A Synthetic 70-Amino Acid Residue Analog of Ribonuclease S-Protein with Enzymic Activity*

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SUMMARY

A 70-residue analog of RNase S-protein was synthesized by the solid phase method. It was obtained by omitting the NH₂-terminus from positions 21 to 28 and the segments 36 to 40, 58 to 73, 87 to 96, and 113 to 114. Four residues were inserted to link the ends formed by the deletions. Half-cystine residues that had not been part of the deletions were replaced by alanine or leucine residues. The synthetic polypeptide was separated by gel filtration into a dimer and a monomer. Both fractions were purified further by ion exchange chromatography.

The dimeric 70-residue S-protein analog had a specific activity of approximately 4% using RNA as substrate. It also cleaved other substrates of RNase A such as 5'- 3'-cytidylyl)-guanosine, 5'- 3'-uridylyl)-guanosine, and polycytidylic acid. The monomer of the 70-residue analog was less active but showed the same substrate specificity as the dimer. It was found that both fractions of the synthetic S-protein analog catalyzed only the transphosphorylation step of the RNase A mechanism and had very little if any activity in the hydrolysis step.

Addition of natural S-peptide or S-protein did not increase the activity in the transphosphorylation reaction but greatly enhanced the reaction rate of the hydrolysis step. In the presence of S-peptide, both monomeric and dimeric 70-residue S-protein analog had approximately 8% activity using cyclic cytidine 2':3'-monophosphate as substrate. The mixtures of monomer and dimer of the synthetic S-protein analog with natural S-protein generated even higher activities (151 and 74%, respectively) against this substrate.

The position of the deletions was decided from a careful inspection of the x-ray structure of RNase S elucidated by Wyckoff et al. (7, 8). The gaps caused by the deletions were closed through insertion of 1 glycine and 3 alanine residues. The fit of these insertions was verified through model building. These considerations led to a 70-amino acid residue S-protein analog (Fig. 1) for which the primary structure of which was synthesized by the solid phase method of Merrifield (9, 10). A preliminary report of this work was published last year (11).

Possible implications of these results for the mechanism of action of RNase A are discussed.

It is known from previous studies on the RNase S-peptide-S-protein system (1, 2) that a number of amino acid residues from the COOH-terminal end of the S-peptide (3, 4) and from the NH₂-terminal end of the S-protein (5) can be missing without decreasing the affinity of the two fragments for each other or diminishing the enzymic activity of the complex. The x-ray structure of RNase A (6) shows that this region of the polypeptide chain (residues 16 to 25) forms a wide loop at the surface of the molecule. The synthesis of the analog described below was undertaken to study the importance of several such exterior loops for the correct folding of RNase to give an active enzyme.

At the outset of this work the objective was to synthesize an analog of S-protein in which several loops and turns that were distant from the active site would be missing, but which would still bind S-peptide to form an enzymically active complex. The position of the deletions was decided from a careful inspection of the x-ray structure of RNase S elucidated by Wyckoff et al. (7, 8). The gaps caused by the deletions were closed through insertion of 1 glycine and 3 alanine residues. The fit of these insertions was verified through model building. These considerations led to a 70-amino acid residue S-protein analog (Fig. 1) for which the primary structure of which was synthesized by the solid phase method of Merrifield (9, 10). A preliminary report of this work was published last year (11).

MATERIALS AND METHODS

Boc-amino acids† were obtained from Schwarz-Mann, Fox Chemical Corp., the Protein Research Foundation (Osaka, Japan), Fluka AG (Buchs, Switzerland), and Bachem Inc. Melting points and Rf values on precoated silica gel thin layer plates were determined for all derivatives. Chlormethylated copoly(styrenes-1% divinylbenzene resin (Bio-Beads SX-1, 200 to 400 mesh, 1.25 mmol of chloromethyl per gm) was obtained from Bio-Rad. Natural RNase A and tRNA (soluble, from baker's yeast) were from Boehringer Mannheim; RNase S (grade XII-S), RNase S-protein (grade XII-PR), RNase S-peptide (grade XII-PE), cyclic 2'-3'-cytidylic acid (sodium salt) and all other nucleotides and dinucleoside phosphates used to study substrate specificity were from Sigma. RNase T₁, RNase I (grade I), and DNA (high molecular

The activating agent, N,N'-dicyclohexylcarbodiimide (13), was coupled in a 5-fold molar excess (1.62 mmol) based on the resin (0.27 mmol of valine per g). The a-amino position of all amine acids was protected by the Boc group. The side chains of aspartic acid, glutamic acid, serine, threonine, and tyrosine were protected by the benzyl group, the e-amino position of lysine was blocked by the carbobenzylox group, and the guanidino side chain of arginine by the nitro group. The imidazole ring of histidine was protected with the tosyl group. Only the thioether side chain of methionine residues by the t-butyl+ ion.

Due to incomplete separation, commercial natural S-peptide and S-protein showed considerable RNase activity using both RNA and cyclic cytidine 2':3'-monophosphate as substrates. In order to achieve a more complete reaction, each coupling step was repeated according to the procedure described in Table I. After Met 79 had been coupled to the peptide resin, the progress of the synthesis was followed by amino acid analysis of acid hydrolysates of small samples (10 to 12 mg) of peptide resin. The progress of the synthesis was followed by amino acid analysis of acid hydrolysates of small samples (10 to 12 mg) of peptide resin, taken after every sixth amino acid coupling. A larger sample (0.25 g) was removed from the reaction vessel after the coupling step was repeated according to the procedure described earlier (12). After Met 79 had been coupled to the peptide resin, all following acid deprotection steps were performed in the presence of 10% anisole (v/v) to prevent alkylation of the thiol side chains of the methionine residues by the t-butyl+ ion. The H-protein analog follows that of the corresponding residues in natural RNase A and does not take into account deletions and insertions. The protected peptide resin (998 mg) was treated under stirring with 12 ml of anhydrous HF in the presence of 2 ml of anisole and most of the anisole were evaporated under vacuum. Traces of anisole and its derivatives were removed by extraction with three 5-ml portions of ether. The deprotected peptide chains were dissolved in water, dialyzed, and lyophilized. The peptide was now completely inactive against both substrates, whereas S-protein was only inactive against cyclic cytidine 2':3'-monophosphate but still had weak activity toward RNA and 5'-cytidylyl-guanosine. The specific activity of purified S-protein using RNA as substrate was 0.15%. These results are discussed later in this paper.

EXPERIMENTAL PROCEDURE AND RESULTS

Solid Phase Synthesis of 70-Amino Acid Residue Analog of S-Protein

The NH2-protected COOH-terminal amino acid, Boc-L-valine, was esterified to the chloromethylated resin by standard procedures (5, 9). The synthesis was started with 2.0 g of Boc Val resin (0.27 mmol of valine per g). The e-amino position of all amino acids was protected by the Boc group. The side chains of aspartic acid, glutamic acid, serine, threonine, and tyrosine were protected by the benzyl group, the e-amino position of lysine was blocked by the carbobenzylox group, and the guanidino side chain of arginine by the nitro group. The imidazole ring of histidine was protected with the tosyl group. Only the thioether side chain of methionine remained unprotected. The NH2-terminal Boc groups of the peptide resin were removed by treatment with 20% (v/v) TFA3 in CH2Cl2. Boc-amino acids were coupled in a 3-fold molar excess (1.62 mmol) based on the amount of COOH-terminal valine covalently linked to the resin. The activating agent, N,N'-dicyclohexylcarbodiimide (13), was used in the same excess and the reaction time was 2 hours. Boc-asparagine and Boc-glutamine were reacted as the p-nitrophenyl esters in dimethylformamide (14) using a 5-fold molar excess (2.70 mmol). The coupling time for the active esters was 10 hours. In order to achieve a more complete reaction, each coupling step was repeated according to the procedure described earlier (12). The NH2-terminal Boc groups of the peptide resin were removed by treatment with 20% (v/v) TFA3 in CH2Cl2. The fractions containing S-peptide and S-protein showed considerable RNase activity using both RNA and cyclic cytidine 2':3'-monophosphate as substrates. In order to achieve a more complete reaction, each coupling step was repeated according to the procedure described earlier (12). After Met 79 had been coupled to the peptide resin, all following acid deprotection steps were performed in the presence of 10% anisole (v/v) to prevent alkylation of the thiol side chains of the methionine residues by the t-butyl+ ion. The H-protein analog follows that of the corresponding residues in natural RNase A and does not take into account deletions and insertions.

