

Aspartic acid-121 functions at the active site of bovine pancreatic ribonuclease

Mary S. Stern and Marilyn S. Doscher*

Department of Biochemistry, Wayne State University School of Medicine, 540 East Canfield Street, Detroit, MI 48201, USA

Received 26 March 1984

The fully active semisynthetic enzyme formed by the non-covalent interaction of residues 1–118 of bovine pancreatic ribonuclease and a synthetic tetradecapeptide containing residues 111–124 of the enzyme has allowed a direct test of the role of aspartic acid-121 in the functioning of the molecule. Replacement of this residue by asparagine results in a derivative that is 4.5% active against cytidine 2',3'-cyclic phosphate at pH 7.0 under standard assay conditions. Further studies with the same substrate at pH 5.8 reveal that the reduced activity results entirely from a diminished catalytic efficiency and not from a decreased affinity for substrate.

<i>Bovine pancreatic ribonuclease</i>	<i>Active site</i>	<i>Semisynthetic enzyme</i>	<i>Chemical modification</i>
---------------------------------------	--------------------	-----------------------------	------------------------------

1. INTRODUCTION

Several lines of evidence have indicated that aspartic acid-121 functions at the active site of bovine pancreatic ribonuclease. Limited peptic digestion removes the 4 COOH-terminal residues of the molecule as a tetrapeptide, leaving a derivative, RNase 1–120, that exhibits only 0.5% of the activity of the native enzyme [1,2]. Although loss of Asp-121 is one feasible basis for the reduced activity, the results of alkylation studies [2,3] in-

troduced the possibility that a subtle conformational change might actually be the cause of inactivation in this derivative. More recently, authors in [4,5] detected by both X-ray and neutron diffraction a hydrogen bond between carboxyl OD1 of Asp-121 and nitrogen NE2 of histidine-119, a well-established and critical active site residue. In 35 species of mammalian ribonucleases Asp-121 is a residue that remains completely constant [6], a situation generally interpreted as indicating functional significance of some sort.

We report here an experimental test of the role of Asp-121 that has involved the preparation and characterization of a semisynthetic derivative of ribonuclease in which Asp-121 has been replaced by an asparagine residue. The semisynthetic system utilized was first developed and explored in [7,8]; this consists of residues 1–118 of the original molecule noncovalently complexed with a chemically synthesized tetradecapeptide comprised of residues 111–124 of the original molecule. The procedure is summarized in fig.1. The complex has a binding constant of $5 \times 10^6 \text{ M}^{-1}$ at pH 6.0 in the presence of substrate and exhibits 98% of the enzymatic activity of native ribonuclease [8].

* To whom correspondence should be addressed

Abbreviations: RNase 1–120, polypeptide composed of residues 1–120 of ribonuclease; RNase 1–118, polypeptide composed of residues 1–118 of ribonuclease; RNase 111–124, synthetic tetradecapeptide composed of residues 111–124 of ribonuclease; RNase 111–124 (Asn-121), synthetic tetradecapeptide composed of residues 111–124 of ribonuclease with Asp-121 replaced by Asn; RNase 1–118:111–124, the non-covalent complex of RNase 1–118 and RNase 111–124; RNase 1–118:111–124 (Asn-121), the non-covalent complex of RNase 1–118 and RNase 111–124 (Asn-121); Boc, *tert*-butoxycarbonyl

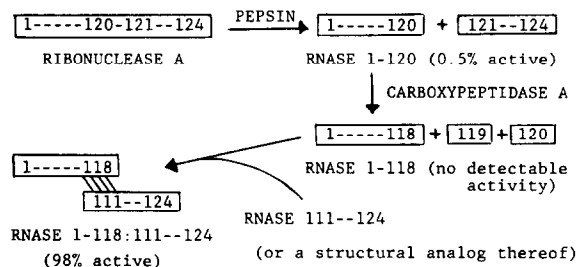


Fig.1. Scheme of semisynthetic ribonuclease system.

2. MATERIALS AND METHODS

RNase A (RAF OLA, salt-free) and pepsin (lot PM36c675, 2700 units/mg) were purchased from Worthington. Carboxypeptidase A (lot 45c8030, 57 units/mg) was from Sigma.

