The Role of Serine-123 in the Activity and Specificity of Ribonuclease

REACTIVATION OF RIBONUCLEASE 1-118 BY THE SYNTHETIC COOH-TERMINAL TETRADECAPEPTIDE, RIBONUCLEASE 111-124, AND ITS O-METHYLSERINE AND ALANINE ANALOGS*

(Received for publication, July 22, 1974)

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SUMMARY

The COOH-terminal tetradecapeptide of ribonuclease A, Glu-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp-Ala-Ser-Val, and two analogs, [Ser(Me)]-RNase 111-124 and [Ala]-RNase 111-124, were synthesized by the solid phase method and were purified to chromatographic and electrophoretic homogeneity. Methods are described for the hydrolysis and quantitative amino acid analysis of peptides containing O-methylserine. The peptides were combined noncovalently with RNase 1-118 and examined for ability to regenerate enzymatic activity in the presence of the substrate poly(C), poly(U), poly(AF), and the nonsubstrate poly(U). The regenerate enzymatic activity in the presence of the substrate poly(C) poly(U), and poly(AF).

The semisynthetic enzyme composed of RNase 1-118 and [Ser(Me)]-RNase 111-124, therefore, shows appreciable selectivity for substrates containing cytosine. It was concluded that a hydrogen bond between the hydroxyl of serine-123 and the C4 carbonyl oxygen of uridine or cytidine-containing substrates is important for both binding and specificity. The carbonyl oxygen at C2 of the competitive inhibitors 3'-uridylic acid and 3'-cytidylic acid was seen to form a good hydrogen bond with the NH proton of the threonine-45 backbone amide. The groups at positions 3 and 4 are either hydrogen donors or acceptors depending on which pyrimidine ring is serving as the base, and the corresponding positions on the protein must, therefore, contain groups that are potentially both acceptors or donors. The electron density maps clearly showed the β-hydroxyl of threonine-45 to be in a position to serve as a hydrogen donor to the amino group at position 4 of uracil or a hydrogen acceptor for a proton of the amino group at position 4 of cytosine. Although NMR studies did not provide direct evidence for these hydrogen bonds, the data were consistent with them and the bonds were incorporated into the proposed structure of the inhibitor-enzyme complexes.

Ward et al. (5) concluded that the same active site on ribonuclease A is responsible for transphosphorylation when the substrate is either polyuridylic acid, polycytidylic acid, or poly(formycin-3'-phosphate). The nucleotides are believed to bind through phosphate and ribose and also the base. With regard to hydrogen bonding of the base, formycin in the syn-conformation can be considered to be a hybrid of cytidine and uridine when they are in the normal anti-conformation. Since poly F assumes the syn-conformation, the ring systems can be aligned so that position 3 of the pyrimidine ring coincides with position 1 of formycin, and position 4 of the pyrimidines coincides with...
position 7 of formycin. By this reasoning the hydroxyls of threonine-45 and serine-123 could each serve as an acceptor in the hydrogen bonds with N\(^1\) and N\(^7\) needed for stabilization of the substrate-enzyme complex. Cyclic formycin-2',3'-phosphate exists in the anti-conformation and cannot form these hydrogen bonds and is not a substrate for the hydrolysis reaction. The proposed hydrogen-bonding scheme for binding of substrates containing cytidine, uridine, or formycin with ribonuclease \(A\) is shown in Fig. 1.

If it is assumed that the serine-123 hydrogen bonds are essential for binding of substrate, then replacement of serine-123 in ribonuclease by an amino acid residue that can serve only as a hydrogen donor should lead to an enzyme that is specific for uridine, and replacement by a residue that can serve only as a hydrogen acceptor should lead to specificity for cytidine or formycin. Replacement by a residue that can serve neither as a donor nor an acceptor should produce an enzyme that does not bind substrates containing any of these bases and there should be no catalytic action on them. Similarly, if threonine-45 were replaced by a hydrogen donor at its \(\beta\) carbon the enzyme should be specific for cytidine, and replacement by a residue that can serve only as a hydrogen acceptor should lead to specificity for uridine or formycin. Again, replacement by a non-hydrogen-bonding residue should give an inactive enzyme. If the postulated hydrogen bonds play only a supplementary role in the binding, only a partial effect on the observed activity would be expected.

\(O\)-Methyl-L-serine provides a selective analog for serine which can serve as a proton acceptor but not as a proton donor. Although it is not isosteric with serine, there appears to be room at position 123 of ribonuclease to accommodate the added methyl group. The predicted hydrogen bonding arrangement for substrate containing cytidine, uridine, or formycin with ribonuclease containing serine-O-methyl ether (Ser(\(Me\))) at position 123 is shown in Fig. 2. Alanine provides an analog of serine which cannot participate as a proton acceptor or donor and its presence at residue 123 of ribonuclease should prevent hydrogen bond formation either with position 4 of cytidine or uridine or with position 7 of formycin (Fig. 2).

To test the role of serine-123 in the binding of substrates by ribonuclease we have made use of the observation (6, 7) that the synthetic COOH-terminal tetradecapeptide of ribonuclease will bind nonequivalently with RNase 1–118 and other shortened, inactive components of RNase to generate nearly full enzymatic activity. This system allows a relatively easy study of the role of residues near the COOH terminus of the enzyme by the synthesis of suitable analogs of the peptide, RNase 111–124. In the present study we have synthesized [Ser(\(Me\))\(^{123}\)]-RNase 111–124 and [Ala\(^{123}\)]-RNase 111–124, in addition to the natural Ser\(^{123}\) sequence, and have measured the enzymatic activity of the complexes formed with RNase 1–118 toward the substrates C > p, U > p, poly(C), poly(U), and poly(AF).
MATERIALS AND METHODS