Cleavage of 70-Residue S-Protein Analog from Polymer Support

The protected peptide resin (998 mg) was treated under stirring with 12 ml of anhydrous HF in the presence of 2 ml of anisole which served as a trap for cations. The temperature was allowed to rise slowly from 0 to 14° over a period of 80 min. Then, HF and most of the anisole were evaporated under vacuum. Traces of anisole and its derivatives were removed by extraction with three 5-ml portions of ether. The cleaved and deprotected peptide was dissolved in four 5-ml portions of TFA and filtered from the resin. The protected peptide resin (998 mg) was treated under stirring with 12 ml of anhydrous HF in the presence of 2 ml of anisole which served as a trap for cations. The temperature was allowed to rise slowly from 0 to 14° over a period of 80 min. Then, HF and most of the anisole were evaporated under vacuum. Traces of anisole and its derivatives were removed by extraction with three 5-ml portions of ether. The cleaved and deprotected peptide was dissolved in four 5-ml portions of TFA and filtered from the resin. TFA was evaporated and 25 ml of 0.05 M NH4HCO3 were added to the remaining peptide. The resulting suspension was rapidly adjusted to pH 8.5 with 1 N NaOH and stirred for 2½ hours at room temperature. After lyophilization, 20 ml of water were added and the suspension was saturated with urea. The pH was kept at 8.5. The insoluble material was centrifuged off, and the solution was dialyzed 8 hours at 4° against distilled water and then lyophilized. The yield of cleaved soluble material was 246 mg or 61.8% of the amount of resin-bound polypeptide chains. Paper electrophoresis of this product is shown in Fig. 2c.

3 The abbreviations used are: TFA, trifluoroacetic acid; TPCK, p-toluenesulfonylami-no-phenylethylchloromethyl ketone; dansyl, 5-dimethylaminonaphthalene-1-sulfonfonyl.
Table I

<table>
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<th>Amino acid</th>
<th>Synthetic 70-residue S-protein analog</th>
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</table>

* Based on the primary structure of the 70-residue S-protein analog as shown in Fig. 1.
* Amino acid ratios are based on the amount of alanine present in the hydrolysate.
* Amino acid ratios are expressed relative to the amount of phenylalanine found.
* Recovery of threonine and serine from resin hydrolysates is known to be low whereas the low yields of methionine and leucine cannot be explained at the moment.
* The batch of natural S-peptide used in this work contained only 4 residues of alanine. Most likely the alanine residue missing was the COOH-terminal alanine that could have been cleaved enzymatically during the preparation of this batch of S-peptide from natural RNase A.

Purification of Cleaved 70-Residue S-Protein Analog

Gel Filtration on Sephadex G-50—The crude synthetic peptide (245 mg) was divided into 4 equal portions which were chromatographed separately. A 61.7 mg sample was dissolved in 5 ml of 0.05 M NH₄HCO₃ buffer. After some insoluble material had been removed by centrifugation, the solution was applied to a Sephadex G-50 column (2.4 × 76 cm) and eluted with 0.05 M NH₄HCO₃. The optical absorption of the column effluent is shown in Fig. 3A. Peak I was a high molecular weight fraction. Since the synthetic S-protein analog did not contain half-cystine residues, the formation of polymers through interchain disulfide bonds between several peptide monomers had to be excluded. Peak II had about the same elution volume (150 to 160 ml) as natural RNase A (155 to 171 ml). Peaks III and IV eluted from 169 (to 190 ml) and from 190 to 211 ml, respectively. After all four portions of the crude cleaved 70-residue peptide had been chromatographed, the fractions comprising identical peaks were
combined and lyophilized. The yields were: Peak I, 18.4 mg (7.5%); Peak II, 29.0 mg (11.8%); Peak III, 20.3 mg (10.7%); Peak IV, 26.5 mg (10.8%). The insoluble material from all four runs weighed 49 mg (20.0%). Since Fractions II, III, and IV were only poorly separated, they were rechromatographed separately as follows. All of the material of Fraction III (26.3 mg) was dissolved completely in 0.05 M NH₄HCO₃ buffer, and the solution was passed through the Sephadex G-50 column. The elution curve is shown in Fig. 3B. The first product to emerge from the column was again a high molecular weight fraction probably newly formed through aggregation of some of the peptide from Peak III during lyophilization following the initial fractionation on Sephadex G-50. Peak III contained most of the peptide and was not yet symmetrical. The fractions with the highest absorptions were pooled (elution volume from 169 ml to 191.5 ml) and lyophilized, and the peptide was obtained in a yield of 16.6 mg or 63.1%. This yield could be raised to 23.2 mg or 88.2% when those parts of the product, eluting at the same volume (i.e. from 169 to 191.5 ml) during rechromatography of Fractions II and IV, were combined with the material from Peak III. Rechromatography of Fractions II and IV gave elution patterns identical with that obtained from Fraction III. The yields of the rechromatography step were 20.9 mg or 72.1% for Fraction II and 19.9 mg or 75.1% for Fraction IV.

When Fractions I and II were heated to 61° for 10 min in 1 M formic acid, then chilled in an ice bath, warmed to room temperature, and applied to a Sephadex G-50 column (2.4 X 76 cm) equilibrated with 1 M formic acid, a partial dissociation of both products was observed (Fig. 4, A and B). Fraction III was treated the same way but no change of the elution volume was found (Fig. 4C). These results suggested that Fraction III was the monomeric form of the 70-residue polypeptide and Fraction II was the dimer (calculated mol wt 15,308), eluting almost at the same position as natural RNase A (mol wt 13,683). Fraction IV probably consisted of deletion peptides derived from incomplete coupling reactions during the assembly of the poly-peptide chain on the resin whereas Fraction I was composed of aggregates of the other fractions. The amino acid composition of the high molecular weight fraction is given in Table I.

Ion Exchange Chromatography on CM-cellulose-Fraction II (the dimer) and Fraction III (the monomer) from Sephadex G-50 were further purified by cation exchange chromatography on CM-cellulose. A 21.2-mg sample of the monomeric 70-residue S-protein analog was dissolved in 2 ml of 0.05 M pyridine acetate, pH 6.5. The insoluble part was centrifuged off and the solution was applied to a CM-cellulose column (2.0 X 18 cm) equilibrated with the same buffer. After a 50-ml eluate had been collected, a gradient was started toward 0.5 M pyridine acetate, pH 6.5. The elution curve is shown in Fig. 5. The fractions comprising the four major peaks were combined and lyophilized, desalted on a Bio-Gel P-2 column (2.4 X 48 cm) in 0.05 M NH₄HCO₃, and were then obtained in the following yields: Peak a (tubes 25 to 31), 4.2 mg or 19.5%; Peak b (tubes 33 to 45), 3.3 mg or 15.5%; Peak c (tubes 61 to 70), 1.6 mg or 7.5%; Peak d (tubes 75 to 89), 5.4 mg or 25.5%.

Small samples of the four products were submitted to paper electrophoresis at pH 2.2 in 2 M formic acid, 4 M in urea. Under these strongly acidic and denaturing conditions the 70-residue S-protein analog (mol wt 7,654, 10 positive charges) was expected to have about the same electrophoretic mobility as natural RNase A (mol wt 13,683, 19 positive charges). It was found that only the position of the material from Peak d agreed with that of RNase A (Fig. 2d). The other three products had moved more slowly indicating that either basic amino acid residues were missing or that the side chains of some of the basic residues were blocked.

Similar results were obtained from CM-cellulose chromatography of the dimeric 70-residue analog. Amino acid analyses of purified monomer and dimer were practically identical and are shown in Table I.

Fig. 4. Gel filtration of the aggregates and of dimeric and monomeric 70-residue analog on Sephadex G-50 after a 10-min incubation of the three fractions at 61° in 1 M HCOOH. The Sephadex G-50 column (2.4 X 76 cm) was equilibrated with the same solvent. A, 2.79 mg of the high molecular weight fraction were incubated in 1 ml of 1 M HCOOH and then applied to the column. About 50% of the aggregated material had dissociated to give dimeric and monomeric product and deletion peptides. R₁ 2.65 mg dimeric 70-residue analog were heated in 1 ml of 1 M HCOOH. 1.28 mg was recovered unchanged, the remainder had been converted to the monomeric form. C, 1.23 mg of monomeric 70-residue analog was incubated in 0.5 ml of 1 M HCOOH.

Fig. 5. Ion exchange chromatography of monomeric 70-residue analog on CM-cellulose. The synthetic material (21.2 mg) was treated with 2 ml of 0.05 M pyridine acetate, pH 6.5, the insoluble part was removed by centrifugation, and the solution was added to the CM-cellulose column (2.0 X 18 cm) which was eluted first with 50 ml of the same buffer. Then the gradient toward 0.5 M pyridine acetate, pH 6.5, was started. The column effluent was collected in 2.4-ml fractions. A 0.2-ml aliquot of each fraction was submitted to alkaline hydrolysis (15) followed by the ninhydrin reaction. The extinctions of the samples were read at 570 nm. After a desalting step on Bio-Gel P-2 the yields of the products were: Peak a, 4.2 mg; Peak b, 3.3 mg; Peak c, 1.6 mg; Peak d, 5.4 mg. The elution pattern of the dimeric 70-residue analog from CM-cellulose was similar. Assays of the monomer and the dimer of the synthetic S-protein analog were performed only with material of Peak d from the CM-cellulose column.
Tryptic Cleavage of Synthetic Monomeric 70-Residue S-Protein Analog and Preparation of Peptide Maps from Digest

Since the 70-residue analog did not contain disulfide bonds, it was immediately susceptible to tryptic digestion. TPCK-treated trypsin (from Serva, Heidelberg, Germany), 0.415 mg, was dissolved in 7.5 ml of 0.2 M NH₄HCO₃ buffer. The trypsin solution (0.075 ml) was added to the purified monomeric 70-residue analog (0.75 mg) and the mixture was incubated for 20 hours at room temperature. The enzyme to substrate ratio was 1:60 (w/w). The reaction was stopped by lowering the pH to approximately 3 with formic acid and the digest was stored frozen.

