Reactivation of des(119-124) Ribonuclease A by Mixture with Synthetic COOH-terminal Peptides; the Role of Phenylalanine-120*

Michael C. Lin, Bernd Gutte, Daniel G. Caldi, Stanford Moore, and R. B. Merrifield
From The Rockefeller University, New York, New York 10021

SUMMARY

Several peptides contained within the amino acid sequence -Glu-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp-Ala-Ser-Val-OH at the carboxyl end of ribonuclease A were synthesized in which phenylalanine-120 was replaced by leucine, isoleucine, or tryptophan: [Leu120]-RNase 111-124, [Ile120]-RNase 111-124, [Trp120]-RNase 111-124, [Leu120]-RNase 115-124, [Ile120]-RNase 115-124, [Trp120]-RNase 115-124. The peptides were examined for their ability to regenerate enzymatic activity when mixed with RNase 1-118 which had been prepared by enzymatic degradation of RNase A. A study of the dissociation constants of the peptide-protein complexes, of the Michaelis constants of the complexes with cyclic 2',3'-cytidylic acid, and of the inhibition constants with 2'-cytidylic acid led to the conclusion that Phe120 plays an important role in binding the peptide and protein and in aligning the catalytic site of the complex, but that it does not have a specific effect on binding of substrate.

There is abundant evidence that phenylalanine-120 plays an important role in the functioning of ribonuclease A. It is clear that it contributes to the stability of the protein and to the enzymatic activity (1). There is also evidence both from nuclear magnetic resonance (2) and x-ray experiments (3) that it may be important for binding of substrate. A further insight into the role of Phe120 should be possible through the total synthesis of suitable analogs of RNase (4), although this synthetic approach would be difficult. Fortunately the problem has been greatly simplified by the recent discovery (5) that the COOH-terminal tetradecapeptide of RNase can bind noncovalently to shortened RNase components with the generation of nearly full enzymatic activity. Small COOH-terminal peptides containing suitable replacements of the residues in this region of RNase can now be synthesized relatively easily and used to examine the role of a particular amino acid in the action of the enzyme. Several such peptide analogs of the natural sequence, -Glu-Gly-Asn-Pro-Tyr-Val-Pro-His-Phe-Asp-Ala-Ser-Val-OH, containing replacements of phenylalanine-120 by leucine, isoleucine, or tryptophan have been synthesized by the solid phase method and are reported here. The peptides were examined for their ability to reactivate partially degraded ribonuclease and for the effect of replacing the aromatic ring of phenylalanine by the aliphatic side chains of leucine or isoleucine or the heterocyclic indole nucleus of tryptophan on the dissociation constants of the resulting complexes.

Experimental Procedure and Results

Materials and Methods

The peptides and proteins were generally prepared (see (4, 6-8)) and tested by the methods described in the accompanying paper (9). Some of the cleaved, crude peptides were purified by free flow electrophoresis on an Elphor FF instrument (Brinkmann). The relative mobilities reported for paper electrophoresis were corrected for endosmotic flow. Tryptophan was determined after 18 hours of alkaline hydrolysis at 110°, according to the method of Hugli and Moore (10); since lysinoalanine was not a possible interferent the chromatographic analysis was performed with 0.35 M sodium citrate buffer, pH 5.28.

Solid-Phase Synthesis of Peptide-Resins

[Leu120]-Peptide-Resins—Boc-Val-resin, 3.00 g (0.27 mmole of valine per g) was placed in the reaction vessel (6) and the steps...

* This work was supported in part by Grants GM 07256 and A-1280 from the United States Public Health Service, and by funds from the Hoffmann-La Roche Foundation.

† Present address, Section of Membrane Regulation, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. 20014.

‡ Present address, Institut für Genetik der Universität Köln, Weyertal 121, Germany.

§ Supported in part by General Research Grant No. RR 05415-10 Subcontract No. 3 from the United States Public Health Service, under the University of Pennsylvania Medical Student Research Training Program

wise synthesis was carried out manually according to the general procedures described before (4, 9). However, in this synthesis N\textsuperscript{o}-Boc, N\textsuperscript{\text{im}}-tosylhistidine was used instead of Boc-histidine (imidazole unprotected). At the stage of the decapptide-resin a 554-mg sample was removed. The remainder was extended to give the Boc-tetradecapeptide-resin containing the sequence Glu-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Leu-Asp-Ala-Ser-Val. The peptide yields (determined from amino acid analyses of acid hydrolysates) were 0.155 mmole per g of protected decapptide-resin and 0.143 mmole per g of protected tetradecapeptide resin. After correcting for the weight increase due to the peptide, the yields were calculated to be 71 and 70\%, respectively. This indicates that the loss of peptide from the resin largely occurred during the early stages of the synthesis and is in agreement with other recent syntheses (9).

