Studies on polypeptides XXXIII*. Semisynthetic Ribonuclease analogues. The role of histidine-119**

J. Serdijn, C. Hoes, J. Raap and K. E. T. Kerling

Gorlaeus Laboratories, Department of Organic Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands
(Received February 22nd, 1980)

Abstract. As part of a structure-activity relationship study of ribonuclease, focussing on the role of histidine-119 in the active centre, two tetradecapeptides were synthesized by the solid-phase method: [His[119]]RNase 111–124 and the corresponding analogue in which His-119 is replaced by L-homohistidine.
While the [Hhis[119]] analogue has almost the same binding capacity towards RNase 1–118 protein as the natural peptide, the resulting complex is devoid of any catalytic activity when RNA or 2',3'-CMP is used as substrate.

Introduction

In most mechanisms put forward for the RNase A catalyzed hydrolysis of phosphodiester bonds histidine residues at position 12 and 119 play an essential role. The RNase S' system of *Richards*[1,2] offers the opportunity to study, among other things, the role of histidine-12 in the active centre by synthesis of *S*-peptide analogues with well defined modifications in this residue[3-8].
RNase 1–118 protein obtained by proteolytic removal of the six *C*-terminal amino acid residues of RNase A is enzymatically inactive. *Merrifield* and coworkers found that on combining this inactive protein with a synthetic RNase 111–124 peptide, a non-covalent complex is formed with almost full enzymatic activity[9,10]. This feature makes it possible to study the specific functions of the amino acid residues near the carboxyl end of RNase A. The functions of Phe-120[11,12] and Ser-123[13] in the enzyme-substrate complex were investigated.
In order to investigate the role of active site histidine-119, we synthesized an RNase 111–124 analogue in which this residue is replaced by L-homohistidine, having an extra methylene group in the side chain. The pK_a^{im} of L-homohistidine does not differ significantly from that of histidine. We therefore expected that the complex formed between RNase 1–118 protein and [Hhis[119]]RNase 111–124 would yield information in particular on the steric requirements of the active site.

Results and discussion

On mixing [His[119]]RNase 111–124 with the RNase 1–118 protein, a high percentage of the RNase activity with yeast RNA (pH 5.0 and pH 7.0), as well as with 2',3'-CMP (pH 6.0) as substrate, was restored. [Hhis[119]]RNase 111–124 did not induce any RNase activity when mixed with RNase 1–118 protein (see Tab.I).
However, [Hhis[119]]RNase 111–124 was capable of competing with [His[119]]RNase 111–124 for binding to RNase 1–118. Only a 5- to 9-fold molar excess was required to give

* Part XXXII, *C. Hoes, J. Raap, W. Bloemhoff* and *K. E. T. Kerling*, Recl. Trav. Chim. Pays-Bas **99**, 99 (1980).

** Abbreviations: The abbreviations for the amino acids and protecting groups are those recommended by the IUPAC IUB Commission on Biochemical Nomenclature, Biochemistry **11**, 1726 (1972). Other abbreviations: RNase A, bovine pancreatic ribonuclease; RNA, ribonucleic acid; 2',3'-CMP, 2',3'-cytidine cyclic phosphate; 3'-CMP, 3'-cytidine monophosphate; BOC_2O, di-*tert*-butyl dicarbonate; DCC, dicyclohexylcabodiimide; DEA, diisopropylethylamine; DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazole; Hhis, 4-(4-imidazolyl)-L-2-aminobutyric acid (L-homohistidine).

