An 8.5-kDa ribonuclease from the extreme thermophilic archaebacterium *Sulfolobus solfataricus*

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Abstract **Protein p3, a ribonuclease we previously isolated from the archaebacterium *Sulfolobus solfataricus* [P. Fusi et al. (1993) Eur. J. Biochem. 211, 305–310], was subjected to complete amino acid sequencing. It consisted of 75 residues, with a calculated M_r of 8582, a pI of 10.1, and had some degree of monomethylation at Lys-4 and Lys-6. p2, a previously sequenced, 62-residue ribonuclease from the same organism, had an identical sequence for 57 consecutive residues starting from the N-terminus. p2 and p3 also showed a striking similarity to five other proteins previously isolated from *Sulfolobus* strains and identified as DNA-binding proteins. However, the C-terminus, 10 residue region of p3 did not show any similarity to these proteins; in contrast, it was significantly similar to stretches in three eubacterial ribonucleases from *Bacillus* strains. No difference between p2 and p3 has so far been detected as regards their catalytic properties. Available data suggest that these molecules have a narrow substrate specificity and probably play specific roles in RNA processing.**

Key words: Ribonuclease; DNA-binding protein; Thermophilic; Archaebacterium; *Sulfolobus solfataricus*

1. Introduction

RNases are molecules of major biological importance. Recently, it has come to light that many more RNases occur in organisms than was previously thought. It has also been discovered that in adddition to sustaining non-specific RNA degradation, they also sustain a variety of specific reactions responsible for RNA processing and turnover [1,2]. However, very little is known about archaebacterial RNases. To our knowledge, until recently only two RNases P had been identified and characterized, one from *Sulfolobus solfataricus* and another from *Haloferax volcanii* [3]. This prompted us to search for RNases from the thermoacidophilic archaebacterium *S. solfataricus*: we found three such enzymes, referred to as p1, p2 and p3 according to the elution order from a Mono-S column [4]. The complete primary structure of p2 was also determined [4]: this showed that the molecule consists of 62 amino acids and is closely related to a class of small proteins previously isolated from *S. acidocaldarius* and *S. solfataricus*, and identified as DNA-binding proteins [5–7]. Particularly, p2 was identical to the protein previously found in *S. solfataricus* [7] except for the replacement of Glu-13 by Gln-13, and the lack of one Lys at the C-terminus.

This paper reports the complete primary structure and an initial biochemical characterization of p3: sequence data show that this molecule consists of 75 residues and has a high degree of similarity to p2. Thus, both p2 and p3 are similar to the above-described class of DNA-binding proteins, although p3 also displays a C-terminus which is closely related to some eubacterial RNases.

While our investigations aim to elucidate the physiological role(s) of these archaebacterial RNases, they might also contribute to a better understanding of protein thermostability. Because of their small size these molecules are ideal tools for structural studies, including NMR and X-ray crystallography.

2. Materials and methods

2.1. Microorganism and growth conditions

S. solfataricus cells (strain MT-4, ATCC 49155) were grown aerobically in a mineral medium as reported [8], using yeast extract (2 g/l) as the carbon source, and collected at the stationary phase.

2.2. Enzyme activity and protein assay

RNase activity was determined as previously described [4], using yeast RNA as substrate. After incubation, undigested RNA was precipitated by a mixture of perchloric acid and lanthanum nitrate and the absorbance at 260 nm was determined in the supernatant. Protein was determined using the Coomassie-blue protein-assay reagent from Pierce (Rockford, USA) and bovine pancreas RNase A as standard protein.

2.3. Enzyme purification

Protein p3 was purified as reported previously [4].

2.4. Amino acid sequence analysis and computer analysis of sequences

Sequence analysis was performed as previously reported [9]. Similarities to the entries in the Swiss-Prot Protein Database were searched by the PC-Gene program.

2.5. Analysis of cleavage products of tRNA incubated in the presence of proteins p2 or p3

20 μg $tRNA^{fMet}$ was incubated at 60°C for 60 min in the presence or the absence of 6 μg of either protein p2 or p3 in 40 mM sodium phosphate, pH 7.8. Samples were further incubated at 37°C for 30 min in the presence of pronase (2 μg). Cleavage products were then resolved in PAGE in the presence of 7 M urea as reported in [10], using a 19% total gel concentration and a 3% concentration of cross-linker. Bands were silver stained according to Morrissey [11].

3. Results and discussion

The amino acid sequence of 64 residues of p3 starting from

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Abbreviations: RNase, ribonuclease; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin.