[Hic\textsuperscript{120}]-Peptide-Resins—In an attempt to evaluate various protecting groups for histidine the [Hic\textsuperscript{120}]-tetradecapeptide was synthesized twice. In the first run His\textsuperscript{120} was introduced as N\textsuperscript{o}.Boc, N\textsuperscript{\text{im}}.DNP-histidine (11, 12) and in the second as N\textsuperscript{o}.Boc, N\textsuperscript{\text{im}}-tosylhistidine (13). The dinitrophenyl and tosyl groups could, therefore, be compared directly for coupling yields, purity of product, and convenience, and they could be indirectly compared with unprotected Boc-histidine that had been used in the synthesis of the [Phe\textsuperscript{120}]-peptides (5, 9).

The synthesis with Boc-His(DNP) began with 3.57 g of Boc-Val-resin containing 0.205 mmole of valine per g. Commercial Boc-his(DNP) was recrystallized from ethanol-petroleum ether before use (12). Since the im-DNP group is stable to IIF, it was removed by thiolysis (11) while the peptide was still attached to the resin (14). Thus, after coupling the last amino acid, Boc-Glu(OBzl), the peptide-resin was washed with DMF three times and was stirred for 30 min at room temperature in 20 ml of a 0.2 M solution of thiophenol (14) in DMF (approximately 10 molar excess). The resin was filtered and washed with DMF and CH\textsubscript{2}Cl\textsubscript{2} and the peptide was then α-deprotected with 50% trifluoroacetic acid-CH\textsubscript{2}Cl\textsubscript{2}. The partially protected tetradecapeptide-resin contained 0.123 mmole of peptide per g, which is equivalent to 0.164 mmole of peptide per g of polystyrene, or 78\% retention of peptide chains during the synthesis.

After this synthesis was completed Stahl and Fridkin (15) recommended the use of much larger (1000-fold) excess of thioldimercaptoethanol or mercaptoacetic acid to obtain quantitative reaction. In order to follow the progress of the thiolysis, they monitored spectrophotometrically. This general method has been found useful for determining the rate and optimal conditions for removal of the DNP group from protected peptide-resins. Thus, the thiolysis of [His(DNP)\textsuperscript{120}]-tetradecapeptide-resins was repeated with 10\textsuperscript{-4} M peptide and 10\textsuperscript{-3} M thiophenol in DMF at 25\° and the deprotection was monitored by continuous spectrophotometric measurement at 337 nm of the by-product, 2,4-dinitrophenyl phenyl sulfide. The reaction closely followed pseudo-first order kinetics (k \textsuperscript{1} = 1.27 \times 10\textsuperscript{-3} sec\textsuperscript{-1}), corresponding to a second order rate constant of 1.27 \times 10\textsuperscript{-1} m\textsuperscript{-2} sec\textsuperscript{-1}. As a control [tosylhistidine-119]-tetradecapeptide-resin was treated in the same way and no change in the absorption at 337 nm was found. It should be noted that the rate of thiolysis of a peptide-resin with mercaptoethanol was found to be very much slower than with thiophenol.

In order to establish the l\textsubscript{max} and the extinction coefficient in DMF a sample of 2,4-dinitrophenyl phenyl sulfide was prepared according to Bost et al. (10). Yield 79\%, m.p. 120–121\° (literature 121\°), l\textsubscript{max} = 337 nm, e\textsubscript{337} = 10,250.

C\textsubscript{13}H\textsubscript{16}O\textsubscript{6}N\textsubscript{8}S

Calculated: C 52.18, H 2.92, N 10.14
Found: C 51.91, H 3.05, N 10.00

From the rate data it can be calculated that the removal of the DNP group during the preparative run was 99.9\% complete within 5 min, assuming the same rate constant for all peptide chains on the resin. The actual experimental condition of 30 min, therefore, represented a large excess of time. The completion of the reaction was verified by amino acid analysis of an acid hydrolysate (during which DNP-histidine is less than 5\% destroyed (17)). Histidine recovery was essentially quantitative and no DNP-histidine was found at the position of the standard, indicating that the thiolysis with thiophenol left less than 1\% of histidine still protected. Hence, it may be concluded that a 10-fold molar excess of thiophenol is quite adequate for quantitative thiolysis in a reasonable period of time, provided that the concentration of the reagents is sufficiently high.

The synthesis with Boc-tosylhistidine began with 10 g of Boc-Val-resin and was used to prepare a series of peptides containing 5 to 14 residues. The deca-, dodeca-, and tetradecapeptides will be described here. The synthesis was the same as described above except for the use of the tosyl protection on histidine and, therefore, the omission of the thiolysis step. At the intermediate stages of the synthesis between the penta- and tetradecapeptides portions of the resin were removed for eventual work-up of the series. The amounts of peptide chains remaining on the resin at the 10. and 14-residue stages were estimated by amino acid analysis to be 78 and 65\%, respectively.