1 *F. M. Richards*, Proc. Natl. Acad. Sci. U.S. **44**, 162 (1958).
2 *F. M. Richards* and *P. J. Vithayathil*, J. Biol. Chem. **234**, 1459 (1959).
3 *F. M. Finn* and *K. Hofman*, J. Am. Chem. Soc. **89**, 5298 (1967).
4 *B. M. Dunn, C. Dibello, K. L. Kirk, L. A. Cohen* and *I. M. Chaiken*, J. Biol. Chem. **249**, 6295 (1974).
5 *O. D. van Batenburg, J. Raap, K. E. T. Kerling* and *E. Havinga*, Recl. Trav. Chim. Pays-Bas **95**, 278 (1976).
6 *O. D. van Batenburg, K. E. T. Kerling* and *E. Havinga*, FEBS Lett. **68**, 228 (1976).
7 *O. D. van Batenburg, I. Voskuyl-Holtxamp, C. Schattenkerk, C. Hoes, K. E. T. Kerling* and *E. Havinga*, Bioch. J. **163**, 385 (1978).
8 *C. Hoes, P. Hoogerhout, W. Bloemhoff* and *K. E. T. Kerling*, Recl. Trav. Chim. Pays-Bas **98**(3), 137 (1979).
9 *M. C. Lin, B. Gutte, S. Moore* and *R. B. Merrifield*, J. Biol. Chem. **245**, 5169 (1970).
10 *B. Gutte, M. C. Lin, C. G. Caldi* and *R. B. Merrifield*, J. Biol. Chem. **247**, 4763 (1972).
11 *M. C. Lin, B. Gutte, D. G. Caldi, S. Moore* and *R. B. Merrifield*, J. Biol. Chem. **247**, 4768 (1972).
12 *R. S. Hodges* and *R. B. Merrifield*, Int. J. Pept. Prot. Res. **6**, 397 (1974).
13 *R. S. Hodges* and *R. B. Merrifield*, J. Biol. Chem. **250**, 1231 (1975).

Table I Effects of the replacement of His[119] on the binding constants of protein/peptide recombination complexes and their enzymatic activity (A^{max}) *relative to natural RNase A.*

Analogue	Substrate 0.075% RNA T 25.2 ± 0.1°C; pH 7.0			Substrate 0.075% RNA T 25.3 ± 0.1°C; pH 5.0			Substrate 0.48 mM 2',3'-CMP T 25.8 ± 0.1°C; pH 6.0		
	A^{max}	$K_b(M^{-1})$	$C_0(nM)$[a]	A^{max}	$K_b(M^{-1})$	$C_0(nM)$[a]	A^{max}	$K_b(M^{-1})$	$C_0(\mu M)$[a]
[His[119]]RNase 111–124	82%	4.3×10^8	6.57	82%	3.3×10^7	18.5	71%	2.0×10^6	0.38
[Hhis[119]]RNase 111–124	–	8.7×10^{7}[b]	19.8	–	6.6×10^{6}[b]	37.0	–	2.3×10^{5}[b]	1.1

[a] C_0 is concentration RNase 1–118.
[b] K_b for Hhis[119] RNase 111–124 calculated from the inhibition experiments.

a 50% inhibition of the activity of [His^{119}]RNase 111–124/RNase-118 complex (Figures 1, 2 and 3).

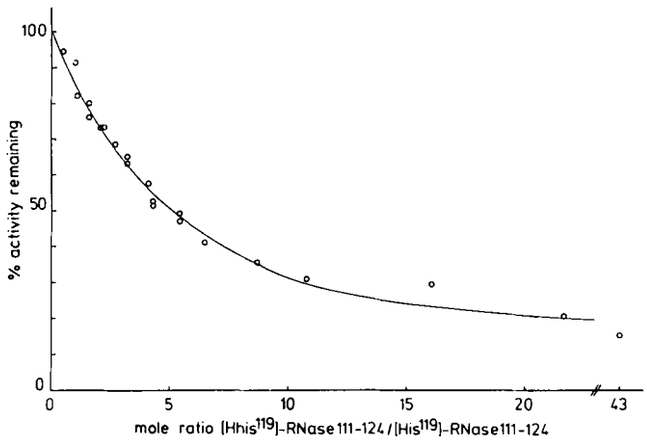

Fig. 1. Inhibition curve generated by addition of increasing amounts of [Hhis^{119}]RNase 111–124 to a 1.8 : 1 [His^{119}] 111–124/RNase 1–118 complex in the presence of yeast RNA at pH 7.0, T 25.0° ± 0.1°C.