All chemicals and solvents used were reagent grade. Dimethylformamide (Spectroquality, Matheson, Coleman, and Bell, East Rutherford, N. J.) was stored over Molecular Sieve type 4A (same supplier). Trifluoroacetic acid, dioxyspropylethylamine, pyridine, and N,N-dimethyl methyl ether and methylene chloride were redistilled prior to use. Boc-amino acids were purchased from Spino Division, Beckman Instruments, Inc., Palo Alto, Calif., Fox Chemical Co., Los Angeles, Calif., or Protein Research Foundation, Japan. Thin layer chromatographic behavior, melting points, and optical purity were checked prior to use. Boc-azide (tert-butyldiazocformate) was purchased from Pierce Chemical Co. Copoly(styrene-1/2-divinylbenzene resin (Bio-Beads S-X1, 200 to 400 mesh) and Aminex 50W-X4 (20 to 29 μm) were from Bio-Rad Laboratories, Richmond, Calif. Bovine pancreatic RNase A (RAF grade) and carboxypeptidase A (COADFP grade) were obtained from Worthington Biochemical Corp., Freehold, N. J., and pepsin (three times crystallized) was purchased from Pentex. Cyclic cystine 2,3,5-triphosphate (sodium salt) and cyclic uridine 2',3'-phosphate (sodium salt) were from Sigma Chemical Co. Polyuridylic acid (potassium salt) and polycytidylic acid (potassium salt) were from Schwarz-Mann. The polyribonucleotide copolymer of formycin and adenosine (1:1) was a gift from Dr. David Ward, Yale University, New Haven, Conn.

Melting points are uncorrected. Thin layer chromatography was performed with Analtech Silica Gel GF plates (0.25 mm); compounds were visualized with 254 nm light, or by spraying with ninhydrin in acetone followed by heating. Deprotection of Boc-amino acids is required before staining with ninhydrin. For that purpose dry thin layer chromatography plates were placed in a closed chamber containing a beaker of fresh, concentrated HCl. After 15 min of exposure to HCl fumes, the plates were air dried, sprayed with ninhydrin, and heated at 110 °C for 10 min. Routine amino acid analyses were performed on a Beckman-Spinco amino acid analyzer model 119; preparative column chromatography with stream division for ninhydrin detection on a Beckman-Spinco amino acid analyzer model 120B. Solid phase reactions were carried out on an automatic Beckman peptide synthesizer model 990.

RNase 1-118 was prepared as previously described by Lin (8). To avoid problems of determining dry weights of proteins and peptides, all quantities of RNase, RNase 1-118, and synthetic COOH-terminal peptides were determined from amino acid analyses. The mean of the molar ratios of all accurately measurable amino acids in the acid hydrolysate was used to calculate the concentration of the protein or peptide.

EXPERIMENTAL PROCEDURE AND RESULTS

N*-t-Butylxycarbonyl-N^4,4'-dimethyl-benzhydryl-L-asparagine-Asn(Mbh) was prepared from Z-Asn as described by König and Geiger (9). t-Asn(Mbh) (19.3 g, 0.054 mol) was suspended in 150 ml of 50% dioxane and Boc-azide (0.081 mol) was added. The pH was adjusted to 9.6 with 4 N NaOH and maintained at this pH with automatic titration equipment for 20 hours at room temperature. The reaction mixture was extracted with 100 ml of ether three times. A 1.0 M solution of citric acid was added to pH 3.1 (200 ml) which precipitated the Boc-Asn(Mbh). The solution was divided by addition of 500 ml of ethyl acetate. The organic phase was separated, and the aqueous phase was extracted with 100 ml of ethyl acetate three times. The 500 ml of organic phase were washed four times with 100 ml of 0.5 M sodium citrate buffer, pH 2.5. Each buffer wash was used to wash the combined 300-ml extractions of ethyl acetate. The combined organic phase was extracted with 60 ml of saturated NaCl solution and concentrated to a small volume. An excess of hexane was added to precipitate the product, which was filtered and dried under high vacuum over P2O5 for 20 hours at room temperature. Yield: 21.3 g (86.4%), m.p. 144.5–145.6°.

C14HsN202

Found: C 62.42, H 6.65, N 6.07

RF values from thin layer chromatography on silica gel (solvent: chloroform-methanol-acetic acid, 85:10:5); N*-Boc-Asn(Mbh), 0.71; Asn(Mbh), 0.21.

N*-t-Butylxycarbonyl-O-methyl-L-serine—O-Methyl-L-serine and Boc-O-methyl-L-serine were prepared from L-serine as described by Hodges and Merrifield (10). The methyl ether function was shown to be stable to the conditions used for acid hydrolysis of peptide bonds and to the acidic conditions used in solid phase peptide synthesis (10). O-Methyl-L-serine-containing peptides were hydrolyzed with 4 N methanesulfonic acid (11, 12).

O-Methylserine Determination by Amino Acid Analyzer—O-Methylserine may be determined quantitatively with the standard conditions of analysis (System B, Table I) where the methyl ether elutes in the identical position of threonine. To separate O-Methylserine from threonine, the incorporation of propanol in the first buffer was required (Fig. 3). However, to maintain the separation on the first six amino acids of the standard mixture, a combination of high temperature (76 °C), 75% propanol, and a lower pH of 2.90 was used (Figs. 3 and 4). These conditions, mainly the high concentration of propanol, worsened the separation of the remaining amino acids of the standard mixture, especially that of glycine and alanine. For this reason the high temperature and the first buffer containing alcohol were switched to 56 °C and pH 3.20 buffer without alcohol as soon as possible rather than introduce the second buffer as pH 4.25. The final

<table>
<thead>
<tr>
<th>A</th>
<th>For mixtures containing threonine</th>
<th>B</th>
<th>For mixtures without threonine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resin</td>
<td>Beckman AA15</td>
<td>Beckman AA15</td>
<td></td>
</tr>
<tr>
<td>Resin height</td>
<td>0.9 × 60 cm</td>
<td>0.9 × 60 cm</td>
<td></td>
</tr>
<tr>
<td>Column temperature</td>
<td>76 °C (40 min)</td>
<td>56 °C (190 min)</td>
<td></td>
</tr>
<tr>
<td>Buffer of (0.2 N sodium citrate)</td>
<td>pH 2.90 (40 min)</td>
<td>pH 3.49 (30 min)</td>
<td></td>
</tr>
<tr>
<td>1st</td>
<td>pH 3.20 (35 min)</td>
<td>pH 4.25 (120 min)</td>
<td></td>
</tr>
<tr>
<td>2nd</td>
<td>pH 3.49 (60 min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd</td>
<td>pH 4.25 (60 min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4th</td>
<td>pH 4.25 (60 min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow rate</td>
<td>65 ml/hr</td>
<td>65 ml/hr</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>35 ml/hr</td>
<td>35 ml/hr</td>
<td></td>
</tr>
</tbody>
</table>

*a Temperature change was instantaneous, with the use of an external Haake water bath for 76 °C and the analyzer bath for 56 °C.

