecules for a rate-limiting quantity of S-peptide was confirmed by the addition of a small quantity of additional S-peptide in a duplicate assay. As illustrated in Fig. 3, such addition of S-peptide restored full enzymic activity.

Carboxypeptidase Degradation of RNase S-Peptide—Previous studies on the digestion of S-peptide by carboxypeptidase (5) have revealed a rapid and complete removal of the first five
amino acids from S-peptide (2 residues of alanine, 2 serine, and 1 threonine) representing Ala-20 through Ser-16. Furthermore, S-peptide is essentially completely resistant to further degradation despite prolonged incubation with fresh aliquots of carboxypeptidase (5). Resistance to cleavage by carboxypeptidase of the aspartyl-serine linkage between residues 14 and 15 is still observed when, after prolonged incubation of a peptide consisting of residues 11 to 18, a fragment comprising residues 11 to 15 is isolated and again exposed to carboxypeptidase (15).

To assess the effects of such digestion on the ability to restore enzymic activity to RNase S-protein, RNase S-peptide (10 mg by weight, uncorrected for moisture and salt) was dissolved in 1 ml of 0.2 M ammonium bicarbonate to which 1 mg of carboxypeptidase had been added. A 200-μl aliquot of the digestion mixture was removed as a control at zero time and diluted with an equal volume of glacial acetic acid.

After 9 hours of incubation, when the removal of the 5 residues, Ala-20 to Ser-16, is complete (as determined in preliminary experiments with S-peptide incubated at concentrations of 8 to 10 mM), the reaction was stopped by addition of 0.8 ml of glacial acetic acid. This 1:1 dilution of the digestion mixture made the concentration of peptide identical in the original control aliquot and the digested S-peptide sample. Analysis of one-fifth of the digest mixture revealed the presence of the following quantities of amino acids (in micromoles): alanine, 3.24; serine, 3.27; and threonine, 1.67. As illustrated in Fig. 2, equal quantities of control and digested S-peptide produced an identical linear increase in the enzymic activity of S-protein with an abrupt maximum. This indicates that, despite extensive digestion, the modified S-peptide retains the same binding affinity and capacity to restore activity with S-protein as the unmodified S-peptide. The minimum volume of the S-peptide solution that restored full enzymic activity to 1 μg of S-protein per ml should contain $0.80 \times 10^{-4}$ μmole of S-peptide on the basis of the molecular weight of the two components. The amount of peptide actually present in this volume of solution, estimated from the micromolar quantity of amino acids released, was $0.80 \times 10^{-4}$ μmole. This agreement indicates the stoichiometrically complete release of the five COOH-terminal amino acids, Ser-16 through Ala-20, from the degraded S-peptide.

The S-peptide derivative (residues 1 to 15) was also examined for its capacity to restore full enzymic activity to the S-protein derivative lacking Val-124 and Ser-123. The data summarized in Fig. 3 show that undegraded and degraded S-peptide are equally effective since the typical linear rise and sharp break in the plot of enzymic activity produced by S-peptide was duplicated with this S-peptide derivative.

**DISCUSSION**

The marked similarity between ribonuclease A and ribonuclease S in terms of enzymic activity and physical properties (3, 14) makes it reasonable to relate observations on RNase S to previous information on structure-function relationships in native ribonuclease A. The results obtained in the present studies on the digestion of S-protein with carboxypeptidase confirm deductions from earlier experiments on the COOH-terminal region of ribonuclease A and add certain additional information about this and other regions of the molecule.