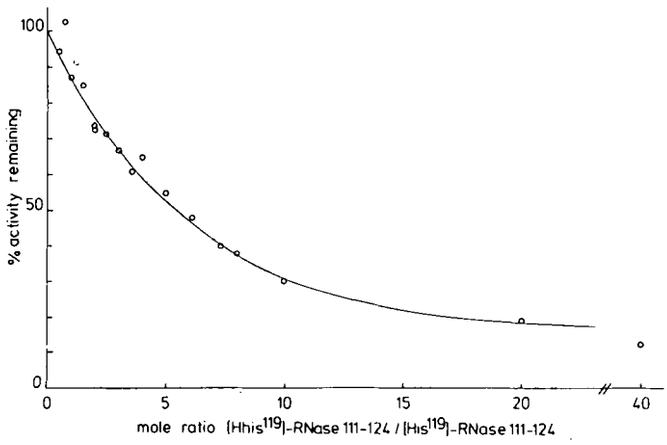

Fig. 2. Inhibition curve generated by addition of increasing amounts [Hhis^{119}]RNase 111–124 to a 4.9 : 1 [His^{119}]RNase 111–124/RNase 1–118 complex in the presence of yeast RNA at pH 5.0, T 25.1° ± 0.1°C.

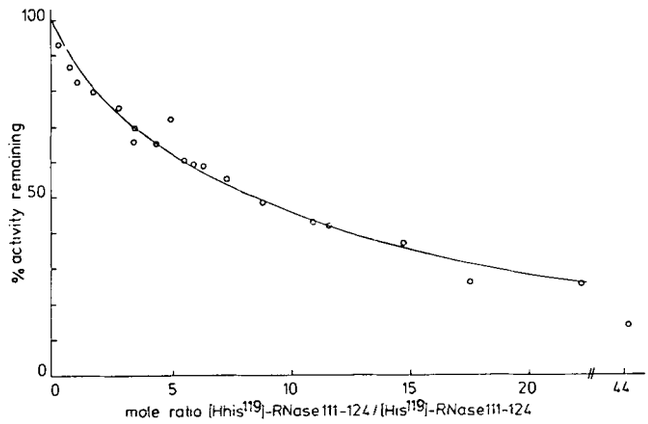

Fig. 3. Inhibition curve generated by the addition of increasing amounts of [Hhis^{119}]RNase 111–124 to a 7.0 : 1 [His^{119}]RNase 111–124/RNase 1–118 complex in the presence of 2',3'-CMP at pH 6.0, T 25.5° ± 0.1°C.

Evidently the binding tendency of the [Hhis^{119}] analogue to RNase 1–118 is only slightly weaker than that of the natural peptide.

We also investigated the capacity of the [Hhis^{119}]RNase 111–124/RNase 1–118 complex to bind substrate by titrating the complex with the inhibitor 3'-CMP. The titration curves for this complex and the natural one are presented in Fig. 4.

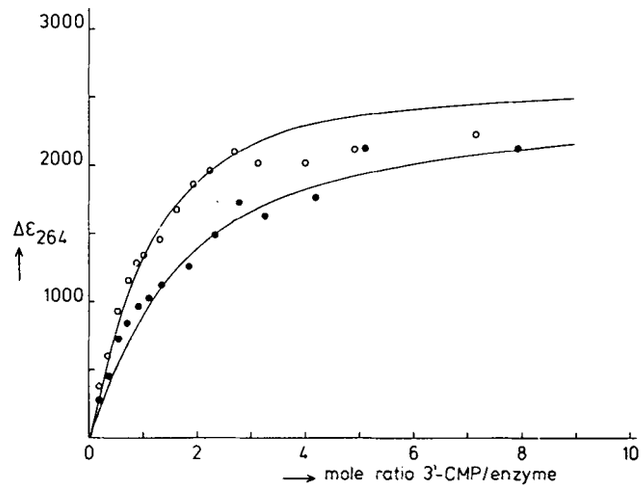

Fig. 4. Complex formation between 3'-CMP and: a) [His^{119}]RNase 111–124/RNase 1–118 (○) (conc. 4.3 × 10^{-5} M, molar ratio peptide/protein 2.3 : 1); b) [Hhis^{119}]-RNase 111–124/RNase 1–118 (●) (conc. 4.1 × 10^{-5} M, molar ratio peptide/protein 6.3 : 1) at pH 5.5, T 20.0° ± 0.1°C.