† The pH 3.49 and 4.25 buffers were prepared by diluting commercial concentrates from Beckman. The pH 2.90 buffer was made by diluting the 3.49 concentrate, adding 1-propanol (75%, 5 ml) adjusted to pH 2.90 with concentrated HCl, and adjusting to the final volume. The pH 3.20 buffer was made in the same way but without added propanol.
Elution time (min)

FIG. 3. Effect of propanol on elution time of amino acids during ion exchange chromatography with 0.2 N sodium citrate buffer, pH 2.90, and a temperature of 76°. A column (0.9 X 60 cm) of Beckman AA15 on a Beckman-Spinco model 120B amino acid analyzer was used. The flow rate of buffer was 66 ml per hour.

FIG. 4. Effect of temperature on the elution time of amino acids during ion exchange chromatography with 0.2 N sodium citrate buffer, 10% propanol, pH 2.90. A column (0.9 X 60 cm) of Beckman AA15 on a Beckman-Spinco model 120B amino acid analyzer was used. The flow rate of the buffer was 66 ml per hour.

FIG. 5. Ion exchange chromatogram of an amino acid standard mixture containing serine-O-methyl ether (30 nmol of each amino acid) on a Beckman model 120B amino acid analyzer. The conditions are described in Table I.

Solid Phase Synthesis of Protected COOH-terminal Peptides of Ribonuclease A—The general procedures of the automated solid-phase method were followed (13-15) but with several changes in detail. The synthesis began with the attachment of the COOH-terminal amino acid, valine, to the resin support and continued with the stepwise addition of protected amino acids until the completed peptide was assembled on the resin. The peptides were then cleaved from the resin, deprotected, purified, and isolated as described below and in Fig. 6.

To 50 g of copoly(styrene-1%-divinylbenzene) resin in 300 ml of chloromethyl methyl ether was added 1.0 ml of SnCl4 in 50 ml of hexane and allowed to react for 1 hour at 0° as described previously (16). A substitution of 0.12 mmol of chlorine per g of resin was determined by Volhard titration (13). The chloromethyl resin (25 g, 3 meq) was esterified with 6 meq of the cesium salt of Boc-valine in 150 ml of dimethylformamide at 50° for 20 hours (17). The substitution of valine on the resin was determined by amino acid analysis of resin hydrolysates and found to be 0.12 mmol of valine per g of Boc-valine resin. This procedure avoided formation of quaternary ammonium sites and any carry over of unreacted chloromethyl sites.

Synthesis of Tetradecapeptides—Boc-amino acid resin (2.0 g, 0.24 mmol of valine) was placed in a small (100 ml) reaction vessel of the automated Beckman 990 peptide synthesizer. The instrument was programmed to perform the remainder of the synthesis automatically with the following reagents and conditions. All amino acids were protected on the alpha-amino position with the Boc-group and the following side chain blocking groups were used: Asp(OBzl), Glu(OBzl), Ser(Bzl), Tyr(Bzl), His(Tos), Asn(Mbh). The Boc groups were removed at each cycle of the synthesis by treatment for 22 min with 40 ml of a solution containing 25% TFA-10% anisole-65% CH2Cl2 (v/v). To avoid dilution of the TFA by solvent retained from the previous CH2Cl2 wash a 2-min preliminary wash with the above solvent was introduced. Following each deprotection step neutralization was carried out with 40 ml of 5% DEA in CH2Cl2.

The couplings were mediated with DCC in CH2Cl2. Boc-amino acid (0.72 mmol, 3-fold excess) in 6.5 ml of CH2Cl2 was
Peptide-resins were hydrolyzed in a mixture of 2 ml of 12 M HCl, 1 ml of acetic acid, and 1 ml of phenol (13), and free peptides were hydrolyzed in 6 M HCl, containing 1 drop of phenol, in evacuated, sealed tubes for 24 hours at 100°. This standard procedure of acid hydrolysis of peptide-resins was not applicable in the case of [Ser(Me)22]-RNase 116-124 peptide-resin due to the instability of the methyl ether of O-methylserine. The following procedure was developed.

Peptide-resins (10 to 20 mg) were suspended in a mixture of 0.5 ml of methanesulfonic acid, 1.2 ml of propionic acid, and 0.3 ml of water, and the tubes were cooled, evacuated, and sealed. They were heated at 110° for 22 hours, and then cooled before opening. Then resin was removed by filtration through glass wool in a Pasteur pipette into a 10-ml volumetric flask and washed with 2 ml of 3.5 N NaOH. The hydrolysate solution was made to volume with water and was now ready for amino acid analysis. The methanesulfonic acid can be used directly without additional purification. The results of acid hydrolysis of a nonapeptide resin ([Ser(Me)22]-RNase 116-124) with the methanesulfonic acid procedure versus the 6 N HCl-acetic acid-water procedure are shown in Table II. These data show that the two methanesulfonic acid-propionic acid-water procedure is as effective as the HCl-acetic acid-pheno1 procedure in removing peptide from the resin. In this procedure the hydrolysate can be placed on the amino acid analyzer column for analysis without the chloroform extractions and removal of solvent, as is required in the procedure with 6 N HCl-phenol-acetic acid.