Removal of valine by carboxypeptidase from ribonuclease A has been shown to have no effect on enzymic activity (4). However, removal of the COOH-terminal tetrapeptide, Asp-Ala-Ser-Val, by limited pepsin digestion of RNase A produced an inactive derivative (2). In attempts to define which of the amino acid residues beyond the dispensable COOH-terminal residue, Val-124, are required for activity, RNase A had been exposed to exhaustive digestion by carboxypeptidase (4). Only traces of serine and alanine were released, and no firm conclusions could be drawn about the essential requirement of these residues for activity (4). In the present studies with RNase S-protein, stoichiometric removal of the COOH-terminal residue, Val-124, was accomplished by carboxypeptidase digestion without reduction in the level of enzymic activity produced when the S-protein derivative was added to 1 molar equivalent of RNase S-peptide. Furthermore, it was possible by carboxypeptidase degradation of RNase S-protein to obtain a homogeneous population of molecules, modified only by the additional removal of Ser-123. Reconstituted RNase S, lacking Val-124 and Ser-123, has only 45% of the enzymic activity characteristic of unmodified RNase S (measured as a maximum velocity), but since considerable enzymic activity remains in the absence of Ser-123, this residue is not directly involved in the catalytic mechanism. Rather, the observed decrease in specific enzymic activity may result from alterations in the conformation of the enzyme secondary to the covalent modifications. Enzymic assays after recombination indicated that S-protein lacking Val-124 and Ser-123 is fully combined with S-peptide at a 1:1 molar ratio of peptide to protein. However, the affinity with which the degraded S-protein binds S-peptide is only about 80 to 90% of that characteristic of the undegraded, native S protein. Further studies to be reported subsequently (see preliminary abstract (16)) indicate that there are substantial alterations in the conformation of RNase S lacking Val-124 and Ser-123. In this connection, Ooi and Scheraga (17) have also related reduced enzymic activity to conformational alterations of ribonuclease derivatives after limited modification of covalent structure by trypsin digestion.

S-Protein lacking Val-124 through Phe-120 was completely inactive even in the presence of a 100-fold molar excess of S-peptide, although it was clear from the competitive binding experiments that this derivative of S-protein could bind S-peptide and hence reassemble the covalent structure of RNase S lacking only the 5 COOH-terminal residues. The lack of activity of this derivative of RNase S, similar to the properties of pepsin-inactivated ribonuclease A lacking the 4 COOH-terminal residues, confirms the essential nature of the COOH-terminal residues in ribonuclease for enzymic activity.

The region of the S-peptide molecule between Ser-16 and Ala-20 appears to be unessential for ribonuclease activity. At molar equivalence, S-peptide, lacking these residues, completely restores enzymic activity to undegraded S-protein. In a preliminary report concerning the restoration of full enzymic activity to S-protein by this S-peptide derivative (18), comparison was made with the results reported by Hofmann et al. (19), who synthesized various porous of the S-peptide molecule. Although the synthetic S-peptide derivative, consisting of residues 1 to 13 (19), and a chemically modified derivative of S-peptide, consisting of residues 1 to 12 plus homoserine at position 13 (20), restore considerable activity to S-protein, the activity characteristics shown at high molar ratios of these 13-amino acid peptide components are characteristic of weakly binding S-peptide. This suggests the possible importance of the dipeptide sequence...
aspartylserine (residues 14 and 15) in the optimal binding of S-peptide to S-protein.

SUMMARY

1. Removal of valine-124 from the COOH terminus of ribonuclease S-protein does not affect the enzymic activity of this derivative after recombination with ribonuclease S-peptide to form ribonuclease S. Complete removal of the next residue, serine-123, produces an RNase S derivative which has only 45% of the enzymic activity of the native molecule, although this modified S-protein combines stoichiometrically with S-peptide, reforming an RNase S' lacking only 2 amino acid residues. With removal of additional amino acids from the COOH terminus of S-protein (Ala-122, Asp-121, and Phe-120), even upon addition of excess S-peptide, enzymic activity is completely absent.

2. The relative binding affinity of the ribonuclease S-protein derivatives for S-peptide was compared with the binding affinity of the unmodified S-protein molecule. S-Protein lacking Val-124 and Ser-123 binds S-peptide with 80 to 90% of the affinity characteristic of undegraded S-protein. This reduced affinity between the modified S-protein and S-peptide may reflect an altered conformation of the reconstituted RNase S' derivative, explaining its lower specific enzymic activity. The S-protein molecule lacking Ala-122, Asp-121, and Phe-120, in addition to Val-124 and Ser-123, while exhibiting weak binding of S-peptide, was totally inactive enzymically.

3. Removal of five amino acids, Ser-16 through Ala-20, from RNase S-peptide was without effect on the ability of the resulting derivative to reconstitute fully active RNase S upon combination with RNase S-protein at molar equivalence. Although these five residues seem unessential for enzymic activity, the neighboring dipeptide sequence, aspartylserine (residues 14 and 15), is probably necessary for optimal association between the NH2-terminal portion of ribonuclease and the remainder of the molecule.

4. The pentadecapeptide derivative of S-peptide was as effective as intact S-peptide in restoring activity to RNase S-protein lacking Val-124 and Ser-123. This reconstituted RNase derivative, devoid of seven of the amino acids normally present in the covalent structure of ribonuclease, retains 45% of the enzymic activity of the native enzyme.

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REFERENCES

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