The results indicate that the binding characteristics of the Hhis^{119} analogue and the natural complex are comparable and of the same order of magnitude (see Tab. II).

Table II Binding constants and maximal extinction change at 264 nm for complexes of 3'-CMP with [His^{119}]- and [Hhis^{119}]-RNase 111–124/RNase 1–118 at pH 5.5, T 22.0 ± 0.1°C. Complex concentration 4.1 × 10^{-5} M.

Semisynthetic enzyme	K_{bind} (M^{-1})	$\Delta\varepsilon_{264}$ (M^{-1} cm^{-1})
[His^{119}]RNase 111–124/RNase 1–118	5.2 × 10^4	−2660
[Hhis^{119}]RNase 111–124/RNase 1–118	2.4 × 10^4	−2480

Evidently the environment of the cytidine chromophore in the two complexes is almost the same. Replacement of histidine-119 by homohistidine does not affect the geometry of the active site in that the substrate binding capacity of the peptide/protein complex is not appreciably lowered.

X-ray data have been interpreted to indicate that the imidazole group in histidine-119, unlike that in histidine-12, has a relatively high mobility[14]. The replacement of histidine-12 by a homohistidine residue[5] or a norhistidine residue[6] in the *S*-peptide/*S*-protein system produced enzymes with substantial activity, while the introduction of an extra methylene group into the side chain of histidine-119 destroys the enzymatic activity. With an extra methylene group in the side chain the arrangement for the catalytic reaction is not easily realized.

[14a] *F. M. Richards* and *H. W. Wijckoff*, The Enzymes, 3rd edn. Vol. 4, pp. 647–806 (1971).

[b] *F. W. Benz* and *G. C. K. Roberts*, Physico-Chemical Properties of Nucleic Acids (*J. Duchesne*, ed.), Vol. 3, pp. 77–138 (1973).

Although further data, obtainable by substituting other groups at position 119, are required for a meaningful discussion on this interesting phenomenon, a tentative explanation may be given, which yields a lead to further experimental investigations. From their calculations *Deakyne* and *Allen* conclude that Asp-121 has little importance in the transphosphorylation step of the reaction catalyzed by RNase A other than electrostatic stabilization of the positive charge on His-119. They point out that the distance between Asp-121 and His-119 makes the existence of a strong hydrogen bond unlikely[15]. However, in [Hhis^{119}]RNase analogue a strong linear hydrogen bond between the imidazole group of Hhis-119 and Asp-121 (or even Glu-111) is quite feasible, as can be deduced from inspection of a CPK model of RNase A. In this way the imidazolyl moiety will be kept away from the active centre, thus explaining the lack of enzymatic activity of the [Hhis^{119}] 111–124/RNase 1–118 complex.
Further investigations to test this supposition and to establish the essential function of His-119 in RNase activity are in progress.