Amino acid intermediates were all obtained from Bachem. They included the α -Boc derivatives of valine, *O*-benzylserine, alanine, phenylalanine, *N*^{im}-DNP-histidine, proline, *O*-benzyltyrosine, glycine, γ -benzylglutamate and the *p*-nitrophenyl ester of asparagine.

Dicyclohexylcarbodiimide (Pierce), 4-dimethylaminopyridine (99%, Aldrich), trimethylamine (Eastman Organics) and trifluoroacetic acid (99%, Pierce) were used without further purification. Pyridine (Baker, AR) was distilled from ninhydrin (1 g/l) before use. Dichloromethane (certified ACS, Fisher) was stored over anhydrous CaCl_2 before use. *N,N*-Dimethylformamide (certified ACS, Fisher) was stored over type 4A molecular sieves (Davison) before use. All other reagents and solvents were the highest grade available and were used without further purification.

2.1. Preparation of RNase 1-118

RNase 1-118 was prepared by successive digestion of RNase A with pepsin and carboxypeptidase A as in [2,3,9]. Stock solutions of RNase 1-118 and of RNase 1-120 were characterized and standardized by amino acid analysis of acid hydrolysates (6 N HCl, 110°C, 22 h, in vacuo) of aliquot samples and were stored at -20°C. Amino acid analyses were performed with a Beckman amino acid analyzer. Specific activity against cytidine 2',3'-cyclic phosphate was determined, directly in the case of RNase 1-120 (product of pepsin digestion) and after the addition of a solu-

tion of RNase 111-124 in the case of RNase 1-118 [10]. At high mole ratios of RNase 111-124, the lot of RNase 1-118 used here exhibited 85% of the activity expected from a corresponding amount of RNase A.

2.2. Synthesis of RNase 111-124 (Asn-121)

The tetradecapeptide was prepared by the use of solid-phase peptide synthetic methods [11-13]. Commercial chloromethylated polystyrene-co-1% divinylbenzene resin (Lab Systems, lot PPMR-45K, 1.09 mmol Cl/g) was converted to the corresponding hydroxymethylated derivative as in [14,15]. Elemental analysis (Galbraith) indicated no Cl in the product. Reaction of a 15.4 g sample of this resin with 2.0 M excess of Boc-L-valine [15] provided a product containing 0.76 mmol valine/g. Acetylation of the remaining hydroxymethyl groups with pyridine-acetic anhydride (1:1, v/v) [16], was followed by standard cycles of deprotection, neutralization and DCC-mediated coupling (except in the case of Asn-121 and -113 where unmediated reaction of the *p*-nitrophenyl ester was utilized to provide the protected 14-residue chain) [13]. The completeness of each coupling step was monitored by the use of the Kaiser test [17]. Repeat coupling was required to complete the addition of Ser-123, Asn-121, Phe-120, His-119, Pro-117, and Asn-113. In the case of Ser-123 and Phe-120 the second coupling was followed by acetylation with pyridine-acetic anhydride (1:1, v/v) [16]. Removal of the *N*^{im}-DNP group from His-119 by thiolysis [18] was followed by acidolysis of the remaining protected groups as well as cleavage from the resin with HBr in trifluoroacetic acid [13]. The crude peptide was purified first by gel filtration in 0.15 M pyridine acetate (pH 5.5) on a 200 × 2.0 cm column of Sephadex G-25, 50-150 μm , followed by ion-exchange chromatography in a gradient of pyridine acetate buffers on a 70 × 2.0 cm column of SP-Sephadex C-25, 40-120 μm (fig.2). Stock solutions of bands A and B from the ion-exchange chromatography were characterized and standardized by amino acid analysis (see table 1) and stored at -20°C.

2.3. Activity determinations

Enzymatic activity was measured spectrophotometrically at 25°C as in [10] using cytidine

2',3'-cyclic phosphate (Schwarz-Mann, lot 616231) as the substrate. Maximal regenerable activity with a given substrate concentration was determined from a series of measurements with a fixed concentration of RNase 1-118 and progressively higher concentrations of tetradecapeptide. Extrapolation of a double-reciprocal plot of observed initial velocity vs mole ratio of peptide to RNase 1-118 provided the desired value (fig.3).