Cleavage of Peptides from Resin—The protected peptide-resins were dried under high vacuum at 25° for 20 hours. To a Daiflon reaction vessel of the HF cleavage apparatus (Toho Co., Osaka, Japan) was added 1 g of resin and 7.5 ml of anisole as a scavenger for cations. The tube was cooled to -78° and 7.5 ml of HF were distilled into the reaction vessel. The temperature was raised to 0° for 60 min. The HF and the bulk of the anisole were then removed under vacuum below 0°, and the residual anisole and by-products were extracted with ether. The cleaved peptide was dissolved in 20 ml of TFA and filtered to remove resin. The TFA was evaporated and the peptide was dissolved in water and lyophilized.

Purification of Peptides—The crude, cleaved peptides (50 to 100 mg) were dissolved in 4.5 ml of pyridine acetate buffer (0.2 M in pyridine), pH 3.6, centrifuged to remove any insoluble material and then applied to an Aminex 50W-X4 (20 to 29 μ) column (1.9 × 85 cm) and eluted with a gradient generated using seven chambers of a Buchler Varigrad (Table III) at a flow rate of 60 ml per hour at 37°. This gradient was used in the separation of [Ser(Me)22]-RNase 111-124. All other peptides were separated with a gradient consisting only of Chambers 2 to 7. The column effluent was stream-divided with the Beckman 120B amino acid analyzer preparative system, and a rate of 6.0 ml per hour was used for the ninhydrin analytical system. Fractions of 0.6 ml were collected. Only one major peak was detected by the ninhydrin reaction in the purification of each peptide and it always represented more than 75% of the ninhydrin-positive material. The lyophilized fractions were submitted to gel filtration on Bio-Gel P-2 (100 to 200 mesh) column (2 × 60 cm) in 0.1 M acetic acid. The peptides purified in this manner were homogeneous on paper electrophoresis at pH 6.5 (pyridine-
To minimize this side reaction, the 2,6-dichlorobenzyl group is now being used routinely as the protecting group for tyrosine. The amino acid compositions of the other impurity peaks indicated the presence of the correct amino acid ratios with no evidence that they were deletion or addition peptides. There is reason to believe (19) that they also arose during the HF treatment rather than during the assembly of the peptide chain. Amino acid analyses of the purified synthetic COOH-terminal peptides are summarized in Table IV.

**Combination of Shortened RNase with Synthetic Peptides and Assay for RNase Activity**—The activity with cyclic cytidine 2',3'-phosphate or cyclic uridine 2',3'-phosphate was determined by a spectrophotometric method similar to that described by Crook et al. (20) and del Rosario and Hammes (21). For the reconstitution experiments, the shortened RNase component (RNase 1-118) and various synthetic peptides were dissolved in 0.02 M Tris-Cl buffer, pH 6.0 or 7.0, aliquots were taken for quantitative amino acid analysis, and the appropriate dilutions were made with 0.1 M Tris-Cl buffer containing 0.1 M NaCl at pH 6.0 or 7.0. To 50 μl of the RNase 1-118 were mixed 50 μl of solutions containing amounts of synthetic peptide (usually 0.2 to 20 eq). After about 30 min, a 25-μl aliquot of the mixture was added to the substrate solution in 0.1 M Tris-Cl buffer with 0.1 M NaCl, pH 6.0 or 7.0. The incubation time was not critical. Measurements of at least six different peptide to protein ratios in duplicate were carried out.

In the 1.5-ml cell of 1-cm path length, 25 μl of enzyme solution were added to 1 ml of substrate solution in the cell, and the solution was mixed by means of a mixing rod, whereas, with the 0.2-cm cell, 25 μl of enzyme solution were added to 400 μl of substrate solution in a test tube and the mixed solution drawn into the flow cell with a syringe. In both cases the recording was commenced within 15 sec from the addition of enzyme. The change in absorbance at 280 nm (due to the difference of extinction between substrate and product) with a constant slit width of 0.4 m was recorded on a Gilford recording spectrophotometer. The substrate solutions were prepared just prior to use, and showed negligible nonenzymatic hydrolysis during the time between preparation and completion of the kinetic runs. Similarly, RNase 1-118 itself showed no hydrolysis of either substrate. The substrate concentrations were determined spectrophotometrically using an extinction coefficient of 9420 M⁻¹ cm⁻¹ at 285.5 nm for cyclic uridine 2',3'-phosphate in 0.1 M Tris-HCl buffer with 0.1 M NaCl, pH 7.0, and 8700 M⁻¹ cm⁻¹ at 271 nm for cyclic cytidine 2',3'-phosphate in 0.1 M Tris-HCl buffer with 0.1 M NaCl, pH 6.0, 2017.

When the COOH-terminal tetradecapeptide of RNase [Ser(Me)²³]-RNase 111-124 was mixed with the inactive RNase 1-118, activity against cyclic cytidine 2',3'-phosphate, at pH 6.0, was almost fully regenerated (90% of the activity of native RNase) (6). This peptide-protein complex was again assayed against C> p as a control for the tetradecapeptide analogs used in this study and found to be 85% active. The maximum activity obtainable for the complex in the presence of excess [Ala²³]-RNase 111-124 or [Ser(Me)²³]-RNase 111-124 was 100% and 48%, respectively, relative to the complex containing the natural peptide sequence, [Ser²³]-RNase 111-124 (Table V).

When cyclic uridine 2',3'-phosphate was the substrate, [Ser²³]-RNase 111-124 and RNase 1-118 gave a maximum activity relative to native RNase A of 85% at pH 7.0. This level of activity was approached at a peptide to protein ratio of about 3 when the concentration of RNase 1-118 was 1.37 × 10⁻⁴ M (Kᵣ = 0.2 × 10⁻⁶ M⁻¹) (Fig. 8). Relative to the above complex, the maximum activities for [Ala²³]-RNase 111-124 and [Ser(Me)²³]-RNase 111-124 were 25% and 38% at pH 7.0 and 42% and 52% at pH 6.0 (Table V).

