Amino acid sequence and S–S bonds of *Penicillium brevicompactum* guanyl-specific ribonuclease

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The primary structure of *Penicillium brevicompactum* guanyl-specific RNase was determined. The enzyme consists of 102 amino acid residues, M_r 10801. The 4 cysteine residues of the RNase are linked in pairs by disulfide bonds: Cys^2–Cys^{10}, Cys^6–Cys^{101}. *P. brevicompactum* RNase structure is similar to RNase T_1; the degree of homology is 66%.

Ribonuclease Penicillium brevicompactum *Covalent structure*

1. INTRODUCTION

Penicillium brevicompactum RNase is specific towards the guanine base at the 3′-end of the hydrolysable 3′,5′-phosphodiester bond in the molecule of RNA [1]. The enzyme catalyses RNA cleavage by transphosphorylation yielding oligo- and mononucleotides with terminal 2′,3′-cyclophosphodiester groups which are then hydrolysed to the corresponding 3′-phosphates.

The pH dependence of the kinetic parameters for Guo-P-Cyt hydrolysis by *P. brevicompactum* RNase was shown to be identical with these dependences for RNase T_1 [2]. Moreover, a great similarity was found in the pH dependences of proton chemical shifts for histidine residues in the active sites of both RNases and their complexes with Guo-3′-P [2,3]. Therefore, the structure of the active sites must be very similar in the two RNases. That is why we decided to compare the complete amino acid sequences of *P. brevicompactum* RNase and RNase T_1.

2. MATERIALS AND METHODS

The work was done using reagents and solvents for the Edman degradation (Beckman) and phenyl[^{14}C]isothiocyanate (125 μCi; Amersham). RNase was isolated from the fungus *P. brevicompactum* [2].

Automatic Edman degradations were performed on a Beckman 890C sequenator according to the 0.1 M Quadrol program no.122974 with polybrene [4]. Proline N-terminal residues were split twice in the course of sequential analysis. The preparations were treated in the reaction cell of the sequenator with fluorescamine as in [5].

PTH-amino acid derivatives were identified by gas-liquid [6] and thin-layer chromatography [7] as well as by amino acid analysis after back hydrolysis of the derivatives with 5 N HI at 150°C for 4 h. Degradation products were converted in 1 N HCl [8].

Proteolysis of reduced and carboxymethylated RNase with staphylococcal protease V8, carboxypeptidase Y or trypsin was performed in 0.1 M ethylmorpholine–acetate buffer at pH 7.8 (9 h), 5.5 or 8.0 (4 h), respectively, at 37°C and an enzyme/substrate ratio of 1:50. In the latter case the protein was also succinylated. The kinetics of protein digestion with carboxypeptidase was followed by analysing aliquots of the reaction mixture on a Biotronik LC 7000 amino acid analyser.

The radioactivity was counted in a toluene-based scintillator on an Intertechnique SL 30 spectrometer.

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3. RESULTS AND DISCUSSION

The conventional method of determining the primary structure of proteins involves specific fragmentation of a polypeptide chain and isolation of overlapping individual peptides followed by the analysis of their structure and reconstruction of the original amino acid sequence. This procedure was used for determining the primary structure of some bacterial and fungal RNases [9–15].

In determining the primary structure of *P. brevicompactum* RNase, we eliminated the most difficult steps of peptide fractionation and purification. Automatic Edman degradation of the whole protein as well as of a mixture of its proteolytic fragments was used.

The primary structure of *P. brevicompactum* RNase is shown in fig.1. It is based on the results of studying the kinetics of RNase digestion with carboxypeptidase Y (residues 100–104) and on the data from automatic sequencing of *S*-carboxymethylated protein (residues 1–62), a mixture of two peptides from the tryptic digest of succinylated protein (residues 78–101), and a mixture of peptides produced by RNase digestion with staphylococcal protease (residues 59/60–78).