[Trp\textsuperscript{120}]-Peptide-Resins—Boc-Val-resin (1.5 g, 0.20 mmole of valine per g) was weighed into the reaction vessel and allowed to swell in CH\textsubscript{2}Cl\textsubscript{2} for 30 min. The synthesis was performed essentially as described for the [Leu\textsuperscript{120}]-peptides with the following modifications: (a) Boc-tryptophan (5-fold excess) was dissolved in 1 ml of DMF, diluted to 10 ml with CH\textsubscript{2}Cl\textsubscript{2}, and added to the resin. After 10 min, 10 ml of CH\textsubscript{2}Cl\textsubscript{2} containing a 5-fold excess of dicyclohexylcarbodiimide were added and the coupling reaction was continued for 5 hours. After Boc-tryptophan had been added to the peptide chain 1% mercaptoethanol was added to the deprotection reagent (20\% trifluoroacetic acid-CH\textsubscript{2}Cl\textsubscript{2}) for all subsequent deprotection steps to prevent acid-catalyzed oxidative decomposition of the indole nucleus (18, 19). Part of the peptide-resin (754 mg) was removed at the decapptide stage and the remaining amount was continued to the tetradecapeptide stage. After removal of the NH\textsubscript{2}-terminal blocking group, 942 mg of side chain-protected tetradecapeptide-resin were obtained. Small samples of the hepta- and tetradecapeptide-resins were hydrolyzed and after quantitative amino acid analysis were found to contain 0.133 and 0.106 mmole of peptide per g of peptide-resin, respectively. This corresponds to a 51\% yield for the heptapeptide and a 44\% yield of the tetradecapeptide based on the original amount of valine attached to the resin. The reason for the greater loss of the peptide chain from the resin during the synthesis of the [Trp\textsuperscript{120}] series compared with that found for the [Phe\textsuperscript{120}], [Leu\textsuperscript{120}], and [Hic\textsuperscript{120}] series has not been established, but it was determined on a model peptide that it was probably not due to the presence of mercaptoethanol during deprotection. Thus, Leu-Ala-Gly-Val-resin was treated for 1
and 6 hours in 20% tritfluoroacetic acid-CH₂Cl₂ with and without addition of 1% mercaptoethanol. The peptides released into the filtrates were hydrolyzed and the extent of cleavage was calculated from amino acid analyses. The rate was 1.3% per hour in the control and was exactly the same in the presence of mercaptoethanol.

**Cleavage of Peptides from Resin**

[Leu<sup>120</sup>]-Peptides—The protected decapeptide-resin (854 mg) was dried under high vacuum at 25° in a Dallion reaction vessel of the HF cleavage apparatus (Toho Co., Osaka, Japan). Anisole (1 ml) was added as a scavenger for cations and the tube was cooled to -78°. HF (10 ml) was distilled into the cold reaction vessel and the temperature was allowed to rise from 0-18° over a period of 80 min. The HF and anisole were then removed under vacuum and residual anisole and by-products were extracted with ether. The cleaved peptide was dissolved in 20 ml of trifluoroacetic acid and filtered from the resin. After the trifluoroacetic acid had been evaporated the peptide was treated with 20 ml of 2% sodium bicarbonate for 2 hours at pH 7.5 in order to reverse any N -> O acyl shift which might have occurred during the HF treatment. The material was lyophilized, dissolved in 6 ml of 0.05 M ammonium bicarbonate, and desalted by passage through a Sephadex G-25 column (2.8 x 40 cm) with 0.05 M ammonium bicarbonate eluant. The peptide fraction was lyophilized. The yield at the cleavage step was 80% for the [Leu<sup>120</sup>]-decapeptide. Paper electrophoresis (C in Fig. 1) in pyridine acetate (0.1 M in pyridine), pH 4.9, showed one main neutral component for the decapeptide at the same position as alanine and three small ninhydrin-positive spots (one cationic and two anionic). The [Leu<sup>120</sup>]-tetradecapeptide was cleaved in the same way in 61% yield. It has one net negative charge at pH 4.9 and showed a main component which moved toward the anode at \( R_{\text{Asp}} = 0.21 \). Several small contaminants were also present (A in Fig. 1).

[Ille<sup>120</sup>]-Peptides—The [Ille<sup>120</sup>]-tetradecapeptide which had been synthesized with DNP coverage of histidine was cleaved in HF as described for the [Leu<sup>120</sup>]-peptides. The final desalting was on a Sephadex G-15 column (2.5 x 35 cm) in 0.05 M ammonium bicarbonate and resulted in the separation of the colorless main peak at 235 ml from a yellow peak at 300 ml which appears to have been due to a by-product of DNP during the thiolysis step. The yield of the HF and Sephadex steps was 102 mg, 56% of the resin-bound peptide. The amino acid analysis of the crude tetradecapeptide showed: His 0.91(1), Asp 2.06(2), Ser 0.80(1), G1u 0.89(1), Pro 2.10(2), Gly 1.50(1), Ala 1.03(1), Val 3.00(3), Ile 0.88(1), Tyr 0.90(1). Silica gel thin layer chromatography in 1-butanol-pyridine-acetic acid-water (15:10:3:12) revealed a main spot of \( R_f 0.47 \) and two small contaminants at \( R_f 0.53 \) (5%) and \( R_f 0.56 \) (2%). Peptide resin of the [Ille<sup>120</sup>] serine made with tosyl coverage of histidine were each cleaved in HF and worked up as described for the leucine peptides. Since the \( N^\text{cm-tosyl} \) group is cleanly removed in HF, the separate thiolysis step required for \( N^\text{cm-DNP} \)-histidine could be avoided.