A 30-µl aliquot of the digestion mixture was applied to a cellulose-precoated thin layer plate (20 × 20 cm, from Schleicher & Schüll, Dassel, Germany) and resolved by electrophoresis in 1.25 M pyridine acetate buffer, pH 6.45, at 400 volts for 90 min in a Camag electrophoresis chamber and by thin layer chromatography in 1-butanol-acetic acid-pyridine-water (30:6:20:24). The chromatogram was sprayed with the ninhydrin-cadmium acetate reagent (16). The peptide map obtained is shown in Fig. 6. Based on the results with performic acid-oxidized natural RNase A (17), six peptides were expected from tryptic cleavage of the synthetic 70-residue S-protein analog. The NH₂-terminal hexapeptide (Leu 26 to Lys 31), the dipeptide Ser 32-Arg 33, and another hexapeptide near the COOH terminus (Thr 99 to Lys 104) should carry one positive charge each, whereas the 34-residue peptide from Asn 34 to Arg 85, the tetrapeptide Glu-Gly-Tyr-Lys, and that peptide 6 were the seryl-arginine dipeptide. A third peptide map containing 37 µl of the digest was developed with the Pauly spray. The positions of the four spots detected agreed with those of peptides 1, 2, 3, and 3a on the chromatogram sprayed with the ninhydrin-cadmium acetate reagent. Peptide 2 was probably the COOH-terminal octadecapeptide with 1 tyrosine and 2 histidine residues, and peptide 3 seemed to be the tetrapeptide Glu-Gly-Tyr-Lys. A severe failure to achieve complete coupling during the synthesis of the region of this tetrapeptide probably gave rise to the formation of peptide 3a. The logarithms of the electrophoretic mobilities, relative to the origin, of spots 4, 5, and 6 were calculated to be 0.556, 0.663, and 0.959. From a logarithmic plot of the mobility versus the molecular weight (19) the two hexapeptides Leu-Asn-Gln-Met-Met-Lys, Thr-Thr-Gln-Ala-Asn-Lys, and the dipeptide Ser-Arg could be assigned to spots 4, 5, and 6, respectively.

Formation of Active Complex Between Synthetic Monomeric 70-Residue S-Protein Analog and Natural S-Peptide

A mixture of purified monomeric 70-residue analog (4 mg) and natural S-peptide (2.25 mg) was dissolved in 1 ml of 0.05 M NH₄HCO₃. After an incubation time of 30 min the solution was placed on a Sephadex G-50 column (2.4 × 76 cm) and eluted with the same buffer. Two fractions were obtained. Fraction I emerged from the column between 161.5 and 181 ml and weighed 3.9 mg after lyophilization. The elution volume of Fraction II was from 202 to 211 ml and the yield of the product was 0.0 mg. Amino acid analyses of acid hydrolyzates of the two Fractions (Table I) showed that the material from Peak I resembled closely a 1:1 molar complex of the synthetic monomeric 70-residue analog with natural S-peptide, whereas Fraction II seemed to be excess S-peptide. This was confirmed by thin layer electrophoresis of samples of both fractions on cellulose-coated plates (20 × 20 cm) in 0.1 M pyridine acetate, pH 5.0. The plates were sprayed with the Pauly reagent. The main amounts of Fraction I and of an RNase S standard moved with identical RHis values (0.31), but in the absence of urea tailing could not be prevented. Unlike RNase S, the complex of monomeric 70-residue analog with natural S-peptide gave an additional spot which had the same electrophoretic mobility as natural S-peptide (RHis = 0.47). This showed that the affinity of S-peptide for the synthetic 70-residue S-protein analog was lower than that for natural S-protein. The position of the product from Peak II agreed with that of natural S-peptide.

Assay of 70-Residue Analog-S-Peptide Complex Using Cyclic Cytidine 2':3'-Monophosphate (C > p) as Substrate

The activities were determined by the spectrophotometric method of Crook et al. (20) and del Rosario and Hammes (21). Cyclic cytidylate (0.91 mg) was dissolved in 20 ml of 0.1 M Tris-acetate, pH 8.0 (0.14 M). To 2 ml of this solution, 0.81 mg (0.06 µmol) RNase S was added in 0.02 ml of the same buffer, and the increase of the optical absorption at 250 nm was measured in a Cary 15 spectrophotometer. The initial slope of the velocity curve for natural RNase S was found to be 2.247 corresponding to 100% specific activity. Then 1.2 mg (0.16 µmol) of synthetic monomeric 70-residue S-protein analog and 0.14 mg (0.06 µmol) of natural S-peptide were dissolved in 0.04 ml of the Tris-acetate buffer. After a 30-min incubation, 2 ml of the substrate solution were added and the change in absorbance at 250 nm followed as before. The curve obtained had an initial slope of 0.171. From this a specific activity of approximately 8% was observed.
Enzymically Active Complex of Synthetic Monomeric 70-Residue S-Protein Analog with Natural S-Protein

Since the binding of S-peptide by the 70-residue S-protein analog was not as strong as that by natural S-protein and the activity generated was rather low, it was expected that upon addition of an equivalent amount of S-protein to an assay mixture containing the 70-residue analog, S-peptide, and cyclic cytidylate as substrate the 70-residue analog would be displaced and the newly formed S-peptide-S-protein complex would exhibit full RNase activity. This increase in the enzymatic activity was indeed observed, but it was larger than calculated for the amount of S-peptide-S-protein complex present.

When a mixture of monomeric 70-residue S-protein analog (1.27 mg, 0.17 μmol) and natural S-peptide (0.13 mg, 0.06 μmol) was assayed against cyclic cytidylate (0.11 mM) in the presence of natural S-protein (0.53 mg, 0.05 μmol) an initial slope of 1.98 was found. In the absence of the synthetic 70-residue analog the initial slope was only 1.60. This suggested an unexpected interaction between synthetic 70-residue S-protein analog and natural S-protein increasing the specific activity against cyclic cytidylate beyond 100%, as compared with an equivalent amount of natural RNase S.

The specific activities generated depended strongly on the ratio of monomeric 70-residue S-protein analog and natural S-protein used. For 1:4, 1:2, and 1:1 molar ratios of the two components, activities were 70%, 151%, and 112%, respectively. The velocity curves obtained from a standard and from a 1:2 molar mixture of 70-residue analog with S-protein are shown in Fig. 7.

To determine zero order rate constants both the 70-residue analog-S-protein complex and natural RNase S were saturated with cyclic cytidylate as substrate, and the reactions were followed at 286 nm for 20 min. From the plot of [S]₀ - [S] against the time (Fig. 8) the rate constants were calculated to be $2.5 \times 10^{-2}$ M s⁻¹ and $1.3 \times 10^{-2}$ M s⁻¹, respectively. The Michaelis constants determined from Lineweaver-Burk plots (Fig. 9) were 0.7 nmol for natural RNase S and 0.48 nmol for the mixture of monomeric 70-residue S-protein analog with natural S-protein using cyclic cytidylate as substrate.

Binding of Substrate Analog Cytidine 3'-Monophosphate (3'-CMP) by Monomeric 70-Residue Analog-S-Protein Complex at pH 8

It is known that pH 5.5 is the optimal pH for the binding of 2' CMP or 3' CMP to RNase A (22). Similarly, guanosine 3' monophosphate is best bound to RNase T₁ at about pH 5 (23). In this work, however, all assays as well as the binding studies with 3' CMP had to be performed at pH 8 because the solubility of the synthetic 70-residue S-protein analog in slightly acidic medium was very low.

Basically, the method of Hummel and Dreyer (24) was followed. Natural RNase S, 2.082 mg (0.152 μmol), and the mixture of monomeric 70-residue analog (0.698 mg or 0.091 μmol) with S-protein (1.750 mg or 0.152 μmol) were dissolved in 0.3 ml of 0.05 M NH₄HCO₃ each and passed separately through a Sephadex G-25 column (25 x 1 cm) using 0.05 M NH₄HCO₃ as eluant. The volumes of the combined protein-containing fractions were 5 ml and 5.4 ml; and the extinctions at 285 nm, a wavelength at which the absorbances of RNase and 3'-CMP are additive (25), were 0.232 and 0.259 A units, respectively.

Then the eluant was made 0.071 mM in 3'-CMP, and the Sephadex G-25 column was equilibrated with the solution of this nucleotide. The same amounts of RNase S and 70-residue analog-S-protein complex were dissolved in 0.05 M NH₄HCO₃ calculation. It should be noted that in this assay the molar ratio of 70-residue analog to S-peptide was 2.67:1 whereas in RNase S the molar ratio of S-protein to S-peptide is 1:1. In a control experiment it was shown that the natural S-peptide used was completely inactive in this assay system.