Experimental section

Materials and methods

TFA, DEA, DMF, CH_2Cl_2 and anisole were redistilled prior to use. BOC_2O was purchased from Fluka AG, Buchs SG, Switzerland. BOC-Aminoacids were obtained from Serva Fein Biochemica, Heidelberg, Germany and Fluka AG, Buchs SG, Switzerland. Aminoacid derivatives were checked for homogeneity by chromatography on Silicagel GF_{254} (Merck), using the following solvent systems: I chloroform/methanol/acetic acid (85/10/5); II *n*-butanol/acetic acid/water (4/1/1); III *n*-butanol/acetic acid/pyridine/water (15/3/10/12); IV methanol. Melting points (uncorrected) were determined using a Büchi apparatus designed by Tottoli. Optical rotation was measured on a Perkin Elmer model 141 polarimeter. Chloromethylated copoly (styrene/1%-divinylbenzene) resin (Bio-Beads S-X1, 200–400 mesh, 0.75 meq Cl/g) was purchased from Bio Rad Laboratories, Richmond, California, USA. For the solid phase synthesis a Schwarz Bioresearch automated peptide synthesizer was used.
Paper chromatography (descending technique) was effected, using Whatman no 1 filter paper in *n*-butanol/acetic acid/pyridine/water (15/3/10/12) for 17 h. High-voltage paper electroforesis was carried out on a refrigerated Pherograph type Mini 65 on Mackery and Nagel paper 214 (analytical) and 827 (preparative); buffers pH 1.7 (acetic acid/formic acid/water 150/50/800), pH 4.8 (acetic acid/pyridine/water 3/4,1/993) and pH 6.4 (acetic acid/pyridine/water 4/100/896). Spots were visualized by Ninhydrin spray (Nh), Reindel Hoppe reagent (RH) after chlorination, Pauly reagent (P) and UV light (254 nm). High performance liquid chromatography (HPLC) columns were packed with Merck Lichrosorb 5 RP 8 and Merck Lichroprep RP 8 5–20 μ. RNase A (RAF grade), pepsin and carboxypeptidase A (COAPMS) were obtained from Worthington Biochemical Corporation, Freehold, New Jersey, USA. Agarose-5′-(4-aminophenylphosphoryl)-uridine 2′(3′)-phosphate was obtained from P.L. Biochemicals Inc. Milkauwee, Wisconsin, USA; Nojax no 28 dialysis tube from Sophyc, Levallois, France. SP-Sephadex C-25, Sephadex G 25 and G 50 were products from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Yeast RNA was obtained from Schwarz, New York, and purified prior to use[16], 2′,3′-CMP and 3′-CMP were from Sigma Chemical Company, St. Louis, USA. Concentrations of peptide and RNA solutions were calculated from the weight dissolved, concentrations of RNase A (ε_{278} 9800 M^{-1} cm^{-1}), RNase-1–118 (ε_{278} 9800 M^{-1} cm^{-1}), 2′,3′-CMP (ε_{284} 3680 and ε_{290} 1260 M^{-1} cm^{-1}) and 3′-CMP (ε_{271} 9400 M^{-1} cm^{-1}) were determined by measuring their absorbance.

Syntheses

BOC-Asn (Mbh) (**1**)

L-Asn (Mbh) was prepared from Z-Asn as described by *König* and *Geiger*[17]. From 0.030 mol L-Asn (Mbh) dissolved in 60 ml 1 N NaOH/DMF (1 : 1), BOC-Asn (Mbh) was prepared by adding 0.045 mol BOC_2O[18], with constant stirring at 45°C. The progress of the reaction was followed by TLC (system I). After 3 h the reaction mixture was worked up as described by *Merrifield*[13]. Yield 85%, homogeneous on TLC (systems I, II and III); m.p. 144–145°C; $[\alpha]_D^{20}$ +52.6 (*c* 2, methanol) (Lit.[13] m.p. 144.5–145°C).

BOC-Tyr(O-2,6-Cl_2Bzl) (**2**)

L-Tyr(O-2,6 Cl_2Bzl) was prepared from L-Tyrosine according to *Erickson* and *Merrifield*[19], BOC-Tyr(*O*-2,6-Cl_2Bzl) was prepared from L-Tyr(*O*-2,6-Cl_2Bzl) as described by *Schnabel*[20]. The product was homogeneous on TLC (systems I, II and III), m.p. 104°C; $[\alpha]_D^{20}$ +10.3 (*c* 2, methanol) and +20.2 (*c* 2, ethanol (Lit.[20] m.p. 108–110°C; $[\alpha]^{D2}$ +21 (*c* 2, ethanol).