[Trp<sup>120</sup>]-Peptides—Protected [Trp<sup>120</sup>]-decapeptide-resin (754 mg) and protected [Trp<sup>120</sup>]-tetradecapeptide-resin (942 mg) were carefully dried in vacuo in separate reaction vessels of the HF cleavage apparatus. Anisole (3 ml) and L-tryptophan (300 mg) were added and 12 ml of HF were distilled into each vessel. The cleavage reaction proceeded for 60 min at 0° with stirring. After evaporation of HF and anisole and extraction with ether the peptides were dissolved in 20 ml of trifluoroacetic acid containing 0.5 ml of mercaptoethanol and filtered from the resin. The solutions were evaporated to dryness and the peptides were treated with 0.05 M ammonium bicarbonate, adjusted to pH 7.5, for 2 hours. After lyophilization the peptides were dissolved in 0.1 M acetic acid and passed through a Bio-Gel P-2 column (2.1 x 46 cm) in the same solvent. The desalted peptides were obtained in yields of 65 and 99 mg. This was 61% of the amount of resin-bound [Trp<sup>120</sup>]-decapeptide and 63% of the amount of resin-bound [Trp<sup>120</sup>]-tetradecapeptide.

**Purification of Peptides**

[Leu<sup>120</sup>]-RNase 115-124 Decapeptide—The crude cleaved decapeptide (30 mg) was dissolved in 1.7 ml of pyridine acetate (0.56 M in pyridine), pH 6.0, centrifuged to remove a small insoluble precipitate, then applied to a CM-cellulose column (1.5 x 12 cm) and eluted with the same buffer. Two peaks were detected by the ninhydrin reaction. After lyophilization they were obtained in the following yields: Peak I, 22.4 mg; Peak II, 2.0 mg. Paper electrophoresis showed the main component from Peak I to be neutral at pH 4.9 as expected, but the fraction still contained small amounts of anionic impurities. The material from CM-cellulose Peak I was dissolved in 1.3 ml of the pH 6.0 pyridine acetate buffer, applied to a Dowex 1 X2 column (2.1 x 16 cm) and eluted with the same buffer. Two well separated ninhydrin-positive peaks were obtained. The lyophilized fractions were submitted to gel filtration on Bio-Gel P-2 (100 to 200 mesh) in 0.1 M acetic acid. The lyophilized material from the main peak weighed 13.6 mg (45% of the crude decapeptide after HF cleavage) and was now homogeneous on paper electrophoresis at pH 4.9 (D in Fig. 1) and at pH 2.0 in 20% acetic acid, \( R_{\text{Asp}} = 0.50 \). It was also homogeneous by thin layer chromatography on Silica Gel G in 1-butanol-pyridine-acetic acid-water (30:30:16:24), \( R_f = 0.70 \). The material from the minor peak of the Dowex I column weighed 3.8 mg and was found to be a mixture of two negatively charged components.
The amino acid analysis of the purified [Leu] decapeptide was: His 1.07(1), Asp 1.09(1), Ser 1.00(1), Pro 1.05(1), Ala 1.00(1), Val 3.00(3), Leu 0.97(1), Trp 0.98(1).

**[Leu]**-RNase 111–124 Tetracapeptide—The crude tetracapeptide (150 mg) was dissolved in 2.5 ml of pyridine acetate (0.165 m in pyridine), pH 5.0, which had been diluted 1:6 with water and was then applied at the middle port (corresponding to tube 24) of the Elphor FT-1 free flow electrophoresis apparatus. The diluted buffer was pumped through the plates at a rate of 128 ml per hour. The buffer circulating through the electrode chambers was undiluted 0.165 m pyridine acetate, pH 5.0. The electrophoresis was run at 2000 volts, 130 ma, 10° for 3 hours. The 48 fractions between the anode and cathode were collected, and spectrophotometric analysis at 250 nm showed three peaks. The main component (Peak II) from tubes 16 to 22 weighed 53.4 mg. It was homogenous by paper electrophoresis (B in Fig. 1) at pH 4.9, \( R_{Asp} = 0.21 \); and at pH 2.0, \( R_{His} = 0.44 \), and also by thin layer chromatography on Silica Gel G in 1-butanol-pyridine-acetic acid-water. The amino acid analysis was: His 0.97(1), Asp 2.10(2), Ser 1.00(1), Glu 1.02(1), Pro 1.92(2), Gly 0.94(1), Ala 1.00(1), Val 3.11(3), Leu 0.97(1), Trp 0.80(1). The over-all yield of [Leu]-tetracapeptide from the original COOH-terminal Boc-Val-resin was 18%. Free flow electrophoresis Peak I (tubes 5 to 15) contained three anionic impurities, and Peak III (tubes 24 to 33) contained a mixture of a neutral component and a portion of the desired tetracapeptide isolated from Peak II.