Fig. 8. Determination of the zero order rate constants of the reaction of natural RNase S and the 70-residue analog-S-protein complex with cyclic cytidine 2':3' monophosphate at pH 8. The substrate concentration used (0.39 mM) was 0.1 mM Tris-acetate, pH 8.0. A gave a linear slope at 286 nm for the first 10 min of the reaction with the half-synthetic complex as enzyme and for the first 15 min with natural RNase S as enzyme. From the molar extinction coefficients of cyclic cytidine 2':3' monophosphate and cytidine 3'-monophosphate at 286 nm in 0.1 M Tris-acetate, pH 8, in 1-cm cuvettes (2564 and 3764, respectively) the amounts of substrate present at time t, [S], was determined. Then [S]₀ - [S] was plotted against the time. O—O, natural RNase S, 31 μg (2.3 nmol), was added to 2 ml of the substrate solution; •—•, synthetic monomeric 70-residue analog, 9 μg (1.2 nmol), and natural S-protein, 26 μg (2.3 nmol), were mixed with 2 ml of the substrate solution. The rate constants found were $1.3 \times 10^{-7}$ M s⁻¹ and $2.5 \times 10^{-7}$ M s⁻¹, respectively.
containing the substrate analog and applied to the column. The proteins were again in 5 ml and 5.4 ml of the eluate and their extinctions at 285 nm were 0.251 and 0.299 A units, respectively. From the increase of the optical absorptions in the presence of 0.071 mM 3' CMP and the known extinction of this nucleotide was followed at 300 nm. From the initial slopes (1.369 for RNase S, 0.49 mg (0.02 pmol) in 2 ml of a 0.1 mM substrate solution was activated by the Kunitz method (26). The pH of the 0.1 mM CH3COONa buffer, however, was raised to 7.8.

Fig. 9. Determination of the Kₐ values of the reaction of natural RNase S and the 70 residue analog S protein complex with cyclic cytidylate as substrate at pH 8. The substrate concentrations ranged from 0.076 to 0.621 mm in 0.1 mM Tris-acetate, pH 8.0. Constant amounts of natural RNase S, 0.49 mg (0.030 μmol), and of the mixture of monomeric 70-residue analog, 0.16 mg (0.02 μmol), with natural S-protein, 0.4 mg (0.035 μmol), respectively, were added to the substrate solutions containing 0.05, 0.075, 0.1, 0.2, and 0.4 mg of cyclic cytidylate per 2 ml of buffer. The initial velocities were measured spectrophotometrically at 286 nm. They were 0.015, 0.022, 0.029, 0.048, and 0.070 A optical density per min for the RNase S standard and 0.050, 0.077, and 0.110 A optical density per min for the mixture of monomeric 70-residue S-protein analog with natural S-protein. From Lineweaver-Burk plots of the results with natural RNase S, O O, and with the half-synthetic complex, ● ●, the Kₐ values were found to be 0.7 and 0.48 mmol, respectively. 

Quantitative Assays with Dimer of 70-Residue S-Protein Analog

RNase A dimers formed through noncovalent intermolecular interactions between partially denatured monomers have full biological activity (28). To study the enzymic properties of the dimeric 70-residue S-protein analog and its complexes with S-peptide and S-protein the following assays were performed.

Cyclic Cytidylate as Substrate—Natural S-protein (0.5 mg, 0.043 μmol) in 2 ml of a 0.1 mM substrate solution was activated by addition of either natural S-peptide (0.1 mg, 0.045 μmol) or dimeric 70-residue S-protein analog (0.06 mg, 0.008 μmol; or 0.30 mg, 0.040 μmol). The specific activities calculated from the initial velocities at 286 nm were 100%, 15%, and 74%, respectively, based on the amount of S-protein. The mixture of dimeric 70-residue S-protein analog with natural S-peptide gave about 8% activity.

5'-3' Cyclic(3' Cytidyl)-guanosine (CpG) as Substrate—Natural RNase S (11 μg, 0.001 μmol) and dimeric 70-residue S-protein analog (0.244 mg, 0.032 μmol) were added to 2-ml portions of a 0.065 mM CpG solution. From the initial velocity of the hypochromic shift at 286 nm the dimeric S-protein analog was found to be 0.6% as active as an equimolar amount of natural RNase S.

RNA as Substrate—To solutions of 1 mg of RNA in 2 ml of 0.1 mM Tris-acetate, pH 8.0, were added 1 μg of natural RNase S and 5 μg of dimeric 70-residue S-protein analog, respectively. From the initial rates measured at 300 nm the specific activity of the dimeric 70-residue analog was calculated to be 4%. No rise of the activity was observed when natural S-peptide or natural S-protein was present in the mixture.

The activities of the 70-residue S-protein analog and its complexes with S-peptide and S-protein against cyclic cytidylate, CpG, and RNA are summarized in Table II.
TABLE III  
Substrate specificity of synthetic 70-residue S-protein analog and its mixtures with natural S-peptide and natural S-protein  

<table>
<thead>
<tr>
<th>Compounds cleaved</th>
<th>Compounds not cleaved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclic cytidine 2':3'-monophosphate*</td>
<td>Cyclic adenosine 2':3'-monophosphate*</td>
</tr>
<tr>
<td>Cyclic uridine 2':3'-monophosphate*</td>
<td>Cyclic guanosine 2':3'-monophosphate*</td>
</tr>
<tr>
<td>5'-3'-Cytidylyl-guanosine*</td>
<td>Cyclic cytidine 3':5'-monophosphate*</td>
</tr>
<tr>
<td>5'-3'-Uridyllyl-guanosine*</td>
<td>Cyclic uridine 3':5'-monophosphate*</td>
</tr>
<tr>
<td>5'-3'-Guanulyl-ctydine*</td>
<td>Cyclic adenosine 3':5'-monophosphate*</td>
</tr>
<tr>
<td>Polycytidylic acid*</td>
<td>Cyclic guanosine 3':5'-monophosphate*</td>
</tr>
<tr>
<td>RNA*</td>
<td>5'-3'-Adenylyl-guanosine*</td>
</tr>
<tr>
<td></td>
<td>5'-3'-Guanyllyl-ctydine*</td>
</tr>
<tr>
<td></td>
<td>Polyadenylic acid*</td>
</tr>
<tr>
<td></td>
<td>Polyadenylic-guanyleic acid*</td>
</tr>
<tr>
<td></td>
<td>5'-3'-([2'-Deoxyadenyllyl])-2'-deoxy-</td>
</tr>
<tr>
<td></td>
<td>guanosine*</td>
</tr>
<tr>
<td></td>
<td>DNA*</td>
</tr>
</tbody>
</table>

* The dimeric 70-residue analog (70n) catalyzed the hydrolysis of approximately 20% of this substrate in 45 hours, in the presence of the monomeric form of the synthetic S-protein analog (70n) the reaction was even slower.

Qualitative Assays with 70-Residue S-Protein Analog

Various cyclic nucleoside phosphates, dinucleoside phosphates, and polynucleotides were employed to determine the substrate specificity of the 70-residue S-protein analog more precisely. The results of these assays are listed in Table III. The chromatograms obtained with CpG, UpG, GpC, polycytidylic acid, and RNA are shown in Fig. 10.

DISCUSSION

Only a few residues or regions of a protein chain are involved in binding and converting the substrate. The function of the largest part of the molecule seems to be to bring the essential residues into optimal position for catalysis through proper folding. For quite some time, protein chemists have been interested in the question of how much of the primary structure, apart from the active site residues, is actually needed for retention of enzymatic activity. One way to approach this problem is the chemical synthesis of the fragment. Since in RNase A only a few single residues can be cleaved from the NH₂- and COOH-terminal end without inactivating the enzyme completely (33-35), a new approach was chosen to look for active substructures of this protein.

It had been shown through synthetic and degradation studies that the region comprising residues 15 to 25 has no structural or functional importance (3-5). This was supported by Barnard et al. (36) who compared the NH₂-terminal sequences of pancreatic RNase from 7 different species and found that the segment from 15 to 24 represents a highly variable part of the polypeptide chain. The secondary structure and location in the molecule of residues 15 to 25 (i.e. loop at the surface remote from the active site) led to the idea of reducing the size of the enzyme drastically by omitting several such superficial loops but leaving the active center and its immediate environment intact. Plans were to synthesize a fragment of RNase S-protein which would still combine and generate activity with S-peptide.

Leucine was chosen as NH₂-terminal amino acid because it allowed hydrophobic interaction with alanine 84 replacing the half-cystine residue of the natural sequence in that position. Residues 36 to 49 and 58 to 73 were omitted and the resulting gaps were closed through insertion of 1 and 2 alanine residues, respectively. The next deletion comprised residues 87 to 96. In the native enzyme, the chain continues from there as an anti-
substrate. C, Polycytidylic acid (Poly-C) and RNA were used as substrates. Poly(C) was digested by both RNase S and the 70n-analog as enzyme had been hydrolyzed to give C-p. B, 5'-5'-(3'-cytidylyl)-guanosine (CpG) and 5'-5'-5'-cytidine (CpG) were used as substrates. GpC was cleaved, as expected, by RNase S, and the products formed were cyclic guanosine 2'-3'-monophosphate (G > p) as an intermediate, guanosine 3'-monophosphate, and cytidine (C). It was also very slowly digested (approximately 5% in 40 hours) by the monomeric 70-residue analog (70n). The only reaction products detected in this case were C > p and cytidine. UpG was cleaved and hydrolyzed by RNase S to give uridine 3'-monophosphate (U-p) and guanosine (G). The 70n-analog, however, catalyzed only the cleavage reaction and the cyclic uridine 2'-3'-monophosphate (U > p) formed was the final product. The small amount of U-p found in this sample was most likely introduced as a contaminant of the UpG substrate. C, Polycytidylic acid (Poly-C) and RNA were used as substrates. Poly(C) was digested by both RNase S and the 70n-analog; (a) after 20 hours the 70n-analog had converted this substrate into a mixture of oligocytidylic acids and C > p whereas with RNase S as enzyme the reaction had gone to completion and the only product obtained was C-p; (b) after 75 hours the 70n-analog had completely cleaved the oligocytidylic acids and the compounds remaining were C > p and C-p in an approximately 2:1 molar ratio. Major components of the digestion of RNA by the 70n-analog were the cyclic pyrimidine nucleoside 2'-3'-monophosphates (the substances with high Rf values). In the presence of natural RNase S, these cyclic products were hydrolyzed giving rise to the formation of the spot with Rf = 0.34 which does not occur in the digest produced by the 70n-analog.