![Table III](http://www.jbc.org/)

**Table III.** Conditions for gradient elution of peptides from Aminex 50W resin

<table>
<thead>
<tr>
<th>Chamber</th>
<th>Pyridine acetate, b (0.2 M, pH 3.6)</th>
<th>Pyridine acetate, b (2.0 M, pH 5.1)</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>270</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>270</td>
<td>60</td>
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<tr>
<td>3</td>
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<td>4</td>
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<td>5</td>
<td>100</td>
<td>20</td>
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</tr>
<tr>
<td>6</td>
<td>50</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>265</td>
<td></td>
</tr>
</tbody>
</table>

* For use in a nine-chambered gradient maker (Buchler Vari-grad).

Relative to the above complex, the maximum activities for [Ala²³]-RNase 111-124 and [Ser(Me)²³]-RNase 111-124 were 25% and 38% at pH 7.0 and 42% and 52% at pH 6.0 (Table V).

![Fig. 7](http://www.jbc.org/)

**Fig. 7.** Chromatographic purification of synthetic [Ser(Me)²³]-RNase 111-124 tetradecapeptide on Aminex 50W-X4. The column, 1.9 × 85 cm, equilibrated with pyridine-acetate buffer (0.2 M in pyridine), pH 3.6, was eluted with the gradient indicated in Table III. The flow rate was 60 ml per hour and fractions were collected at 6-min intervals. The effluent was monitored by automatic ninhydrin analysis at 570 nm.

Acetic acid-water, 100:3:900, by volume) and pH 1.8 (formic acid-acetic acid-water, 1:3:45, by volume). High voltage electrophoresis was carried out at 60 volts per cm for 45 min. The peptides were visualized by ninhydrin spray and the Pauly reagent for the detection of histidine. The separation of [Ser(Me)²³]-RNase 111-124 on Aminex 50W-X4 is shown in Fig. 7. The gradient used consisted of the seven-chambered gradient shown in Table III and the pH profile as shown in Fig. 7. All fractions indicated were pooled and lyophilized. The yields of each fraction were determined by amino acid analysis of acid hydrolysates; Peak I, 0.16 μmol, 0.9%; Peak II, 12.4 μmol, 76.5%; Peak III, 0.64 μmol, 3.9%; Peak IV, 0.71 μmol, 4.3%; Peak V, 0.99 μmol, 6.1%; Peak VI, 1.36 μmol, 8.4%. Fraction VI was deficient in tyrosine; however, analysis of the acid hydroylase on a 0.3-cm column of Beckman PA35 resin at 87° with pH 7.0 buffer as described by Erickson and Merrifield (18) indicated that the peptide contained 1.36 μmol of 3-benzyltyrosine. The product arises during the HF cleavage of the peptide from the resin as a rearrangement product of O-benzyltyrosine (18).

To minimize this side reaction, the 2,6-dichlorobenzyl group is now being used routinely as the protecting group for tyrosine. The amino acid compositions of the other impurity peaks indicated the presence of the correct amino acid ratios with no evidence that they were deletion or addition peptides. There is reason to believe (19) that they also arose during the HF treatment rather than during the assembly of the peptide chain. Amino acid analyses of the purified synthetic COOH-terminal peptides are summarized in Table IV.

![Diagram](http://www.jbc.org/)

**Diagram**

- For use in a nine-chambered gradient maker (Buchler Vari-grad).

The molarity is the total pyridine concentration. The pH was adjusted with acetic acid.
### TABLE IV

Amino acid analyses of synthetic tetradecapeptides

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>[Ser(^{123})]-RNase 111-124</th>
<th>[Ser(Me)(^{123})]-RNase 111-124</th>
<th>[Ala(^{123})]-RNase 111-124</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val</td>
<td>2.98 (3)</td>
<td>3.04 (3)</td>
<td>3.01 (3)</td>
</tr>
<tr>
<td>Ser</td>
<td>0.89 (1)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ser(Me)</td>
<td>0 (0)</td>
<td>0.97 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ala</td>
<td>1.04 (1)</td>
<td>1.04 (1)</td>
<td>1.99 (2)</td>
</tr>
<tr>
<td>Asp</td>
<td>2.04 (2)</td>
<td>1.95 (2)</td>
<td>1.97 (2)</td>
</tr>
<tr>
<td>Phe</td>
<td>0.98 (1)</td>
<td>1.03 (1)</td>
<td>1.04 (1)</td>
</tr>
<tr>
<td>His</td>
<td>1.03 (1)</td>
<td>0.97 (1)</td>
<td>0.98 (1)</td>
</tr>
<tr>
<td>Pro</td>
<td>2.00 (2)</td>
<td>1.93 (2)</td>
<td>2.00 (2)</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.00 (1)</td>
<td>1.01 (1)</td>
<td>1.02 (1)</td>
</tr>
<tr>
<td>Gly</td>
<td>1.06 (1)</td>
<td>1.04 (1)</td>
<td>1.06 (1)</td>
</tr>
<tr>
<td>Glu</td>
<td>0.98 (1)</td>
<td>1.01 (1)</td>
<td>1.05 (1)</td>
</tr>
</tbody>
</table>

* Peptides containing Ser(Me) were hydrolyzed in 4 N methanesulfonic acid for 22 hours at 110° in sealed tubes. The mixture was cooled, neutralized with an equal volume of 0.5 N NaOH and, made to volume with water, and amino acid analysis was performed as described in Table I. Ser\(^{123}\)- and Ala\(^{123}\)-containing peptides were hydrolyzed in 6 N HCl containing one drop of phenol for 22 hours at 110°.

### TABLE V

Activity, dissociation constants, and Michaelis constants for peptide-protein complexes

<table>
<thead>
<tr>
<th>Components</th>
<th>C&lt;sub&gt;DP&lt;/sub&gt;, pH 6.0</th>
<th>C&lt;sub&gt;DP&lt;/sub&gt;, pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide</td>
<td>Protein</td>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(mM)</td>
</tr>
<tr>
<td>[Ser(^{123})]-RNase 111-124</td>
<td>RNase A</td>
<td>1.2</td>
</tr>
<tr>
<td>[Ser(Me)(^{123})]-RNase 111-124</td>
<td>RNase A</td>
<td>1.5</td>
</tr>
<tr>
<td>[Ala(^{123})]-RNase 111-124</td>
<td>RNase A</td>
<td>2.0</td>
</tr>
<tr>
<td>[Ser(^{123})]-RNase 111-124</td>
<td>RNase A</td>
<td>8.3</td>
</tr>
<tr>
<td>[Ser(Me)(^{123})]-RNase 111-124</td>
<td>RNase A</td>
<td>6.6</td>
</tr>
<tr>
<td>[Ala(^{123})]-RNase 111-124</td>
<td>RNase A</td>
<td>4.3</td>
</tr>
</tbody>
</table>

* In the determination of K<sub>d</sub>, K<sub>m</sub>, or A<sub>max</sub> of a complex containing a peptide analog, the complex containing the natural peptide sequence, [Ser\(^{123}\)]-RNase 111-124, was always assayed as a control.