BOC-Hhis(DNP) (**3**)

Prepared as described by *van Batenburg*[21,5] with one alteration. L-Hhis (1.7 g, 0.010 mol) was dissolved in 10 ml 1 N NaOH and 5 ml 1 M $NaHCO_3$. BOC_2O[18] (0.025 mol) in 10 ml *tert*-butanol was added at room temperature, with constant stirring. The progress of the reaction was followed by TLC (system III). The product was homogeneous on TLC (systems I and III), m.p. 163–166°C; $[\alpha]_D^{20}$ +5.1 (*c* 2, methanol) and +25.0 (*c* 0,9, ethyl acetate). (Lit.[5] m.p. 164–165°C; $[\alpha]_D^{22}$ +25.2 (*c* 0.9 ethyl acetate)).

[Hhis^{119}]RNase 111–124 (**4**)

The solid phase method of *Merrifield*[22,23] was chosen to synthesize the peptide. The chloromethylated resin (10 g, 7.5 meq Cl) was esterified to the extent of 0.35 mmol of BOC-Valine per gram, using potassium *tert*-butoxide and BOC-Valine in DMSO at 80°C for one hour, according to *M. W. Monahan* and *C. Gilon*[24]. The α-amino position of each amino acid was blocked with the BOC group, applying the side chain blocking groups: Asp(OBzl), Glu(OBzl), Ser(Bzl), Tyr(O-2,6-Cl_2Bzl), Hhis(DNP), Asn(Mbh). Starting with 1 g BOC amino acid resin, the synthesis was carried out according to the scheme described by *Hodges* and *Merrifield*[13] with the following alterations. The BOC groups were removed with TFA/anisole/CH_2Cl_2 (30/10/60 v/v). BOC-Hhis(DNP) was dissolved in DMF and the coupling of this residue was mediated with DCC/HOBt[25] (1/1). The completeness of each coupling step was controlled by means of the ninhydrin test of *Kaiser*[26].
After removal of the N^{im}-DNP group of the peptide with thiophenol[27], the tetradecapeptide resin was deprotected and cleaved with liquid HF (~ 15 ml, 1 h, 0°C), containing 1 ml anisole, according to *Hodges* and *Merrifield*[13]. Isolation of the peptide yielded about 300 mg crude product. The peptide was dissolved in 1% acetic acid, centrifuged to remove any insoluble material and desalted on Sephadex G25 (column 1.6 × 125 cm, 1% acetic acid, 22 ml/h). The fractions of the main peak were lyophilized, yielding 250 mg slightly yellow material.
Analysis of this crude peptide by HPLC on a RP 8 column showed one major and several minor components (Fig. 5a). Analytical paper electroforesis (1500 V) at pH 1.7, pH 4.8 and pH 6.4 also showed one major component (Nh^+, P^+) and several contaminants (Nh^+, P^+).

---

[15] *C. A. Deakyne* and *L. C. Allen*, J. Am. Chem. Soc. **101**, 3951 (1979).
[16] *W. A. Klee* and *F. M. Richards*, J. Biol. Chem. **229**, 489 (1957).
[17] *W. König, R. Geiger*, Chem. Ber. **103**, 2041 (1970).
[18] *V. F. Podznew*, Khim. Prir. Soed. **6**, 764 (1974); translated in English: Chem. of Nat. Compds. 782 (1977).
[19] *B. W. Erickson* and *R. B. Merrifield*, J. Am. Chem. Soc. **95**, 3750 (1973).
[20] *E. Schnabel*, Justus Liebigs Ann. Chem. **702**, 188 (1967).
[21] *O. D. van Batenburg* and *K. E. T. Kerling*, Int. J. Pept. Protein Res. **8**, 1 (1976).
[22] *R. B. Merrifield*, J. Am. Chsm. Soc. **85**, 2149 (1963).
[23] *R. B. Merrifield*, Adv. Enzymol. **32**, 221 (1969).
[24] *M. W. Monahan* and *C. Gilon*, Biopolymers **12**, 2513 (1973).
[25] *W. König* and *R. Geiger*, Chem. Ber. **103**, 788 (1970).
[26] *E. Kaiser, R. L. Colescott, C. D. Bossinger* and *P. L. Cook*, Anal. Biochem. **34**, 595 (1970).
[27] *G. R. Marshall, W. Vine* and *P. Needle*, Proc. Natl. Acad. Sci. U.S. **67**, 1624 (1970).