**[His]**-RNase 111–191 Tetracapeptide—An 88 mg sample from Sephadex Peak I of the crude peptide made from DNP-protected histidine was dissolved in 3.5 ml of 1 N acetic acid which had been brought to pH 2.05 with formic acid. It was applied through Port Number 1 (corresponding to tube 7) of the free flow electrophoresis apparatus and separated in the same solvent at 1750 volts, 190 ma, 120 ml per hour buffer flow rate for 130 min. One main fraction with a peak at tube 17 was found, together with a shoulder. A narrow cut of only tubes 16, 17, and 18 was made. The lyophilized product weighed 28.6 mg. Paper electrophoresis in 20% acetic acid, pH 2, showed a single sharp spot at \( R_{His} = 0.39 \). Thin layer on Silica Gel G in 1-butanol-pyridine-acetic acid-water revealed a sharp spot at \( R_{F} = 0.47 \) and approximately 2% of a contaminant at \( R_{F} = 0.53 \). Amino acid analysis showed: His 1.00(1), Asp 2.14(2), Ser 0.62(1), Gly 1.02(1), Pro 2.17(2), Gly 1.07(1), Ala 1.00(1), Val 3.00(3), Leu 0.93(1), Trp 0.93(1). A small sample of the [His]-tetracapeptide which had been synthesized with tyrosylhistidine was purified in a similar way and had similar properties.

**[Trp]**-RNase 111–124 Tetracapeptide—A 16 mg sample of the crude tetracapeptide was chromatographed on a column, 2.1 x 15.5 cm, of DEAE-cellulose with pyridine acetate (0.56 m in pyridine), pH 6.0. Three ninhydrin-positive peaks were found. Paper electrophoresis indicated that the main peak was still contaminated by traces of a more anionic impurity and it was, therefore, rechromatographed on the same column to remove them. The contents of the main peak were lyophilized, pooled through a Bio-Gel 2 column in 0.1 m acetic acid. Lyophilization of the peptide components gave 2.4 mg from Peak I, 6.9 mg (53%) from Peak II, and only a trace of material from Peak III. The purified peptide was neutral by paper electrophoresis at pH 4.9, and in 20% acetic acid \( R_{His} = 0.52 \). The \( R_{F} \) was 0.61 by thin layer chromatography on Silica Gel G in the 1-butanol-pyridine-acetic acid-water system. Amino acid analysis: His 1.02(1), Asp 1.11(1), Ser 0.85(1), Pro 1.00(1), Ala 1.00(1), Val 3.05(8), Trp 0.78(1), Trp 0.99(1).

**[Trp]**-RNase 111–124 Tetracapeptide—A 13 mg sample of crude decapeptide was fractionated on a CM-cellulose column (2.1 x 17 cm) in 0.56 m pyridine acetate buffer, pH 6.0, as described for the [Leu]-peptide. Three ninhydrin-positive fractions were detected. They were each passed through a Bio-Gel 2 column in 0.1 m acetic acid. Lyophilization of the peptide components gave 2.4 mg from Peak I, 6.9 mg (53%) from Peak II, and only a trace of material from Peak III. The purified decapeptide was neutral by paper electrophoresis at pH 4.9, and in 20% acetic acid \( R_{His} = 0.52 \). The \( R_{F} \) was 0.61 by thin layer chromatography on Silica Gel G in the 1-butanol-pyridine-acetic acid-water system. Amino acid analysis: His 1.02(1), Asp 1.11(1), Ser 0.85(1), Pro 1.00(1), Ala 1.00(1), Val 3.05(8), Trp 0.78(1), Trp 0.94(1).

**Determination of Extent of Racemization of Histidine in Synthetic Peptides**

The recent discovery by Windridge and Jorgensen (20) of rather extensive racemization during the coupling of Boc-N-benzyl-L-histidine or Boc-L-histidine by a variety of activating reagents in solution or, especially, in solid-phase prompted us to examine and compare the amount of d-histidine present in the synthetic peptides described here. The method of Manning and Moore (21) was used in which the amino acids in acid hydrolysates of the peptides were converted to diastereomeric reagents in solution or, especially, in solid-phase prompted us to examine and compare the amount of D-histidine present in the synthetic peptides described here. The method of Manning and Moore (21) was used in which the amino acids in acid hydrolysates of the peptides were converted to diastereomeric mixtures by reaction with L-glutamic acid N-carboxyanhydride and then separated by ion exchange chromatography.

For example, 35 mg of [tosylhistidine][Ile]RNase 115–124 decapetide-resin were cleaved with HF, 1 hour, 0°, and the liberated peptide, after extraction with trifluoroacetic acid and evaporation, was hydrolyzed in a sealed, evacuated tube with 6 N HCl for 24 hours at 110°. The hydrolysate, containing approximately 5 μmoles of each amino acid, was treated with 10.5
ng (20% excess) of glutamic acid N-carboxyanhydride in a pH 10.2 borate buffer. The mixture of diastereomers was separated and analyzed on the Beckman model 120B amino acid analyzer on a column, 0.9 × 13 cm, of PA-35 resin with 0.35 M sodium citrate, pH 4.25, as the eluant. L-Glu-d-His emerged at 55 min and L-Glu-L-His at 110 min, and the other amino acid derivatives were well separated from these. As little as 2 nmoles of the L-D isomer could be detected in the presence of 2000 nmoles of the L-L isomer.