3. RESULTS AND DISCUSSION

Fractionation of the gel-filtered, crude RNase 111-124 (Asn-121) on SP-Sephadex provided two bands, A and B, both of which had amino acid compositions after acid hydrolysis corresponding to that expected for RNase 111-124 (Asn-121) (fig.2 and table 1). The maximal regenerable activity obtainable with band A or B was determined under standard assay conditions by measuring the initial rate of hydrolysis of cytidine 2',3'-cyclic phosphate at progressively higher mole ratios of the material in A or B to RNase 1-118 and extrapolating a double-reciprocal plot of the data to an infinite mole ratio (fig.3). Compared to the value obtained with the parent semisynthetic com-

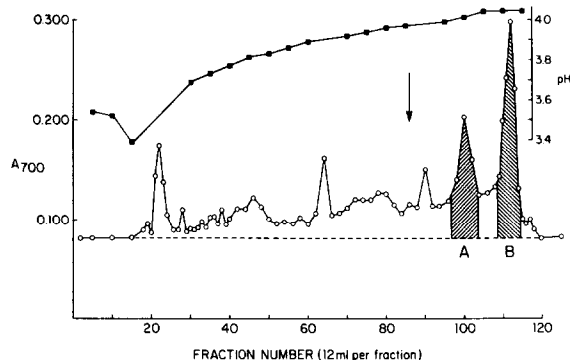


Fig.2. SP-Sephadex chromatography of gel-filtered RNase 111-124 (Asn-121). Column, 70 × 2.0 cm SP-Sephadex C-25 (40-120 μm); initial buffer, 550 ml of 0.05 M pyridine acetate (pH 3.55); limiting buffer, 550 ml of 0.25 M pyridine acetate (pH 4.08); at arrow, elution by undiluted limiting buffer began. Flow rate, 36 ml/h; temperature, 55°C. Folin-Lowry determinations on dried aliquots of fractions (○—○); pH of selected fractions (■—■). Amino acid analyses (see table 1) and assays of activity in the presence of RNase 1-118 (see text and fig.3) were performed on fraction pools A and B.

Table 1

Amino acid compositions^a of bands A and B from the ion-exchange chromatography of RNase 111-124 (Asn-121) on SP-Sephadex

Amino acid	Band A	Band B	Residues 111-124 of RNase ^b
Histidine	0.97	0.81	1
Aspartic acid	2.24	2.09	2 ^c
Serine	1.27	1.14	1
Glutamic acid	0.96	1.03	1
Proline	1.92	1.77	2
Glycine	1.09	1.09	1
Alanine	1.10	1.17	1
Valine	3.00	3.07	3
Tyrosine	0.87	0.86	1
Phenylalanine	0.89	0.90	1

^a Values were determined after 22 h hydrolysis and are given as molar ratios. No corrections for hydrolytic losses or incomplete hydrolysis have been applied

^b From [23]

^c In ribonuclease one residue is aspartic acid and the second is asparagine; in RNase 111-124 (Asn-121) both residues are asparagine

plex, i.e., RNase 1-118:111-124, band A generated 4.5% activity and band B, 4.8% activity.

The presence of more than one band of peptide material having an amino acid composition, after acid hydrolysis, corresponding to RNase 111-124 (Asn-121) reflects the presence of partially deblocked product in the material placed on the SP-Sephadex column. By analogy with effluent patterns from previous syntheses, band A should be the completely deblocked peptide. In any event, only one acid-labile side chain block, viz., the *O*-benzyl block on Ser-123, could conceivably affect the activity of the complex.

We may conclude, therefore, that substitution of Asp-121 by asparagine in RNase 1-118:111-124 has caused a substantial reduction in the rate of substrate turnover in this enzyme as measured under standard assay conditions.

The kinetic behavior of the complex formed by the material in band A and RNase 1-118 was further characterized by the measurement at pH 5.8 of the initial rates of hydrolysis of cytidine 2',3'-cyclic phosphate as a function of substrate

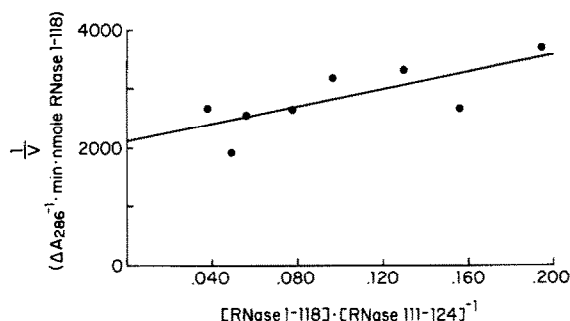


Fig.3. Reciprocal of the initial rate of hydrolysis of cytidine 2',3'-cyclic phosphate under standard assay conditions as a function of progressively higher mole ratios of RNase 111-124 to RNase 118 (mole ratio varied between 5.1 and 25.7). The concentration of RNase 1-118 was 0.181 μ M throughout the series. The points are the mean of 3 determinations and the line is the least-square analysis of the data.

concentration. These measurements revealed that the reduced activity results entirely from a diminished catalytic efficiency and not from a decreased affinity for substrate.