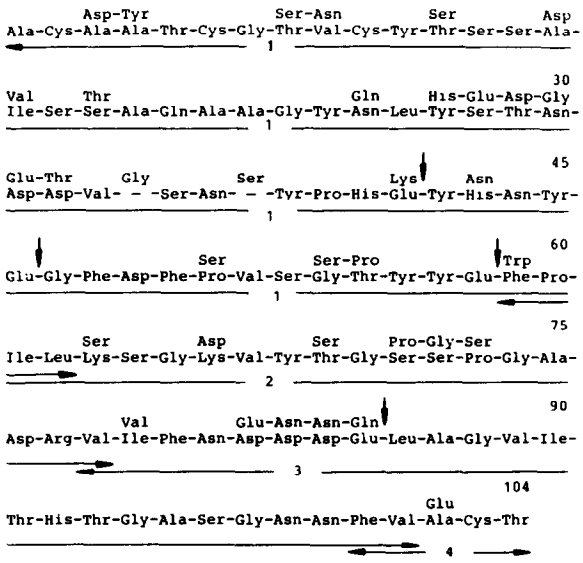

Fig.1. Amino acid sequence of *P. brevicompactum* RNase. Amino acid residues of RNase T_1 different from those of *P. brevicompactum* RNase are shown in the upper line. The residues are numbered according to the amino acid sequence of RNase T_1. (1–4) The structures determined in sequencing the *S*-carboxymethylated protein, the products of its proteolysis with staphylococcal protease and limited trypsinolysis as well as in analysing the kinetics of the protein digestion with carboxypeptidase Y. The sites at which RNase is digested with staphylococcal protease are indicated with arrows.

Some comments on the structure determination of the central RNase region (residues 59–78) must be made. RNase contains 4 Glu residues located at positions 41, 46, 58 and 85 of the polypeptide chain. The presence of Pro^{60} in the vicinity of Glu^{58} is typical of this protein segment structure. An equimolar mixture of amino acids (Ala, Tyr, Gly, Phe and Leu) was found in degradation products after RNase proteolysis with staphylococcal protease followed by one cycle of Edman degradation of the nonfractionated peptide digest; this finding indicates that RNase is cleaved in a highly specific manner at Glu residues (fig.1). In this step, the peptide mixture was treated with fluorescamine directly in the reaction cell of the sequenator. It eliminated all peptides containing primary α-NH_2 groups from the subsequent automatic degradation. The structure of the polypeptide chain in the region of residues 60–78 was then identified in the course of 19 successive cycles of degradation.

Thus, *P. brevicompactum* RNase contains 102 amino acid residues: Asp_7, Asn_7, Thr_9, Ser_{11}, Glu_4, Gln_1, Pro_4, Gly_{10}, Ala_{11}, Cys_4, Val_7, Ile_4, Leu_3, Tyr_9, Phe_5, Lys_2, His_3, Arg_1 (M_r 10801). The 4-Cys of the protein are linked in pairs by disulfide bonds. These data were confirmed by studying the automatic Edman degradation of intact nonmodified RNase.

The native RNase undergoes qualitatively 'normal' sequencing just as the *S*-alkylated protein, the only difference being that the yield of degradation products noticeably decreases, and cannot be identified at cycles 2, 6 and 10 using the techniques employed here (fig.2).

One complete cycle of automatic Edman degradation was carried out on the intact protein to localize S–S bonds in RNase. Phenyl[^{14}C]isothiocyanate was then added to the reaction cell at the coupling step of cycle 2 and, after washing the reagent off, the sequencing was continued according to the standard program. Analysis of fractions containing anilinothiazolinone amino acid derivatives split (fig.2) shows that the major part of the radioactively labeled material appears at cycle 10

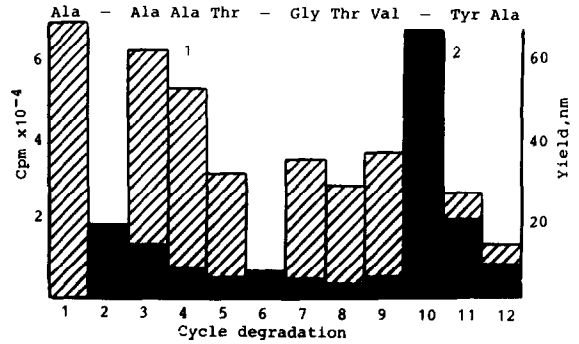

Fig.2. Analysis of products obtained by the Edman degradation of *P. brevicompactum* intact unmodified RNase. (1) Amino acid yield (nm), (2) radioactivity of fractions (1/10 part). [^{14}C]Phenylisothiocyanate was added at the second cycle of degradation.

of the stepwise protein degradation. This finding, as well as the absence of ^{14}C-labeled products at cycle 6, indicates unambiguously that Cys^{2} and Cys^{10} are linked with an S–S bond as are Cys^{6} and Cys^{101}.

Comparison of the primary structures of *P. brevicompactum* RNase and RNase T_1 shows that these two guanyl-specific enzymes are very similar. Taking account of two deletions in the amino acid sequence of *P. brevicompactum* RNase in positions 34 and 36, the maximal homology of the two RNases is 66%. Differences between the structures are consistent with the scheme of point mutations of structural genes which, to a first approximation, are uniformly distributed along the molecule. The following amino acid residues are invariant in the active sites of the two RNases: His^{40}, Glu^{58}, Arg^{77} and His^{92}.

We believe that the modified procedure used here for analysing amino acid sequences will become a potent tool for studying the primary structures of other microbial RNases.

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