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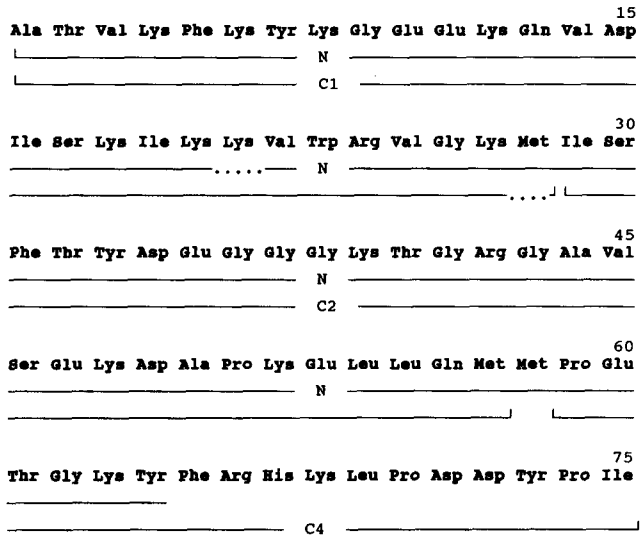

Fig. 1. Primary structure of p3 from *S. solfataricus*. N and C indicate the amino acid sequence of the entire molecule and of CNBr peptides, respectively. The Edman degradation was performed on 1 nmol of the entire protein and 0.5–1 nmol aliquots of each peptide.

the N-terminus was determined by subjecting the intact protein to Edman degradation. The complete sequence was determined by CNBr fragmentation, which allowed unambiguous identification of Lys-21 and sequencing of the C-terminal portion of the molecule. The primary structure is shown in Fig. 1. p3 consists of 75 residues, for an M_r of 8582 and a pI of 10.1, a value close to that calculated for p2 (10.2). The amino acid composition of p3 was similar to that previously determined by amino acid analysis [4]. As with p2 [4], a partial methylation of Lys-4 and -6 was found: the degree of monomethylation, estimated from the peak height on the chromatogram of the PTH-analyser, was 31% for Lys-4 and 30% for Lys-6.

The sequence of p3 was identical to that of p2 over a length of 57 residues starting from the N-terminus, and, like p2, also showed a striking similarity to five other proteins previously isolated from *Sulfolobus* strains and identified as DNA-binding proteins [6,7] (Fig. 2). The most significant differences among these molecules were detected at the C-termini: in particular, p3 has a stretch of 10 residues at the C-terminus, not present in any other related protein. Furthermore, no similarity was found between its N-terminus over a length of 65 residues and any other sequence deposited in data banks. However, the

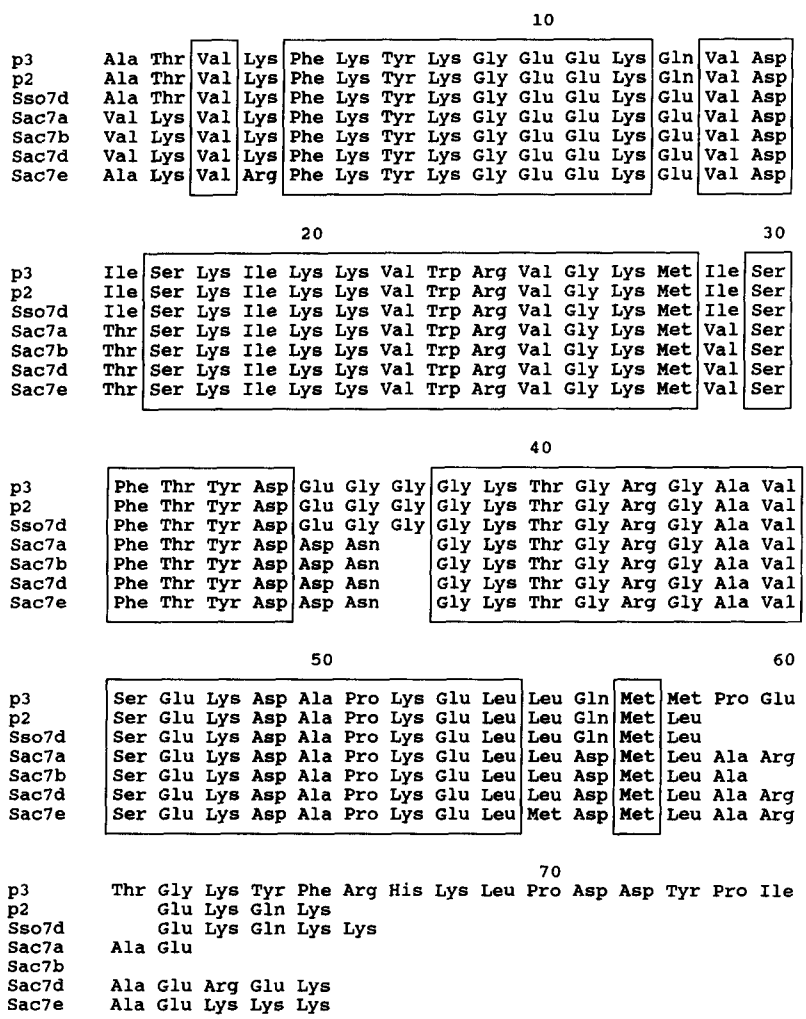

Fig. 2. Comparison of the amino acid sequence of proteins p2 and p3 from *S. solfataricus* with 7a, 7b, 7d and 7e DNA-binding proteins previously isolated from *S. solfataricus* (Sso) [7] and *S. acidocaldarius* (Sac) [6]. Residues conserved in all five proteins are boxed.