Thus, 38 residues were deleted and 4 residues were inserted bringing the total number of amino acids in the S-protein analog to 70. The synthetic product still contained all those regions of the parent molecule known to be crucial for binding of substrate and catalysis, but 34 residues distant from the active site including all four disulfide bonds were missing. The calculated molecular weight of the 70-amino acid residue peptide was 7654, that is 66.5% of that of S-protein or 55.9% of that of RNase A. Fig. 1 shows the primary structures of the synthetic analog and of natural S-protein. In Fig. 11 the tertiary structures of the 70-residue S-protein analog and its parent molecule are compared assuming that, despite the changes in the amino acid sequence, the synthetic S-protein analog has retained the ability to fold in the same fashion as the corresponding regions of natural S-protein. Although there is at present no direct evidence for this, the conformation of the 70-residue analog is presented that way to illustrate the structural differences between native S-protein and its synthetic analog.

Some of the physicochemical properties of the 70-residue peptide may be explained by its amino acid composition. Table IV reveals a relatively high content of aliphatic hydrophobic residues in the 8-protein analog as compared with that in native S-protein or RNase A. This was probably the reason for the low solubility of the synthetic product at slightly acidic pH and its strong tendency to aggregate.

Solid Phase Synthesis of 70-Residue S-Protein Analog—Some procedures and amino acid derivatives employed for the total synthesis of RNase A by the solid phase method (5) were changed in this work. To remove the Boc groups, a solution of 20% (v/v) TFA in CH2Cl2 was used instead of a 50% mixture. The decreased concentration of TFA was probably advantageous for the stability of side chain protecting groups such as the benzyl residue blocking the phenolic hydroxyl group of tyrosine or the parallel pleated sheet. In order to allow a reverse turn to form between glutamic acid 86 and tyrosine 97 a glycine residue was incorporated. Finally, half-cystine 110 of the natural sequence was replaced by alanine and residues 113 and 114, part of a reverse turn, were omitted, and the distance between the two ends formed was bridged by directly linking glycine 112 with tyrosine 115.

To remove the Boc groups, a solution of 20% (v/v) TFA in CH2Cl2 was used instead of a 50% mixture. The decreased concentration of TFA was probably of advantage for the stability of side chain protecting groups such as the benzyl residue blocking the phenolic hydroxyl group of tyrosine or the parallel pleated sheet. In order to allow a reverse turn to form between glutamic acid 86 and tyrosine 97 a glycine residue was incorporated. Finally, half-cystine 110 of the natural sequence was replaced by alanine and residues 113 and 114, part of a reverse turn, were omitted, and the distance between the two ends formed was bridged by directly linking glycine 112 with tyrosine 115.
Fig. 11. Comparison of the tertiary structures of the 70-residue S-protein analog (left) and natural RNase S-protein (right) assuming that the polypeptide chain of the synthetic S-protein analog folds in the same way as the corresponding regions of the parent molecule. In the 70-residue analog, amino acids 21 to 25, 36 to 40, 53 to 73, 87 to 96, 113 and 114 were omitted; and 4 residues (gray circles) were inserted to bridge the gaps caused by the deletions. The half-cystine residues were either part of the omissions or they were replaced by hydrophobic residues. In the natural S-protein, the open circles represent the sulfur atoms of the disulfide bonds. RNase S-protein was reproduced, as seen through a stereo viewer, from Dickerson and Geis (47) with permission of the authors.

Table IV
Amino acid composition of RNase A, S-protein, and synthetic 70-residue S-protein analog

<table>
<thead>
<tr>
<th></th>
<th>RNase A</th>
<th>S-protein</th>
<th>70-residue analog</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp⁺</td>
<td>5</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Asn⁺</td>
<td>10</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Thr</td>
<td>10</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Ser</td>
<td>15</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>Gly⁺</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Gly</td>
<td>7</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Pro</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Gly⁺</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Ala</td>
<td>12</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Cys⁺</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Val⁺</td>
<td>9</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Met⁺</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>His⁺</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Leu⁺</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Tyr⁺</td>
<td>6</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Phe</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>His</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Lys</td>
<td>10</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Arg</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>124</td>
<td>104</td>
<td>70</td>
</tr>
</tbody>
</table>

- Charge at neutral pH: +8
- Amides: 17
- Gly, Pro: 7
- Aliphatic hydrophobic residues: 30
- S-S bonds: 4
- Aromatic residues: 9

benzoxycarbonyl residue blocking the ε-amino function of lysine. These protecting groups are known not to be completely resistant to 50% (v/v) TFA in CH₂Cl₂ (37). For the incorporation of the 3 histidine residues the Boc N⁹-tosyl derivative (38) was chosen because the tosyl group could be removed simultaneously with the cleavage of the peptide from the resin by anhydrous HF. A higher coupling rate and a reduced tendency to racemize during the coupling reaction (39, 40) made Boc-N⁹-tosyl-histidine superior to the N°-protected form for the use in solid phase peptide synthesis. A precaution taken to protect the thioether group of methionine was the addition of 10% anisole (v/v) to each 20% (v/v) TFA-CH₂Cl₂ deblocking mixture after the first methionine residue had been added to the peptide chain on the resin in order to prevent the formation of sulfonium ions through alkylation at the sulfur atoms. The yield of methionine calculated from amino acid analyses of acid hydrolysates of the purified synthetic 70-residue analog was usually between 60 and 70%, the remainder was recovered as methionine sulfoxide. Part of this may have been formed during the synthesis and work up, another part may have formed during the acid hydrolysis of the peptide.

The protected 70-residue polypeptide resin was obtained in a 40% yield based on the amount of COOH-terminal valine bound to the polymer support at the beginning of the synthesis. This is in relative agreement with the 17% yield found after the 123 deprotection and coupling cycles of the solid phase synthesis of RNase A (5, 41). The major part of these losses must have occurred during the early stages of the synthesis because it was shown that at the level of the hexapeptide resin the retention of peptide chains was already as low as 71% (12), and in an extreme case only 51% of the chains remained on the resin at the heptapeptide level (40). Side reactions such as carboxylic acid-catalyzed formation of diketopiperazines during the second coupling step (42) probably contributed mainly to this substantial loss of peptide chains from the solid support during the early steps of the synthesis. The reduction of the concentration of TFA from a 50 to a 20% (v/v) solution in CH₂Cl₂ seemed, in the long run, to have only a minor effect, if any, on the stability of the ester bond linking the peptide to the resin.

In Table I an amino acid analysis of the protected 70-residue peptide resin is shown. The ratios obtained are in some cases not in agreement with the expected composition. This is not surprising because the analysis was made from the crude synthetic product. In addition, amino acids like serine and threonine usually give low values from acid hydrolysates of peptide resins. The ratios found for methionine and leucine were also too low. However, this cannot be explained at the moment.

Cleavage of 70-Residue S-Protein Analog from Polymer Support

—Treatment with anhydrous HF cleaved the synthetic product from the resin and at the same time removed all protecting groups. The yield of the cleavage step was 62%. When fully protected RNase resin was treated under identical conditions, only 41% of the peptide chains were removed from the solid support. This yield, however, could be raised to 63% when the partially cleaved polypeptide resin was submitted to anhydrous HF a second time (5). To keep the possible side reactions in liquid HF at a minimum, the cleavage of the protected 70-residue peptide resin was not repeated. It should be noted that also short peptides were removed from the resin with only about 60% yield (12, 40). Therefore, the accessibility of the ester bond linking the peptide chains to the resin seems to depend rather on the physical properties of the polymer support than on the length of the peptide.
44), caused by the loss of N'-benzyloxycarbonyl protecting

Thus, branching of the polypeptide chain at lysine residues (43,

blocking reagent, must also be considered as possible sources of

tions, known to occur in 20 to 50% (v/v) TFA-CHK12 as de-

through deletion of one of the neutral amino acids, but side reac-

ture obtained was resolved by electrophoresis and thin layer

by trypsin. Therefore, only six tryptic peptides were expected

that the bond between lysine 41 and proline 42 is not hydrolyzed

from the sequence determination of RNase A (17) it was known

Ionomeric WResidue S-Protein Analog—The 70 residue analog

make an additional purification step feasible.

substrate analog column would have removed more of the by-

shorter than the sequence of the desired product. It is possible

some extent.

analog were not yet homogeneous at this stage. Most likely

they still contained a number of protein chains slightly longer or

Purification of Cleaved 70-Residue S-Protein Analog—In Table

V the yields of the synthesis and purification steps are sum-

marized. Through deletion of several loops from the surface of the

molecule the hydrophobic core of the 70-residue analog be-

came more exposed to the solvent than natural S-protein thus

favoring the formation of aggregates through hydrophobic inter-

actions. This aggregation and the limited solubility were the

main reasons for the relatively low over-all yield (1.2%) of puri-

fied monomeric and dimeric 70-residue S-protein analog based on

the amount of COOH-terminal valine originally esterified to the

resin.