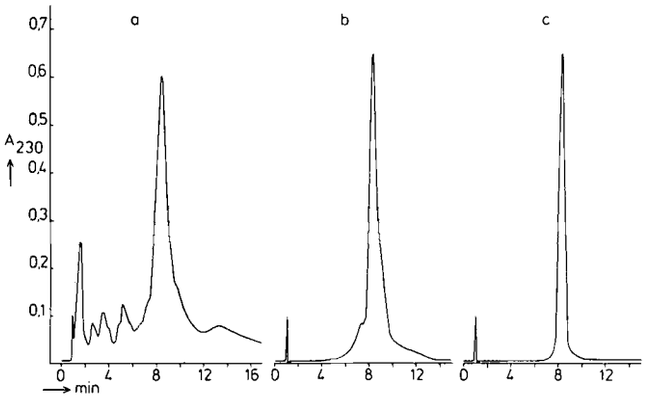

Fig. 5. Analysis of [Hhis[119]]RNase 111–124 by HPLC, in different stages of the purification.
Column: 10 × 0.46 cm 5 μm Lichrosorb RP8; mobile phase 20% acetonitrile/80% 0.005 M NH_4OOCH pH 3.9; flow rate 1.4 ml/min; UV detection at 230 nm. *a)* crude peptide (after G 25 Sephadex); *b)* peptide after preparative HPLC; *c)* peptide after preparative electrophoresis.

Since purification of the [Hhis[119]]RNase 111–124 on SP Sephadex C25, using several different elution systems, gave very poor results, we purified the peptide by preparative HPLC. The peptide was dissolved in 1 ml of an ammonium formate buffer (0.005 M, pH 3.9) and applied to a preparative RP 8 column (1.8 × 50 cm) in portions of 200 μl, eluent ammonium formate (0.005 M, pH 3.9)/acetonitrile 80/20 (v/v), flow 7.5 ml/min. The main peak, after evaporation of the acetonitrile, was lyophilized and analysed by HPLC (RP 8) (Fig. 5b). Analysis by paper electrophoresis showed a homogeneous product at pH 4.8 and pH 6.4. However, paper electrophoresis at pH 1.7 showed a major (~ 90%, Nh^+, P^+) and a minor (~ 10%, Nh^+, P^+) spot.
Preparative electrophoresis was carried out at pH 2.5 (3% acetic acid, 1300 V, 4 h). After drying, the paper was extracted with 3% acetic acid and the main component was lyophilized. The peptide was submitted to gel filtration on Sephadex G50 in 1% acetic acid (22 ml/h) and again lyophilized. Yield 55 mg (10%). The peptide thus purified proved to be homogeneous on HPLC (RP 8) (Fig. 5c), on paper electrophoresis at pH 1.7, pH 4.8 and pH 6.4 and on paper chromatography (BAPW, Rf 2.5 × His). Amino acid analysis: Asp 2.01 (2), Ser 0.96 (1), Glu 1.00 (1), Pro 2.08 (2) Gly 1.00 (1), Ala 0.96 (1), Val 3.02 (3), Tyr 0.96 (1), Phe 0.99 (1), Hhis 1.01 (1). Peptide content 93%.

[His[119]]RNase 111–124 (**5**)

Prepared and purified as (**4**), yield 60 mg (11%). The peptide was homogeneous on HPLC (RP 8), paper electrophoresis pH 1.7, pH 4.8 and pH 6.4 and paper chromatography (BAPW). Amino acid analysis: Asp 2.03 (2), Ser 0.96 (1), Glu-1.00 (1), Pro 2.06 (2), Gly 1.00 (1), Ala 0.98 (1), Val 3.04 (3), Tyr 0.96 (1), Phe 1.01 (1), His 0.97 (1) Peptide content 85.5%.