### Michaelis Constants and Dissociation Constants

The dissociation constant (K<sub>d</sub>) for the peptide-protein complex and the maximum regenerable activity (A<sub>max</sub>) were obtained graphically by the method of Berger and Levit (22). Activity (in absorbance change per min) was determined for various concentrations of peptide at a constant protein concentration. These activities were compared to the activity of RNase taken as 100% at an identical protein concentration. The data were then plotted using the equation:

\[
P_{0} \left( \frac{A_{\text{max}}}{A} - 1 \right) = [C]_{0} \left( 1 - \frac{A}{A_{\text{max}}} \right) + K_{d}
\]
where \([P_0]\) is total concentration of peptide from amino acid analysis; \([C_0]\), total concentration of protein from amino acid analysis; \(A\) and \(A_{max}\), the activity and the activity at saturation, respectively.

It should be emphasized that the dissociation constants obtained in this paper are for the peptide-protein complex in the presence of substrate. The results of these recombination experiments with the synthetic COOH-terminal peptides and RNase 1-118 are summarized in Table V.

The Michaelis constants \((K_m)\) and turnover numbers \((k_2)\) for the reconstituted ribonucleases, were determined for substrates cyclic cytidine 2',3'-phosphate and cyclic uridine 2',3'-phosphate and compared with the values for native RNase A. The concentration of U > p varied from 0.13 to 4.7 mM in the 1-cm cell and 1.5 to 18.2 mM in the 0.2-cm cell. The concentration of C > p varied from 0.2 to 1.5 mM in the 1-cm cell. The \(\Delta A_{286}\) was determined for seven different substrate concentrations and duplicate determinations were carried out at each substrate concentration.

The enzyme activity was determined spectrophotometrically with the complex between RNase 1-118 and [Ala\(^{23}\)]-RNase 111-124 was estimated to be 19, showing that the presence of alanine at position 123 introduced an additional factor of 3.9 in the selectivity of the enzyme for C > p.

The activity with poly AF was determined spectrophotometrically by observing the increase in ultraviolet absorption at 295 nm at 25° and a slit width of 0.3. In a 1.5-ml cell of 1-cm path length was added 1 ml of substrate solution (1 optical density unit at 257 nm) in 0.1 M Tris-HCl containing 0.1 M NaCl at pH 6.0 in a manner similar to that described for C > p and U > p assays. The recorder was set for a full scale absorbance of 0.100 unit. The concentration of enzyme was 0.17 × 10^{-8} M (1.27 pg per ml) in the reaction vessel. The activity of RNase 1-118 plus [Ser\(^{23}\)]-RNase 111-124 was taken as 100% and the activities of the other peptides were compared to this reference. The results are shown in Table VI.

The activity with poly(C) and poly(U) was determined spectrophotometrically by observing the increase in ultraviolet absorption at 285 nm for poly(U) at and 291 nm for poly(C) at 25° and a slit width of 0.6. In a 1.5-ml cell of 1-cm path length was added 1 ml of substrate solution (1 optical density unit at 257 nm) in 0.1 M Tris-HCl containing 0.1 M NaCl at pH 7.0. The substrates poly(U) (25 mg) or poly(C) (25 mg) were dissolved in 2 ml of the above buffer and 50 μl of these solutions were used for each assay. The peptide-protein complexes were prepared as described above and a 25-μl aliquot was added to the substrate solution. The RNase 1-118 concentration used was 10.7 × 10^{-9} μmol per ml for poly(U) and poly(C). The recorder was set for full scale absorbance of 0.1 and 0.8 unit for poly(U) and poly(C), respectively. The maximum activity of RNase 1-118 plus [Ser\(^{23}\)]-RNase 111-124 was taken as 100% and the activities of the other peptides were compared to this reference. Full activity was regenerated with the Ala\(^{23}\) analog when poly(C) and poly(AF) were substrates, but for poly(U) the activity was only 50% as high as for the natural Ser\(^{23}\) sequence. The complex of [Ser(Me)\(^{23}\)]-RNase 111-124 and RNase 1-118
was between 38 and 55% for all three polynucleotide substrates. The results are shown in Table VI.

**DISCUSSION**

The system of hydrogen bonds shown in Fig. 1 offered a reasonable mechanism for the interaction of specific substrates with pyrimidine binding sites of ribonuclease. It was based primarily on x-ray studies (1–3) and accounted in molecular and spatial terms for the known specificity of this enzyme. The data indicated a good hydrogen bond between the NH of threonine-45 and the carbonyl at C° of the pyrimidines. The N—H—O bond was nearly straight, although not in the plane of the pyrimidine ring. The x-ray evidence was also considered to be satisfactory for a hydrogen bond between threonine-45 serving as an acceptor for the N°—H of uracil or as a donor to the N° of cytosine. The bonds were not quite straight but were within acceptable limits, and the angle could be improved by slight rotation of the side chain of threonine-45 around the C°—C³ bond. On the other hand, the bond which might be formed between serine-123 and the NH₂ group in position 4 of cytosine was markedly nonlinear. Richards et al. (2) concluded that it would not be of appreciable strength and that substantial motion of serine-123 would be required to straighten this bond, but they pointed out that the serine residue is near the COOH-terminus of the molecule, in an area not well defined in the electron density map, so such a motion is possible although not yet proved. From an inspection of their data, the corresponding H² bond with uracil substrates appears to be better and may be of more importance.