Dissociation of the aggregates and fractionation of the result-

ing mixture of low molecular weight compounds (Fig. 4A) would

have increased the yield of the biologically active fractions to

same extent.

It is clearly recognized that monomeric and dimeric 70-residue

analog were not yet homogeneous at this stage. Most likely

they still contained a number of protein chains slightly longer or

shorter than the sequence of the desired product. It is possible

that affinity chromatography on a Sepharose-bound S-protein or

substrate analog column would have removed more of the by-

products, but the small amount of material available did not

make an additional purification step feasible.

Preparation of Peptide Maps from Tryptic Digest of Synthetic

Monomeric 70-Residue S-Protein Analog—The 70-residue analog

contained six bonds susceptible to tryptic cleavage. However,

from the sequence determination of RNase A (17) it was known

that the bond between lysine 41 and proline 42 is not hydrolyzed

by trypsin. Therefore, only six tryptic peptides were expected

from the 70-residue S-protein analog. The peptide mixture

obtained was resolved by electrophoresis and thin layer

chromatography. Spraying of the plates with the ninhydrin-

cadmium acetate solution (16) revealed the presence of seven

peptides (Fig. 6).

Peptide 3a, the weak spot, was probably a failure sequence of

the tetrapeptide Glu-Gly-Tyr-Lys. It could have formed

through deletion of one of the neutral amino acids, but side reac-

tions, known to occur in 20 to 50% (v/v) TFA-CHCl3 as de-

blocking reagent, must also be considered as possible sources of

heterogeneity of peptides synthesized by the solid phase method.

Thus, branching of the polypeptide chain at lysine residues (43,

44), caused by the loss of N'-benzyloxy carbonyl protecting

groups, and intramolecular rearrangement of O-benzyl tyrosine to

yield 3-benzyl tyrosine (37) have been observed to some extent.

The presence of seven spots on the peptide map instead of the

expected six does not mean that only the synthesis of the region

of one of the tryptic peptides failed to give a uniform product.

The limiting factor to detect very small amounts of impurities

was the sensitivity of the staining reagents used. On the thin

layer plate sprayed with the ninhydrin-cadmium acetate solu-

tion, 13 nmol of the tryptic digest had been applied. Peptides

present at a concentration below 1.5 to 2 nmol (i.e. 12 to 15 mol

%) would not have been visible. The plate stained with the

Itano reagent (18) contained 3.3 nmol of the tryptic hydrolysate.

Impurities with a concentration smaller than 0.2 nmol (or about

6 mol %) would have gone undetected.

It has become apparent from this work that sequences sub-

stantially shorter than the 124-residue polypeptide chain of

RNase A can retain at least part of the specific activity of this

enzyme. In this connection the question may be asked whether

the RNase A activity of the first synthetic enzyme (5) was more

or less due to the presence of several such biologically active

fragments. However, the results obtained upon tryptic treat-

ment of synthetic RNase A and 70-residue S-protein analog

clearly indicated a structural difference between the two prod-

ucts. Native RNase A is known to be very stable to tryptic

attack (45). A trypsin-resistant fraction with high specific

activity could be isolated also from partially purified synthetic

RNase A (5), whereas the enzymatic activity of the 70-residue

analog was rapidly destroyed by tryptic digestion.

Active Complex between Synthetic 70-Residue S-Protein Analog

and Natural S-Peptide—The original goal of this work was to

synthesize an analog of RNase S-protein substantially shorter

than the parent molecule which would still form an enzymically

active complex with S-peptide. Gel filtration studies on Sepha-

dex G-50 showed that the synthetic S-protein analog could bind

natural S-peptide. The resulting complex, however, was not

very strong and dissociated partially upon thin layer electro-

phoresis at pH 5.0. Under these conditions, natural RNase S

was completely stable. Both monomeric and dimeric 70-residue

S-protein analog gave about 8% activity using cyclic cytidine

2':3'-monophosphate as substrate when they were mixed with

natural S-peptide in a 3:1 molar ratio. The weaker binding

between synthetic 70-residue analog and natural S-peptide was

probably the reason for the low enzymic activity of this complex

against cyclic cytidylate. The reduced affinity of S-peptide for

the 70-residue S-protein analog cannot yet be explained con-

vincingly. Table VI shows that almost all amino acid residues

of the S-protein chain involved in hydrogen bonding and close

backbone contacts with S-peptide were still present in the 70-

residue S-protein analog. The hydrophobic area formed by

Ala 4, Ala 5, and Phe 8 from the NH2 terminus and Val 116, Pro

backbone contacts with S-peptide were still present in the iO-

protein could also exist when the latter was replaced by its synthetic

analog. The hydrophobic contacts of the S-protein chain involved

inamide nitrogen of proline 42 and the NH of tyrosine 25 (46, 48).

It has become apparent from this work that sequences sub-

stantially shorter than the 124-residue polypeptide chain of

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against cyclic cytidylate. The reduced affinity of S-peptide for

the 70-residue S-protein analog cannot yet be explained con-

vincingly. Table VI shows that almost all amino acid residues

of the S-protein chain involved in hydrogen bonding and close

backbone contacts with S-peptide were still present in the 70-

residue S-protein analog. The hydrophobic area formed by

Ala 4, Ala 5, and Phe 8 from the NH2 terminus and Val 116, Pro

backbone contacts with S-peptide were still present in the iO-

protein could also exist when the latter was replaced by its synthetic

analog. The hydrophobic contacts of the S-protein chain involved

inamide nitrogen of proline 42 and the NH of tyrosine 25 (46, 48).
residue S-protein analog in the transphosphorylation reaction.

Thus, the 70-residue S-protein analog contained all of those residues of the parent molecule known to be important for the binding of S-peptide and for the enzymic activity. However, the altered primary structure most likely led to some changes of the tertiary structure so that the essential residues were probably not in their most favorable positions. This may have decreased the affinity between the two components and consequently reduced the activity generated by the complex. The impurities still present in the synthetic product probably also contributed to the deteriorated enzymic properties of the 70-residue analog-S-peptide complex.

In conclusion, it can be said that S-peptide activated both the monomer and the dimer of the S-protein analog to catalyze the hydrolysis step of the RNase A action. It had no measurable effect, however, on the activity of monomeric and dimeric 70-residue S-protein analog in the transphosphorylation reaction (Table I).

**Generation of Activity in Hydrolysis Step by Mixtures of Monomeric or Dimeric Synthetic 70-Residue S-Protein Analog with Natural S-Protein**—When a mixture of S-peptide, monomeric 70-residue S-protein analog, and natural S-protein was assayed using cyclic cytidylate as substrate the activity regenerated exceeded that shown by a control containing exactly the amount of RNase S that was expected to form. It could be demonstrated that the increased activity against cyclic cytidylate was solely due to a strong interaction between the synthetic S-protein analog and its parent molecule that did not require the presence of S-peptide.

The mixture of monomeric 70-residue S-protein analog with natural S-protein was most active in the hydrolysis reaction when the two components were used in a 1:2 molar ratio. This could mean that the enzymically active species was a ternary complex consisting of one 70-residue analog and two S-protein subunits. The activities measured against cyclic cytidine 2':3'-monophosphate as substrate were as high as 151% based on the amount of S-protein present in the sample and in the RNase S standard. This number should perhaps be corrected because the hypothetical complex formed by 2 molecules of S-protein and 1 molecule of 70-residue S-protein analog would contain three intact substrate binding sites whereas the equivalent amount of RNase S (i.e., 2 molecules of the enzyme) could bind only 2 substrate molecules. Thus, the activity generated per binding site would be the same for the half-synthetic as well as the natural complex.

When dimeric 70-residue S-protein analog was combined with natural S-protein different results were obtained in this assay system. Maximal activity (74%) was found when the two compounds were added to cyclic cytidylate in a 1:1 molar ratio. Probably, the dimer of the 70-residue analog did not bind natural S-protein as efficiently as the monomer so that the active site residues, whether they were located on one or on both components of the complex, could not assume their most favorable position for catalysis.