For a control to correct for racemization occurring during cleavage and hydrolysis, a mixture of L-histidine and Val-resin was treated with HF and hydrolyzed with 6 N HCl as before. The control contained 2.8% D isomer while the [His(DNP)]-decapeptide-resin made with tosyl histidine gave rise to 3.1% D-histidine, indicating that very little if any racemization (0.3%) occurred during the synthesis (Table I). Since more racemization is expected to occur during hydrolysis of peptides than of free amino acids, the control is not entirely adequate and the corrected number can be considered to be a maximum value. The accuracy of these measurements is estimated to be about 0.5%. The decapeptide-resin made with tosylhistidine, but containing phenylalnine at position 120 gave 3.5% D-histidine, or 0.7% racemization after correction for the hydrolysis control. The [Ile120]-decapeptide-resin, which had been synthesized with DNP-histidine, gave rise to 3.4% D-histidine after thiolysis, HF cleavage, and hydrolysis, showing that the DNP-derivative was also relatively resistant to racemization. In contrast, it was found that the [Phe120]-decapeptide-resin made with unprotected histidine gave rise to 51% of D isomer. This finding of approximately 2.3% racemization during coupling with histidine containing a free imidazole group is somewhat lower than that reported by Windridge and Jorgensen (20) for their model dipeptide but confirms their general conclusions. The results were well separated from these. As little as 2 nmoles of the DNP which reduces the basicity of the imidazole ring.

Combination of Shortened RNase with Synthetic Peptides and Assay for RNase Activity

The activity measurements were performed spectrophotometrically as described in the accompanying paper (9). The results obtained with the peptide-protein complexes were plotted by the method of Berger and Levit (22) as discussed previously (9), to obtain the dissociation constants, \( K_d \) (Table II) and the maximum regenerable activities, \( A_{\text{max}} \), which are expressed in Table III as percentage of the effect of an equimolar amount of native RNase A.

In the case of the tryptophan analogs, where the enzymatic activity was too low to obtain reliable values for the dissociation constant by the direct procedure, competitive binding experiments were used. Thus, the activity of a solution containing 1.23 × 10^{-4} M RNase 1-118, 8.8 × 10^{-4} M [Phe120]-RNase 115-124 (\( K_d = 1.5 × 10^{-3} \) M), and 10^{-3} M cyclic cytidylate in 0.1 M Tris-HCl, pH 6.0, was compared with similar solutions containing in addition either 9.9 × 10^{-4} or 19.8 × 10^{-4} M [Trp120]-RNase 111-124 tetradecapeptide. Taking the original activity as 1.00, the activities in the competition experiments were 0.95 and 0.66. From these data the dissociation constant for the complex between [Trp120]-RNase 111-124 and RNase 1-118 was calculated to be 3.5 × 10^{-3} M.

**Michaelis Constants and Inhibitor Dissociation Constants**—The Michaelis constants for the reconstituted ribonucleases, determined for the substrate cyclic 2',3'-cytidylate, were compared with the value for native RNase. The substrate concentration varied from 0.1 to 0.9 mM and the enzyme activity at pH 6 was calculated from spectrophotometric measurements.

### Table I

<table>
<thead>
<tr>
<th>Component</th>
<th>Racemization after acid hydrolysis</th>
<th>Racemization after acid hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine (control)</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>[His][119], [Phe][119]-RNase 115-124 decapetide-resin</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>[His(DNP)][119], [Ile][119]-RNase 115-124 decapetide-resin</td>
<td>5.1</td>
<td>5.1</td>
</tr>
<tr>
<td>[His][120], [Ile][120]-RNase 115-124 decapetide-resin</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>[His][120], [Phe][120]-RNase 115-124 decapetide-resin</td>
<td>3.1</td>
<td>3.1</td>
</tr>
</tbody>
</table>

### Table II

<table>
<thead>
<tr>
<th>Component of complex</th>
<th>( K_d )</th>
<th>( K_d ) for C2p</th>
<th>( K_d ) for C2p</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase A............</td>
<td>0.7 × 10^{-3}</td>
<td>0.9 × 10^{-3}</td>
<td>0.9 × 10^{-3}</td>
</tr>
<tr>
<td>[Phe120]-RNase 111-124 + RNase 1-118</td>
<td>2.0 × 10^{-7}</td>
<td>0.7 × 10^{-3}</td>
<td>0.6 × 10^{-3}</td>
</tr>
<tr>
<td>[Ile120]-RNase 111-124 + RNase 1-118</td>
<td>2.5 × 10^{-6}</td>
<td>1.0 × 10^{-4}</td>
<td>1.0 × 10^{-4}</td>
</tr>
<tr>
<td>[Ile120]-RNase 111-124 + RNase 1-118</td>
<td>4.0 × 10^{-9}</td>
<td>1.2 × 10^{-5}</td>
<td>1.1 × 10^{-5}</td>
</tr>
</tbody>
</table>

### Table III

<table>
<thead>
<tr>
<th>Component of complex</th>
<th>Maximum activitya</th>
<th>Maximum activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Phe120]-RNase 111-124</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>[Leu119]-RNase 111-124</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>[Ile120]-RNase 111-124</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>[Trp120]-RNase 111-124</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>[Phe120]-RNase 111-124</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>[Ile120]-RNase 111-124</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>[Ile120]-RNase 111-124</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>[Leu120]-RNase 111-124</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>[Ile120]-RNase 111-124</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>[Trp120]-RNase 111-124</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

a Maximum regenerable activity expressed as percentage of the effect of an equimolar amount of native RNase A.
at 286 nm. The data for the [Leu]$^{120}$- and [Ile]$^{130}$-tetradecapeptide complexes with RNase 1-118 are shown in Fig. 2.