Characterization of the histidine proton NMR spectrum of the complex in a manner analogous to that recently reported for the parent RNase 1-118:111-124 [19] should assist in defining the immediate basis for the reduced activity. If suitable crystals can be grown, X-ray diffraction analysis will be applied, as it has been both to RNase 1-118:111-124 and the catalytically defective Leu-120 derivative [20-22].

ACKNOWLEDGEMENT

This work was supported in part by a grant-in-aid (NIH 05384-22) from Wayne State University School of Medicine.

REFERENCES

- [1] Anfinsen, C.B. (1956) *J. Biol. Chem.* 221, 405-412.
- [2] Lin, M.C. (1970) *J. Biol. Chem.* 245, 6726-6731.
- [3] Lin, M.C., Stein, W.H. and Moore, S. (1968) *J. Biol. Chem.* 243, 6167-6170.
- [4] Wlodawer, A. and Sjölin, L. (1981) *Proc. Natl. Acad. Sci. USA* 78, 2853-2855.
- [5] Wlodawer, A., Bott, R. and Sjölin, L. (1982) *J. Biol. Chem.* 257, 1325-1332.
- [6] Beintema, J.J. and Lenstra, J.A. (1982) in: *Macromolecular Sequences in Systematic and Evolutionary Biology* (Goodman, M. ed.) pp.43-73, Plenum, New York.
- [7] Lin, M.C., Gutte, B., Moore, S. and Merrifield, R.B. (1970) *J. Biol. Chem.* 245, 5169-5170.
- [8] Gutte, B., Lin, M.C., Caldi, D.G. and Merrifield, R.B. (1972) *J. Biol. Chem.* 247, 4763-4767.
- [9] Puett, D. (1972) *Biochemistry* 11, 1980-1990.
- [10] Murdock, A.L., Grist, K.L. and Hirs, C.H.W. (1966) *Arch. Biochem. Biophys.* 114, 375-390.
- [11] Merrifield, R.B. (1963) *J. Am. Chem. Soc.* 85, 2149-2154.
- [12] Doscher, M.S. (1977) *Methods Enzymol.* 47, 578-617.
- [13] Barany, G. and Merrifield, R.B. (1979) in: *The Peptides* (Gross, E. and Meienhofer, J. eds) vol.2, pp.1-284, Academic Press, New York.
- [14] Gisin, B.F. and Merrifield, R.B. (1972) *J. Am. Chem. Soc.* 94, 6165-6170.
- [15] Wang, S.S. (1975) *J. Org. Chem.* 40, 1235-1239.
- [16] Markley, L.D. and Dorman, L.C. (1970) *Tetrahedron Lett.* 1787-1790.
- [17] Kaiser, E., Colescott, R.L., Bossinger, C.D. and Cook, P.I. (1970) *Anal. Biochem.* 34, 595-598.
- [18] Shaltiel, S. and Fridkin, M. (1970) *Biochemistry* 9, 5122-5127.
- [19] Doscher, M.S., Martin, P.D. and Edwards, B.F.P. (1983) *Biochemistry* 22, 4125-4131.
- [20] Lin, M.C., Gutte, B., Caldi, D.G., Moore, S. and Merrifield, R.B. (1972) *J. Biol. Chem.* 247, 4768-4774.
- [21] Sasaki, D.M., Martin, P.D., Doscher, M.S. and Tsernoglou, D. (1979) *J. Mol. Biol.* 135, 301-304.
- [22] Doscher, M.S., Martin, P.D. and Edwards, B.F.P. (1983) *J. Mol. Biol.* 166, 685-687.
- [23] Wyckoff, H.W. and Richards, F.M. (1971) in: *The Enzymes* (Boyer, P.D. ed.) 3rd edn, vol.4, pp.647-806, Academic Press, New York.