Preparation of RNase 1–118 (**6**)

Bovine pancreatic ribonuclease A was incubated with pepsin, as described by *Lin*[28]. The RNase 1–120 protein was purified on SP Sephadex C25 (column 1.6 × 51 cm), using a sodium phosphate buffer, 0.13 M pH 6.45, as eluent. The RNase 1–120 protein fractions were pooled and desalted by dialysis (48 h) followed by gel filtration on Sephadex G25 (column 1.6 × 125 cm) with 5% acetic acid as eluent.

To remove both His-119 and Phe-120, the RNase 1–120 protein was incubated with carboxypeptidase A[29]. The amount of liberated histidine and phenylalanine was detected by amino acid analysis.
After desalting and lyophilizing the RNase 1–118 protein was applied to agarose-5′-(4-aminophenylphosphoryl)-uridine 2′(3′)-phosphate[30,31] (column 0.6 × 15 cm) using ammonium acetate, 0.1 M pH 5.2, as eluent (18 ml/h), to remove any traces of RNase A. An acid hydrolysate had Phe 1.9 (2) and His 3.2 (3).

Enzymic Assay and substrate binding

The capacity of the two tetradecapeptides to combine with the RNase 1–118 and to restore enzymatic activity was determined with both yeast RNA (in 0.1 M NaOAc pH 5.0 and 0.05 M Tris HCl pH 7.0) and 2′,3′-CMP (in 0.05 M Tris/0.05 M NaOAc/0.1 M NaCl pH 6.0) as substrate.
To 10 μl of RNase 1–118, dissolved in buffer, on a spoon shaped rod, were added 10 μl of peptide solutions of varying concentrations (0.2–15 equivalents). After 10 minutes the mixtures were transferred into a 3 ml quartz cuvette (1 cm path length), containing 3 ml of 0.075% RNA solution. Using a Perkin Elmer 3 T spectrofotometer, (90–110% Transmittance scale), the change in transmittance at 297 nm was recorded. All solutions were freshly prepared prior to use.
With 2′,3′-CMP as substrate the same procedure was followed.
The increase in absorbance at 285 nm was measured using a Varian Cary 219 spectrofotometer (0.0–0.1 absorbance scale). The initial velocities were determined as described by *Crook*, *Mathias* and *Rabin*[31].
The capacity of the [Hhis[119]]RNase 111–124/RNase 1–118 complex to bind substrate was determined by titrating a solution of the complex with the inhibitor 3′-CMP. Using the method of *Hummel*[33], the binding constants between substrate and semisynthetic enzyme were determined from the negative maximum at 264 nm[34] in the UV difference spectra.
From the experimental data the best fitting curves and their parameters were determined by the method of *Waltz*[35].

Acknowledgements

Our sincere thanks are due to Prof. Dr. *E. Havinga* and Miss *C. Schattenkerk* for their continual interest and valuable discussions. We thank Dr. *B. Kraal* (Analytical Division of the Department of Biochemistry, Leiden) and Dr. *S. Visser* (The Netherlands Institute for Dairy Research (NIZO), Ede) for performing the amino acid analyses, Dr. *W. Bloemhoff* for technical assistance during the HPLC work and Mrs. *S. Amadio* for linguistic revision of the manuscript.

[28] *M. C. Lin*, *W. H. Stein* and *S. Moore*, J. Biol. Chem. **243**, 6167 (1968).
[29] *M. C. Lin*, J. Biol. Chem. **245**, 6726 (1970).
[30] *M. Wilchek* and *M. Gorecki*, Eur. J. Biochem. **11**, 461 (1969).
[31] *I. M. Chaiken* and *H. C. Taylor*, J. Biol. Chem. **251**, 2044 (1971).
[32] *E. M. Crook*, *A. P. Mathias* and *B. R. Rabin*, Biochem. J. **74**, 234 (1960).
[33] *J. P. Hummel*, *D. A. Verploeg* and *C. A. Nelson*, J. Biol. Chem. **236**, 3168 (1961).
[34] *M. J. Ettinger* and *C. H. W. Hirs*, Biochemistry **7**, 3374 (1968).
[35] *D. G. Anderson*, *G. G. Hammes* and *F. G. Walz*, Biochemistry **7**, 1637 (1968).