The dissociation constants \( K_m \) for the inhibitor, 2'-cytidylic acid were determined from activity data in a similar way. The data from one experiment in which 2'-cytidylic acid was 5 \( \mu \)M and cyclic 2',3'-cytidylic acid ranged from 0.1 to 0.9 mM are shown in Fig. 3.

**Fig. 2.** Michaelis constant, \( K_m \) determination for RNase and reconstituted RNases. \( [S] \) is the concentration of cyclic cytidylic acid. The velocity, \( V \), has units of \( A_{286} \) per min. Enzymic activity was measured with substrate concentration 0.1 to 0.9 mM in Tris acetate buffer (0.1 M Tris), pH 6.0, with 0.1 M NaCl at 25°C. 1, native RNase A (0.36 pM); 2, RNase 1-118 (5 pM) plus [Leu]$^{120}$-RNase 111-124 (10.5 pM); 3, RNase 1-118 (3.1 pM) plus [Ile]$^{120}$-RNase 111-124 (30 pM).

**Fig. 3.** Dissociation constants, \( K_i \), for inhibitor, 2'-cytidylic acid. \( [S] \) and \( V \) are the same as defined in the legend to Fig. 2. The condition for assay is also the same as described. 1, obtained with native RNase A (0.36 pM); 2, with RNase A (0.36 pM) in the presence of 5 \( \mu \)M 2'-cytidylic acid; 3, with RNase 1-118 (5 \( \mu \)M) plus [Ile]$^{120}$-RNase 111-124 (57 \( \mu \)M); and 4, with RNase 1-118 (5 \( \mu \)M) plus [Ile]$^{120}$-RNase 111-124 (57 \( \mu \)M) in the presence of 5 \( \mu \)M 2'-cytidylic acid.

**DISCUSSION**

Several lines of evidence have indicated that Phe$^{120}$ plays a role in stabilizing ribonuclease. For example, the resistance to trypsin is markedly dependent on the presence of the phenylalanine residue. Native RNase is very resistant to trypsin digestion and RNase lacking the last four amino acids is only slightly susceptible to the enzyme. However, when Phe$^{120}$ is also removed RNase is extensively digested under the same conditions (1). These results indicated that when Phe$^{120}$ is removed native RNase loses its compact structure. Similar conclusions can be reached by examining the transition temperature of these proteins. The transition temperature, as measured by the change in ultraviolet absorption, is about 61°C for native RNase. When the COOH-terminal tetrapeptide is removed it decreases to 45°C and when Phe$^{120}$ is also cleaved it drops to 34°C. Likewise, it can be recalled that RNase 1-120 still retains low but definite enzymatic activity, while the further loss of Phe$^{120}$ removes all RNase activity (1). Although these results show a stabilizing role for Phe$^{120}$ and a dependence of enzymatic activity on its presence they do not indicate whether or not Phe$^{120}$ is involved in substrate binding.

The results of nuclear magnetic resonance (2) and x-ray crystallography (3) on RNase S- and RNase A-inhibitor complexes have focused attention on a possible role for the aromatic ring of Phe$^{120}$ as a part of the binding site for the pyrimidine ring of RNase substrates (23). In the x-ray model, the aromatic ring of Phe$^{120}$ appears to be close to the cytosine ring of 3'-cytidylic acid, although the planes of the two are clearly not parallel.

The nuclear magnetic resonance peak of an aromatic group on the enzyme (presumably that of Phe$^{120}$) showed an upfield shift when nucleotides were bound, whereas the pyrimidine C-6 and C-5 proton resonances moved downfield. This suggested (2) that these two aromatic rings in the enzyme-inhibitor complex interact with each other, but that they are not coplanar.

The synthetic approach was chosen to obtain further information about the function of Phe$^{120}$ and the newly discovered COOH-terminal peptide-protein system (5) was used for the experiments. Replacement of the Phe$^{120}$ residue in the COOH-terminal peptide of RNase by amino acids with hydrophobic, but nonaromatic, side chains was expected to answer the question of whether an aromatic ring at that position in the enzyme is essential for binding substrates and inhibitors and for preserving the biologically active conformation of the enzyme. For that reason leucine and isoleucine were chosen to replace phenylalanine in the synthetic COOH-terminal RNase peptides. In addition, tryptophan was selected as a bulky aromatic replacement.