The mechanism of action of the 70-residue analog-S-protein mixtures is not understood at present. Most RNase mechanisms proposed so far involve 2 histidine residues (His 12 and His 119) and, with lesser certainty, 1 lysine residue (Lys 41) as the catalytically essential groups of the enzyme (for a summary, see Ref. 46). Neither natural S-protein nor the synthetic 70-residue analog contained the NH2-terminal portion of the native enzyme, including histidine 12, and both were inactive in the hydrolysis step. But on combination of the two products, high specific activity was generated against cyclic cytidine 2':3'-monophosphate. The conclusion must be that either histidine 12 does not take part in the catalysis of the hydrolysis reaction or it can be replaced in its function by a group with similar pK value when a subunit other than the S-peptide portion is bound properly by S-protein. The former would mean that all essential residues are located in the S-protein and that the only role of the NH2-terminus of RNase A or the S-peptide of RNase S is to hold the rest of the molecule in the enzymically active conformation or to contribute to the binding of the substrate or both. Removal of the S-peptide part would cause the active conformation to change, weaken the interactions with the substrate, and leave the S-protein with little or no biological activity. The mechanisms of action put forward by Wang (49) and Hammes (50) would support such a structural role of the NH2-terminal end of RNase because they require only one histidine residue for the proton transfer in both the transphosphorylation and the hydrolysis step. The postulated protonation of a free equatorial oxygen of the phosphate moiety of the substrate (49) could be performed by the c-NH3+ group of Lys 41 and did not necessarily require the presence of a 2nd functional histidine residue. If His 12 would serve as a positively charged binding locus (50) its replacement by an amino acid residue in a similar ionization state located on a peptide which can interact with S-protein should not be impossible. Inactivation of the enzyme through carboxymethylation (51, 52) or iodination (53) of His 12 would then have primarily sterical reasons causing the binding constant of the S-peptide-S-protein complex to decline and probably also preventing the substrate from entering the active site. The inactivation of RNase S, however, observed upon replacement of the

<table>
<thead>
<tr>
<th>Hydrogen bonding</th>
<th>Close backbone contacts</th>
</tr>
</thead>
<tbody>
<tr>
<td>His 12-CO---HN-Val 47</td>
<td>Ala 4, Ala 5, Phe 8 near Pro 117, Val 118</td>
</tr>
<tr>
<td>Asp 14-NH---OC-Val 47</td>
<td>Arg 10, Gln 11 near Arg 33, Asn 34</td>
</tr>
<tr>
<td>Ser 16-NH---OC-His 48</td>
<td>His 12, Met 13, near Val 47, His 48,</td>
</tr>
<tr>
<td>Arg 10-CO---HN-U-Ar 33</td>
<td>Asp 14, Ser 10</td>
</tr>
<tr>
<td>Gln 11-CO---HN6-Asn 44</td>
<td>Gln 49, Ser 50</td>
</tr>
<tr>
<td>His 12-NH---OC-Thr 45</td>
<td></td>
</tr>
<tr>
<td>Met 13-CO---HN6-U-Ar 33</td>
<td></td>
</tr>
<tr>
<td>Asp 14-CO---HN6-His 48</td>
<td></td>
</tr>
<tr>
<td>Asp 14-β-COO---HO-Tyr 25</td>
<td></td>
</tr>
</tbody>
</table>

* These data were taken from the work of Richards and Wyckoff on the x-ray structure of RNase S (46) and from the stereo drawings by Dickerson and Geis (47).
imidazole ring of His 12 by the pyrazole ring (54, 55) would rather lend support to a more functional role of the side chain of this histidine residue in the catalytic mechanism of RNase.

If His 12 is essential for the action of RNase, the high specific activity of 70-residue analog-S-protein mixtures against cyclic pyrimidine nucleoside 2':3'-monophosphates could only be explained by the formation of a complex in which the function of His 12 was assumed by a residue with similar acid-base properties.

Natural S-protein does not dimerize under the assay conditions described under “Experimental Procedure and Results.” The 70-residue S-protein analog with its increased hydrophobic character, however, had a much stronger tendency to bind to S-protein or to a 2nd molecule of its own species. At present, the mode of binding of the synthetic S-protein analog to natural S-protein as well as the nature of the active site residues of the complex are completely unknown. It is also unclear whether the substrate is bound mainly by the S-protein and the 70-residue analog is merely providing the missing catalytic group or whether the role of the two components of the complex is reversed. It seems that the deletion of the three loop sequences 36 to 40, 58 to 73, and 87 to 96 would have made His 119 and Lys 41 of the 70-residue S-protein analog more accessible so that S-protein could bind in this area and constitute an active site different from that of RNase A or S.

The primary structure of RNase A (56, 57) shows that the nearest neighbors of the 4 histidine residues of this enzyme are different in every case. However, the pK value of the functional side chain of an amino acid residue is also determined by the environment of that group in the three-dimensional structure of the protein. Thus, possible conformational changes in the 70-residue analog induced by the deletions may have caused a shift of the pK value of 1 or several of the histidine residues. On formation of the complex with S-protein, further changes of the pK values of imidazole side chains of both components could be expected. It is conceivable, therefore, that 1 of the histidines of the 70-residue analog S-protein complex became almost indistinguishable from His 12 in RNase A or S with respect to pK value and the position in the active center. These considerations apply also to the ionization states of other functional side chains.

In RNase A, the unique alkylation of His 12 and His 119 by iodoacetate at pH 5.5 (51) leads to inactivation of the enzyme and the extent of this reaction is a measure of the correct alignment of the 2 histidine residues in the active site. Carboxymethylation of the 70-residue analog-S-protein complex under the same conditions may have revealed whether the 2 His 119 residues of the complex were identical with each other and with His 119 in RNase A and whether, through the interactions between synthetic analog and natural S-protein, a histidine residue had evolved that resembled His 12 of the native enzyme in its reactivity toward iodoacetate. However, the 70-residue analog was almost insoluble at pH 5.5, and at higher pH values the reaction with iodoacetate is known to be less specific (58).

**Kinetics of Monomeric 70-Residue Analog-S-Protein Complex**—

All measurements had to be performed at pH 8 because of the very limited solubility of the synthetic material in more acidic medium. At this pH, maximal binding of substrate and maximal reaction rates could not be expected.

Binding studies with cytidine 3'-monophosphate and the determination of Michaelis constants using cyclic cytidylate as substrate showed that the half-synthetic complex bound both substrate analog and substrate tighter than natural RNase S. Thus, when the mixture of monomeric 70-residue analog and S-protein was used as enzyme, the cyclic cytidylate was more readily available for catalytic hydrolysis than in the complex with RNase S. This may have been the reason for the increased specific activity of the half-synthetic complex.

The lower $K_m$ value of this complex indicated that half-maximal reaction velocity was achieved at a lower substrate concentration than in the presence of natural RNase S as enzyme (0.48 mM and 0.7 mM, respectively; see Fig. 9). Furthermore, at pH 8 neither S-protein nor its 70-residue analog interacted with cytidine 3'-monophosphate whereas a 1.7:1 molar mixture of the two products bound 0.5 mol of this substrate analog. Under the same conditions, the molar ratio of RNase S and 3'-CMP was found to be 1:0.13. The difference in the zero order rate constants of the hydrolysis of cyclic cytidylate (2.5 $\times$ 10$^{-7}$ M$^{-1}$ s$^{-1}$ for the monomeric 70-residue analog-S-protein complex and 1.3 $\times$ 10$^{-7}$ M$^{-1}$ s$^{-1}$ for natural RNase S) was in agreement with the more productive binding of the substrate and the higher specific activity shown by the half-synthetic complex as compared with RNase S.

**Activity of Synthetic 70-Residue S-Protein Analog in Transphosphorylation Step**—Apart from the activity of its mixtures with natural S-peptide or S-protein in the hydrolysis step, the synthetic S-protein analog alone catalyzed the transphosphorylation reaction to a considerable extent. Table II shows that the dimer of the 70-residue analog was more active in the first step of the RNase A action than the monomer.

In this connection, a possible intrinsic activity of S-protein in the transphosphorylation step must be discussed. Since the binding between S-peptide and S-protein is very strong (the dissociation constant of the complex in the presence of substrate was found to be 7 $\times$ 10$^{-7}$ M (50)), it may be very hard to remove last traces of the smaller component by precipitation or chromatography methods. Contamination of S-protein by minute amounts of S-peptide would then first be noticed by an activity in the transphosphorylation step because the phosphodiester cleavage proceeds at a considerably faster rate than the hydrolysis of the cyclic 2':3'-intermediates.

In this work, commercial S-protein was purified by column chromatography on Bio-Gel P-6 in 1 M CH$_3$COOH and repeated precipitation with CH$_3$COONa (2) and was then found to have a residual activity of 0.1% using RNA as substrate. Since the analytical methods available today are not sensitive enough to detect traces of S-peptide bound to S-protein, the fact that this very low activity was still caused by the presence of a small amount of S-peptide-S-protein complex, cannot be ruled out. When the purified natural S-protein was reduced with excess mercaptoethanol in concentrated urea solution and then reacted with iodoacetamide, amino acid analysis showed that all 8 half-cystine residues had been converted to N-carboxamidomethyl-cysteine residues. This S-protein derivative had no detectable activity in the transphosphorylation reaction. If the weak activity shown by the unmodified S-protein was intrinsic, the introduction of the eight rather bulky S-carboxamidomethyl groups may have caused conformational distortions leading to inactivation.