Serine-123 cannot be essential to the catalytic mechanism of the enzyme since RNase S lacking valine-124 and serine-123 still has substantial catalytic activity (24). This means that serine can play only a supplementary role in binding substrate. We have found that the elimination of valine 121 and serine 123 from the synthetic tetradecapeptide prevented the generation of catalytic activity when the peptide was mixed with RNase 1–118. This loss appears to be associated with binding of the peptide to the protein.

Based on the work from Reich’s laboratory (5) the same functional groups on the enzyme can be invoked to explain the activity of ribonuclease toward polymers containing formycin. By superimposing the phosphate groups of poly nucleotides of formycin, cytidine, or uridine, it is possible to align the bases so that position 1 of formycin coincides with position 3 of the pyrimidines, and position 7 of formycin coincides with position 4 of the pyrimidines. The N—H and N°—H groups are both donors that can potentially form hydrogen bonds with the oxygen atoms of the threonine-45 and serine-123 side chains. Even though the N° of formycin could be an acceptor and the NH of the threonine amide is a potential donor, they assumed from models that this hydrogen bond would not be sterically favored and their scheme of hydrogen bonds for formycin contained only two such bonds rather than three as proposed for pyrimidine substrates. However, for discussion purposes, all three bonds are indicated in Fig. 1.

It is believed that substrates of ribonuclease can bind at several sites, including not only the phosphate, sugar, and base of the 3’ nucleotide adjacent to the bond being split but also at sites on other nucleotides. Therefore, the contribution of any one hydrogen bond to the total binding energy need not be large, and in general might be expected to contribute only in a quantitative rather than a qualitative way. Since the strength of a hydrogen bond depends critically on the precise alignment of the component atoms a considerable range of binding energies can also be expected. A scheme such as shown in Fig. 1 is, therefore, an oversimplification of the actual situation and cannot accurately predict the strength of substrate binding. Nonetheless, it does give a basis for planning experiments to test the general proposal and for evaluating the importance of the hydrogen bonds that it suggests.

One way to test the proposal is through the chemical synthesis of ribonuclease molecules in which certain residues are replaced by modified residues with different hydrogen bonding capabilities. At the present time the effort required to prepare the necessary number of analogs by total synthesis is excessively large. The synthetic approach to the problem can be greatly simplified by resorting to a semisynthetic enzyme in which a protein complex formed by noncovalent interaction of a natural protein component with a synthetic peptide component is studied. The S-peptide-S-protein complex constitutes one such system, and the complex formed from RNase 1–118 and the COOH-terminal tetradecapeptide, RNase 111–124, constitutes another. Fortunately, the latter system contains the serine-123 residue that is of interest in the present context.

**Ribonuclease catalyzes two reactions: a transphosphorylation reaction in which the 3′-5′-diester linkage of a dinucleotide or polynucleotide, is converted to a cyclic 2′,3′-phosphate with liberation of the hydroxyl at the 5′ position of the adjacent nucleotide, and a hydrolysis reaction in which pyrimidine cyclic nucleoside-2′,3′-phosphates are opened to the corresponding 3′-nucleotides. Since the strength of binding of these substrates with ribonuclease is different, the contribution of the proposed hydrogen bonds may be different, and it was necessary to test the effect of the reconstituted enzyme analogs both on the polynucleotides, poly(C), poly(U), and poly(AF), and on the cyclic phosphates, C> P and U> P.**

### Table VI

<table>
<thead>
<tr>
<th>Components of Complex</th>
<th>Poly AF</th>
<th>Poly C</th>
<th>Poly U</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide:Protein Molar Ratio</td>
<td>a_&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Peptide:Protein Molar Ratio</td>
<td>a_&lt;sub&gt;max&lt;/sub&gt;</td>
</tr>
<tr>
<td>[Ser&lt;sup&gt;123&lt;/sup&gt;]-RNase 111–124</td>
<td>RNase 1–119</td>
<td>26:1</td>
<td>100</td>
</tr>
<tr>
<td>[Ser(Me)&lt;sup&gt;123&lt;/sup&gt;]-RNase 111–124</td>
<td>RNase 1–119</td>
<td>24:1</td>
<td>38</td>
</tr>
<tr>
<td>[Ala&lt;sup&gt;123&lt;/sup&gt;]-RNase 111–124</td>
<td>RNase 1–119</td>
<td>28:1</td>
<td>101</td>
</tr>
</tbody>
</table>

* This is the maximum activity obtainable in the presence of excess peptide, relative to the complex containing the natural peptide sequence [Ser<sup>123</sup>]-RNase 111–124.

---

From the data presented in this paper several general conclusions can be drawn. It is clear that the substitution of O-methylserine or alanine for serine at position 123 of the tetradecapeptide causes a marked decrease in the specific activity of the resulting complex with RNase 1-118 when either U > p or poly(U) is the substrate. This observation fits the hypothesis presented in the introduction and in Figs. 1 and 2 that a hydrogen bond between serine-123 and the carbonyl oxygen at position 4 of uracil-containing substrates is important for binding of such substrates to the enzyme. The fact that the reconstituted enzymes were not completely inactive indicates that this interaction is not absolutely essential, but that it contributes significantly to the enzyme-substrate binding energy. The data show that the interaction is important both for substrates undergoing the transphosphorylation step (poly(U)) or the hydrolysis step (U > p).

The data with cytosine-containing substrates stand in rather sharp contrast to the results with uracil substrates. The presence of a hydrogen bond between position 123 of the enzyme and the amino group at position 4 of the cytosine ring was completely dispensable. Thus, with either C > p or poly C as substrate, the complexes of RNase 1-118 with [Ala]RNase 111-124 or [Ser]RNase 111-124 were equally active. These results do not agree with the hypothesis because the alanine residue could not form a hydrogen bond with cytosine and such a bond could not be contributing significantly to the binding energy of this enzyme-substrate interaction. It was initially predicted that serine-O-methyl ether residue at position 123 of the enzyme would be able to form a hydrogen bond with the amino group at C4 of cytosine and should be approximately as active as the enzyme with serine itself. The finding that the analog with Ser(Me) was only 47% as active cannot be explained on the basis of poor hydrogen bond formation since the results with the AlaRNase 111-124 showed that this bond is not required for full activity. The lower activity can probably be explained on sterics grounds. The presence of the extra bulk of a methyl group in place of a proton could interfere with both the binding of the peptide to the protein and the binding of the substrate to the peptide-protein complex.