It was found that the replacement analogs containing either leucine or isoleucine bound with decreased affinity to the shortened RNase proteins and generated correspondingly lower enzymatic activity (Tables II and III). Thus, the [Leu]$^{120}$-decapeptide and the [Leu]$^{120}$-tetradecapeptide combined with RNase 1-118 to give 10 and 15% maximum activity while the corresponding [Ile]$^{120}$-peptides gave 9 and 19% activity, respectively. These results are to be compared to 70 and 98% for the natural [Phe]$^{120}$-peptides. The calculated \( K_d \) for [Leu]$^{120}$-RNase 111-124 was 12 times greater than that for the natural [Phe]$^{120}$-sequence, and for [Ile]$^{120}$-RNase 111-124 it was 20 times larger. The aromatic side chain of Phe$^{120}$, therefore, is not essential for binding the peptides to the protein and can be replaced by hydro-
phobic aliphatic side chains, but it clearly makes a marked difference in the strength of the binding.

The main function of the amino acid side chain of residue 120 appears to be to fit into a hydrophobic pocket of the protein, and the benzene ring of phenylalanine is better than the isobutyl side chain of leucine or the sec-butyl chain of isoleucine. The proper fit of this residue contributes to the physical stability of the protein and probably serves to orient His119 in the most favorable position for catalysis. The effect of these substituents on the pK of the histidine is not yet known.

The relatively low activity generated with the [Leu<sup>140</sup>] and [Ile<sup>120</sup>]-tetradecapeptides and RNase 1-118 correlates with the diminished reactivity of His<sup>119</sup> in these peptide-protein complexes toward alkylation by iodoacetate. Under the alkylation conditions described earlier (9, 24), in which 0.7 residue of histidine 119 was alkylated in the complex between [PhelzO]-tetradecapeptide and RNase 1-118, only about 0.1 residue of N-carboxymethylhistidine is formed when leucine or isoleucine is substituted.

When the mixtures of [Trp<sup>120</sup>]-decapeptide or [Trp<sup>120</sup>]-tetradecapeptide with RNase 1-118 were assayed toward cyclic N<sub>1</sub>-carboxymethylhistidine is formed when leucine or isoleucine was present at position 120 (Table II). Although the indole side chain of tryptophan provided an aromatic ring, it was presumably too large to replace the benzene ring of phenylalanine in the hydrophobic pocket of RNase and therefore did not allow the catalytically active conformation to form. The main reason for the very low enzymatic activity of the [Trp<sup>120</sup>]-peptide-protein complexes is poor binding. The dissociation constant of 3.5 × 10<sup>-8</sup> M for the complex between [Trp<sup>120</sup>]-tetradecapeptide and RNase 1-118 indicates about 175 times weaker binding than in the corresponding complex with the natural [Phe<sup>120</sup>]-tetradecapeptide. The question of whether Phe<sup>120</sup> is involved in binding of substrate or inhibitor could be tested by examining the effect of replacement peptides on the Michaelis constants of the complexes with the substrate cyclic 2'-3' -cyclic acid. The K<sub>m</sub> value for complexes with the tetradecapeptides containing Phe<sup>120</sup>, Leu<sup>120</sup>, and Ile<sup>120</sup> were 0.7 × 10<sup>-9</sup>, 1.0 × 10<sup>-9</sup>, and 1.2 × 10<sup>-9</sup>, respectively, and for native RNase itself K<sub>m</sub> was 0.7 × 10<sup>-9</sup> (Table II). Since the values all fall within the experimental error of these measurements it can be concluded that the replacement of Phe<sup>120</sup> by Leu<sup>120</sup> or Ile<sup>120</sup> does not appreciably influence the binding of substrate. If residue 120 is involved in substrate binding it is a role which is not unique or specific for phenylalanine but one which can be assumed also by leucine or isoleucine. A similar conclusion was evident from the dissociation constants, K<sub>d</sub>, for the inhibitor 2'-cyclic acid when either phenylalanine or isoleucine was present at position 120 (Table II).

We conclude that Phe<sup>120</sup> is important for binding the peptide to the protein and for binding the COOH terminus of RNase A to the remainder of the enzyme, with a resulting increase in the stability of the molecule. Peptides in which Phe<sup>120</sup> is replaced by leucine, isoleucine, or tryptophan do not bind as tightly and, even when present in large excess relative to RNase 1-118, do not produce full activity. This may result from inexact alignment of the catalytically active site or from effects such as a slightly altered pK of His<sup>119</sup>, but in any case it appears that it is not due to a change in the strength of the binding of substrate.

Acknowledgments—We wish to thank Dr. William H. Stein for his continued interest and valuable discussions of this work. We also appreciate the excellent technical assistance of Miss Sandra Walsh, Miss Anita Bach, and Miss Joan Clarke.

REFERENCES

Reactivation of des(119–124) Ribonuclease A by Mixture with Synthetic COOH-terminal Peptides; the Role of Phenylalanine-120
Michael C. Lin, Bernd Gutte, Daniel G. Caldi, Stanford Moore and R. B. Merrifield


Access the most updated version of this article at http://www.jbc.org/content/247/15/4768

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/247/15/4768.full.html#ref-list-1