	66						75
p3	Arg	His Lys Leu Pro	Asp	Asp Tyr	Pro		Ile
	17						25
B.a.	Tyr	His Lys Leu Pro	Asn	Asp Tyr	---		Ile
	16						24
B.i.	Tyr	Lys Arg Leu Pro	Asn	Asp Tyr	---		Ile
	17						25
B.c.	Tyr	His Lys Leu Pro	Asp	Asn Tyr	---		Ile

Fig. 3. Sequence alignment of p3 with eubacterial RNases. B.a., *Bacillus amyloliquefaciens* [12]; B.i., *Bacillus intermedius* (12]; B.c., *Bacillus circulans* [13]. Residues conserved in at least three RNases are boxed.

C-terminus showed a significant sequence similarity to some eubacterial RNases (Fig. 3), one of which is the extensively characterized barnase (*Bacillus amyloliquefaciens* RNase). In this enzyme, the segment 17–25 shown in Fig. 3, mainly occurs as a loop between two α-helices (residues 6–18 and 26–34, respectively) [14], and does not seem to be directly involved in catalysis [15].

This sequence similarity, along with the known ribonucleolytic activity of p3, provides further evidence to support its identification as a RNase. Furthermore, preliminary structural data obtained by NMR analysis (Dr. Lucia Zetta, personal communication) show that p2 also shares the structural model of the RNA-recognition motif found in a family of proteins which bind specifically to several RNA molecules [16]. In fact, p2 consists of four consecutive antiparallel β-strands carrying three resi-dues (Trp-23, Phe-31 and Tyr-33) on adjacent strands: these aromatics form a cluster which is thought to participate in ring-stacking interactions with nucleotide bases of RNA [16]. In addition, the enzyme produced by a synthetic p2-encoding gene expressed in *Escherichia coli*, was endowed with RNase activity and was catalytically indistinguishable from the native enzyme [17]. The possibility that the activity detected in preparations of pure p2 from *S. solfataricus* was due to contaminants could therefore be ruled out. All these data strongly support our proposed identification of p2 and p3 as RNases.

Fig. 4. Hydrolysis of $tRNA^{fMet}$ from *E. coli* by proteins p2 or p3 from *S. solfataricus*. 20 μg $tRNA^{fMet}$ was incubated at 60°C for 60 min in the presence or the absence of 6 μg of either protein in 40 mM sodium phosphate, pH 7.8. Samples were incubated at 37°C for a further 30 min in the presence of pronase (2 μg). Cleavage products were then resolved in PAGE in the presence of 7 M urea. Lane 1, $tRNA^{fMet}$; lane 2, $tRNA^{fMet}$ incubated in the absence of any RNase; lane 3, $tRNA^{fMet}$ plus p2; lane 4, $tRNA^{fMet}$ plus p3. For further details see section 2.

Further investigation is, of course, required to obtain full understanding of the physiological role(s) played by these molecules. It appears surprising that two different functions may be sustained by these single, small-sized proteins. On the other hand, their DNA-binding capacity appears to be well established, although findings so far have only shown non-specific binding [5,7].

As regards their RNase activity, proteins p2 and p3 might be responsible for some process of RNA maturation, suggested by their low specific activity values and inability to attack homopolyribonucleotides [4]. Their narrow substrate specificity also supports this idea. It was shown that on incubation of $tRNA^{fMet}$ with either RNases few fragments were generated and there were no substantial differences between the degradation patterns produced by the two enzymes (Fig. 4). On the other hand, we could not discriminate between the catalytic properties of the two molecules: the pH-activity profiles were indistinguishable, with a broad pH optimum in the range 6.7–7.6; they were both positive to Kunitz assay; they both hydrolyzed 2′-3′ cyclic nucleotides and were insensitive to Mg^{2+}, phosphate and EDTA (data not shown). Furthermore, there was no difference in thermostability, with complete retention of activity up to 80°C (data not shown).

To gain more insight into the physiological role(s) of p2 and p3, a thorough kinetic characterization is being performed in our laboratory. Current investigations also aim to clarify how expression of the genes encoding these proteins is regulated; this covers the possibility of alternative splicing, which may account for their shared N- and diverging C-termini. Also, research is currently underway for other genes coding for p2 and p3 related proteins in *S. solfataricus*.

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