The activity of monomeric and dimeric 70-residue S-protein analog in the first step of the RNase A action was demonstrated spectrophotometrically and by thin layer chromatography (Fig. 10). Both compounds also seemed to catalyze the hydrolysis of cyclic cytidine 2':3'-monophosphate at an extremely slow rate (in the presence of the dimer approximately 20% of the substrate was hydrolyzed in 45 hours, see Table III), whereas no reaction was observed when cyclic uridine 2':3'-monophosphate was added as substrate or had been formed as an intermediate. Monomer
and dimer of the synthetic S-protein analog cleaved RNA at a faster rate than dinucleoside phosphates such as CpG. Although the two products differed in their specific activities in the transphosphorylation step, they were indistinguishable from each other and from RNase A with respect to substrate specificity. This suggested that the conformation of the binding site of the 70-residue analog was to a high degree identical with that of native RNase A in spite of different primary structures of the two proteins.

The synthetic S-protein analog contained all of those amino acid residues of the parent molecule that are considered to be involved in the orientation, binding, and conversion of the substrate. These residues are Lys 41, Asn 44, Thr 45, His 119, Phe 120, Asp 121, and with less certainty Ser 123 (46).

In this context, a brief comment should be made about a possible interplay of catalytically essential groups at the COOH terminus of RNase A. His 119 is most likely taking part in the proton transfer reactions of the RNase mechanism. There is evidence that the pH value of the imidazole side chain of His 119 may be controlled by an interaction with the C=OOG group of Asp 121. The x-ray structure of RNase S (7, 46) does not show unambiguously whether such an interaction is actually taking place, but synthetic studies making use of the enzymically active tivRNase(1-118)-RNase(111-124) complex (60) have clearly demonstrated that an acidic group is needed in position 121 to preserve the biological activity of this protein-peptide system. When Asp 121 in the peptide component was replaced by asparagine the activity of the complex dropped to about 2% as compared with that of native RNase A using cyclic cytidine 2',3'-monophosphate as substrate (47).

It is possible that through slow deamidation of asparagine 121, occurring during the synthesis, cleavage from the resin, and work-up of this peptide analog, enough aspartic acid residues were formed to account for the residual activity. Since Asp 121 seems to be so crucial for the functioning of this enzyme, a proton and charge transfer mechanism, cleavage from the resin, and work-up of this peptide analog, enough aspartic acid residues were formed to account for the residual activity. Since Asp 121 seems to be so crucial for the functioning of this enzyme, a proton and charge transfer system similar to that described for chymotrypsin (61) cannot be excluded.

The interactions proposed to exist between the S-peptide portion of RNase S and the substrate or substrate analog (i.e. hydrogen bonds of the side chains of His 12 and Gln 11 with the phosphate moiety of the bound nucleotide, the latter probably via a solvent molecule (46)) could not occur in the complexes of natural S-protein or synthetic 70-residue S-protein analog with suitable nucleotides. Their absence, however, probably only weakened the binding but did not abolish it completely.

The 70-residue S-protein analog showed considerable activity in the transphosphorylation step but had very little if any activity in the hydrolysis step. The catalysis of the two reactions may not necessarily follow the same pathway (62, 63). This could mean that in the synthetic 70-residue analog the two activities have been separated because amino acid residues essential for the second step were missing or were in an unfavorable position and the necessary conformational change could not be induced in the absence of the NH terminus of the enzyme. Richards and Wyckoff (46) suggested that a minor pathway for Step 1 not involving His 12 which would also explain the activity of natural S-protein and synthetic 70-residue S-protein analog in the transphosphorylation reaction may exist. They based their assumption on the observation by Bernfield (94) that S-protein showed reduced synthetic activity (the reverse reaction of Step 1) but much more markedly reduced Step 2 activity as compared with intact enzyme. Although the mixtures of the 70-residue analog with cyclic cytidylate were incubated and chromatographed under conditions slightly different from those used by Bernfield (see Fig. 10 legend), it seemed certain that the synthetic S-protein analog had lost both the hydrolytic and the synthetic activities of RNase A since only about 5% of the substrate was hydrolyzed in 12 hours and no oligocytidylic acids could be detected under ultraviolet light after that time.

The results of this work seem to support the hypothesis that somewhat different mechanisms exist for the two steps of the RNase A action, the first one not requiring His 12 while for the second this residue may be important. As can be seen from Table II, addition of natural S-peptide or S-protein to either the monomeric or dimeric synthetic 70-residue analog did not increase the activity in the transphosphorylation reaction whereas, particularly in the presence of S-protein, very high hydrolytic activities were regenerated. This could mean that the site catalyzing Step 1 of the RNase A mechanism was severely damaged in the 70-residue analog and could not be repaired when either S-peptide or S-protein was added. In contrast, the site catalyzing the hydrolysis step was obviously reconstituted with great precision. Thus, the amino acid residues taking part in the catalysis of transesterification and hydrolysis were probably partly different.

The specific activity of the dimeric 70-residue analog using RNA as substrate was low (4%) but quite remarkable. It should be considered that this activity was derived from a synthetic product that was not 100% pure. Furthermore, one or a few of the residues purposely omitted may have been important structurally; their loss could account for most of the decrease of the activity in the transphosphorylation step. If correct, even pure natural S-protein should possess substantial Step 1 activity. Table II, however, shows that both S-protein and monomeric 70-residue S-protein analog were only about 0.1% as active as RNase S using RNA as substrate, whereas the dimeric 70-residue analog exhibited increased Step 1 activity. This indicated that natural S-protein as well as the monomer of the synthetic S-protein analog contained all of the groups essential for the catalysis of the transphosphorylation reaction but that the conformation of the active site of the two products allowed only very weak binding or cleavage of the substrates. Aggregation of two monomers of the 70-residue analog, however, may have caused steric changes toward a more active conformation in one or both subunits of the dimeric complex. Another possibility could be the formation of active dimers from monomers with little or no enzymic activity in such a way that the active site of the complex was located partly on one component, partly on the other, similar to the active dimers obtained through hybridization of inactive N\(^{3}\)His(Cm)-12 RNase with inactive N\(^{3}\)His(Cm)-110 RNase as described by Crestfield and Fructer (65).

It is interesting in this context that, with polyformycin as substrate, RNase A also catalyzed only the transphosphorylation step and no hydrolysis of the cyclic phosphate intermediate was observed (62). Views differ as to whether or not this is an indication for a difference in the pathway of the two reaction steps (46, 62).

**Substrate Specificity of Synthetic 70-Residue S-Protein Analog**—The substrate specificity of monomeric and dimeric 70-residue analog in the transphosphorylation step agreed well with that of native RNase A (Table III). RNA, polycytidylic acid, CpG, and UpG were cleaved whereas ApG, dApG, and DNA did not react. Two exceptions were found among the substrates...
assayed. Polyadenylic acid which is digested by RNase A at a slow rate was completely stable to depolymerization by the monomer or the dimer of the synthetic S-protein analog. On the other hand, GpC which is a substrate for RNase T1 and is not cleaved by RNase A was digested very slowly by the monomeric 70-residue analog to give G > p and cytidine. The dimer of the 70-residue S-protein analog had no effect on this dinucleoside phosphate. Probably, the conformation of the active site of the dimer was more closely related to that of RNase A than that of the monomer, and consequently, the substrate specificities of dimer and RNase A were in better agreement.

CONCLUSIONS

The work presented here is a new approach to structure-function studies in proteins. It has been shown that a large part of the primary structure of RNase A (43.5% of the amino acid residues or 44.1% of the molecular weight) could be omitted without loss of substrate specificity in the transphosphorylation step and with a considerable retention of specific activity even when His 12, regarded by many authors as one of the essential residues, was missing.

The omission of 38 residues including the four disulfide bonds and the insertion of 4 residues closing the gaps caused by the deletions did not seem to affect severely the folding of the hydrophobic core of S-protein. The good agreement of the substrate specificities of 70-residue S-protein analog and RNase A in the depolymerization step suggested that at least the conformation of the "active site" of the synthetic analog was probably very similar to that of the corresponding regions in native RNase A.

Thus, the 70-residue analog is the first example of a synthetic peptide with a remarkable conservation of the tertiary structure of the active site despite rather drastic changes of the primary structure of the parent compound. In nature, however, several examples for this phenomenon have been found already. Thus, chymotrypsin (61) and subtilisin BPN' (67) have the same active site configuration involving an aspartic acid, a histidine, and a serine residue although the folding of the remainder of the molecules and their primary structures are very different from each other. These two proteases thus have different binding sites and substrate specificities but probably very similar catalytic mechanisms. Another example of this kind is the coenzyme-binding portions of the four dehydrogenases that have been analyzed by x-ray crystallography (68-71). Despite the wide variations in the sequences, the structure of the sites interacting with NAD+ has been conserved to a high degree. From this it was concluded (71) that the various dehydrogenases could be related by an evolutionary model involving gene duplication.

The two major prerequisites to the work presented in this paper were met. First, the position of the active site and the x-ray structure of RNase were known; and second, the 70-residue S-protein analog synthesized by the solid phase method was sufficiently pure to make some reliable statements about its altered enzymic properties.

The shortcomings of this work, in particular a certain degree of heterogeneity of the 70-residue polypeptide, are clearly recognized. However, the synthetic methods will be further improved and at the same time the number of proteins, the detailed x-ray structures of which have been elucidated, will be growing, so that the investigations described here for RNase could be extended to other proteins. This may enhance our knowledge about the evolution of enzymes and their mechanisms of action and may lead to products with new interesting properties.

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