The results with cytosine and uracil substrates can be rationalized in terms of the combined strength of all the hydrogen bonds involved. The assumption can be made that the total energy needed for adequate substrate binding will be equivalent to only two hydrogen bonds per pyrimidine. Since the bond with the carbonyl at position 2 is the same for both pyrimidines, the difference would appear to reside at position 3 of the rings. We would then have to postulate that the binding with the N7-H of uracil serving as a donor to Thr40 is quite weak, whereas the one with the Thr40 hydroxyl serving as a donor to N7 of the cytosine ring is strong. In the former case, an additional contribution from the H bond at position 4 would be needed while in the latter it would not. The data with formycin require a different explanation since only two hydrogen bonds have been proposed for substrate binding. The results with [Ala]RNase 111-124 (Table VI) have shown, however, that one of these is not necessary. Therefore, in the absence of further information we can rationalize the results by supposing only one strong bond, between the N7-H and the oxygen of the threonine-45 hydroxyl, is sufficient for binding this substrate. It must be recalled, however, that the formycin ring is only approximately isosteric with the pyrimidine rings of cytidine and uridine and that the potential hydrogen bonding groups are not in exactly the same relative positions. Thus it is possible that a reorientation of the ring could allow the formation of the hydrogen bond between the N7 at position 2 and the NH of the threonine backbone, making again a total of two effective bonds. We can also suggest that other binding energy is contributed by the larger aromatic ring system of formycin. In any case, the present data have shown that bond between the C- NH2 of formycin and serine-123 is not required.

The observed specific activities of the complexes are functions both of the binding of the peptide component with the protein component and of the binding of substrate with the resulting peptide-protein complex. Furthermore, the strength of peptide-protein binding depends on the presence of substrate. For that reason dissociation constants and Michaelis constants have been separately determined (Table V). Most instances the maximum regenerable activity decreases when the binding constant (Kd) for the peptide-protein complex decreases, but as expected there is not a strict quantitative correlation. With the assumption (23) that k2 >> k1, values for Kd have been taken as a measure of the strength of binding of the substrates to the peptide-protein complex. Thus, the observed Amax (25%) for the complex containing the AlaRNase with U > p at pH 7 can be attributed to a decrease in substrate binding (Kd increased 6-fold, whereas the k1 was not appreciably changed).

From the ratio of k2: Kd for C > p versus k1: Kd for U > p, it was shown that RNase A and the complex of [Ser]RNase 111-124 with RNase 1-118 have selectivities for the hydrolysis of C > p versus U > p of 4.6 to 5.0. The presence of alanine at position 123 in the complex introduced an additional factor of 3.9 in the hydrolysis step in favor of C > p. The Kd and k1 for the crucial chain-splitting transphosphorylation step have not yet been determined. However, the data under the conditions given here show that, with poly(C) as substrate, the complexes containing Ser and Ala are equally active, while with poly(U) as substrate the Ala-containing complex was only half as active, indicating that the alanine complex has some selectivity for cytosine-containing substrates, not only in the hydrolysis step, but also in the transphosphorylation step.

The purity of these synthetic tetradecapeptides is quite good and we have no reservations from this source in our interpretation of the data. The peptides were chromatographically and electrophoretically homogeneous and gave excellent amino acid analyses after acid hydrolysis. The Aminex ion exchange column is very selective as judged by the clean separation of six peptides derived from the [SerMe]RNase 111-124 synthesis (Fig. 7). The flat base-line of this chromatogram permitted the detection of as little as 0.5% of peptide impurities. The main peak contained the desired peptide and accounted for 76% of the total peptide in the initial unpurified cleavage product. All five of the minor by-products were also tetradecapeptides, one of which was shown to contain the 3-benzyltyrosine derivative and thus differed from the parent peptide by only a single benzyl group. We believe that HF-catalyzed rearrangements of aspartic acid and glutamic acid residues are responsible for the remaining by-products (19). Progress has been made in the elimination of these side reactions.

In reaching the conclusions concerning the role of serine-123 in the substrate binding and specificity of ribonuclease we must remember that the data are derived from a semisynthetic enzyme complex rather than intact ribonuclease itself. It seems quite reasonable to extrapolate from the two-component enzyme to the native single chain enzyme because of the known similarities in specific activity and substrate specificity, for example the maximum activity obtained with the complex of [Ser]RNase 111-124 and RNase 1-118 was 85% of that for RNase A with
both substrates U\textsuperscript{>}P and C\textsuperscript{>}P and the $K_m$ values for the complex were $1.5 \times 10^{-3}$ M for C\textsuperscript{>}P, $1.6 \times 10^{-2}$ M for U\textsuperscript{>}P, pH 6.0, and $11 \times 10^{-3}$ M for U\textsuperscript{>}P, pH 7.0, compared to $1.2 \times 10^{-2}$ M, $0.86 \times 10^{-3}$ M, and $9.7 \times 10^{-3}$ M, respectively, for RNase A. However, there are no data to prove that the conformations are identical. X-ray data, such as those for RNase A and RNase S, showing that the COOH-terminal region of the RNase 1–118 + 111–124 complex is essentially identical with that of RNase A would allow the extrapolation to be made with greater confidence. The total synthesis of [Ala\textsuperscript{123}]-RNase 1–124 would be the most direct test. We can predict, from the data presented in this paper, that this enzyme would be more specific than RNase A for the cleavage of RNA at bonds adjacent to cytosine in preference to uracil.

Acknowledgments—We are grateful to Dr. David Ward, Yale University, for a generous gift of the polyribonucleotide copolymer of formycin and adenosine, and we wish to thank Miss Anita Bach for her assistance throughout this study.

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