Amino acids and peptides. LVII. Synthetic peptide with a sequence of ribonuclease from *Sulfolobus solfataricus*, SSR(1–62), does not function as an RNase

Shima Joshi[a], Yuko Tsuda[b], Noriyuki Shintomi[b], Hiroyuki Kondo[b], Yasuhiro Nishiyama[c], Masanori Iwama[d], Kazuko Ohgi[d], Masachika Irie[d], Yoshio Okada[b,*]

[a] *STAR Biochemicals, Torrance, CA 90501-1722, USA*
[b] *Kobe Gakuin University, Nishi-ku, Kobe 651-2180, Japan*
[c] *Seikei University, Musashino-shi, Tokyo 180-0001, Japan*
[d] *Hoshi College of Pharmacy, Shinagawa-ku, Tokyo 142-8501, Japan*

Received 18 October 1999; received in revised form 22 January 2000

Edited by Shozo Yamamoto

Abstract The 62 residue peptide, SSR(1–62), whose sequence corresponds to that of ribonuclease (RNase) from *Sulfolobus solfataricus*, and its related peptides, SSR(1–22) and SSR(10–62), were chemically synthesized and their RNase activity and DNA-binding activity were examined. The RNase activity assay using yeast RNA or *t*RNA^{fMet} as substrate showed that the synthetic peptide SSR(1–62) did not hydrolyze yeast RNA or *t*RNA^{fMet}. These data were not consistent with previous reports that both the native peptide isolated from *S. solfataricus* [Fusi et al. (1993) Eur. J. Biochem. 211, 305–311] and the recombinant peptide expressed in *Escherichia coli* [Fusi et al. (1995) Gene 154, 99–103] were able to hydrolyze *t*RNA^{fMet}. However, the synthetic SSR(1–62) exhibited DNA-binding activity. In the presence of synthetic SSR(1–62), the cleavage of DNA (plasmid pUCRh2-4) by restriction endonuclease (*Eco*RI) was not observed, suggesting that synthetic SSR(1–62) bound to DNA protected DNA from its enzymatic digestion. Neither SSR(1–22) nor SSR(10–62) prevented DNA from being cleaved by a restriction enzyme. These findings strongly suggest the importance of not only the N-terminal region of SSR(1–62) but also the C-terminal region for DNA-binding. Circular dichroism spectroscopy of synthetic SSR(1–62) indicated a β-sheet conformation, in contrast with synthetic SSR(1–22), which exhibited an unordered conformation.

© 2000 Federation of European Biochemical Societies.

Key words: Circular dichroism spectroscopy; DNA-binding activity; P2; RNase activity; Synthetic SSR(1–62); *Sulfolobus solfataricus*

1. Introduction

Ribonuclease (RNase) is an enzyme that catalyzes the hydrolysis of phosphodiester bonds in an RNA chain. More RNases occur in organisms than previously thought. However, little is known about archaebacterial RNases. Only two RNases P, which are involved in *t*RNA processing, have been identified and characterized thus far, one from *Sulfolobus solfataricus* and the other from *Haloferax volcanii* [1]. Fusi et al. isolated three proteins with RNase activity, referred to as P1, P2 and P3, from the thermoacidophilic archaebacterium *S. solfataricus* [2]. Protein P2 consists of 62 amino acid residues and shows no significant similarity to any known class of RNases; in particular, P2 is deficient in His residues, which are essential for catalysis in all of the RNases known to date. On the other hand, the complete amino acid sequence of P2 revealed a high sequence similarity to that of the 7 kDa DNA-binding proteins [3–5]. In fact, protein P2 and P3 demonstrated non-specific DNA-binding activity [2]. The first question coming from the identification of those proteins is what mechanism is involved when they function as RNases. The second one is which region of the peptide is involved in RNase activity. Furthermore, most surprisingly, it was found that they exhibit two different functions; RNase activity and DNA-binding activity. These questions motivated us to investigate the structure-activity relationship of P2. In our previous study [6,7], attempts were made to synthesize peptide fragments related to SSR(1–62): SSR(57–62), SSR(52–62), SSR(47–62), SSR(44–62), SSR(37–62), SSR(33–62), SSR(27–62) and SSR(17–62) by both solution and solid-phase methods. However, those truncated peptides showed neither RNase activity nor DNA-binding activity, suggesting the importance of the N-terminus region to manifest RNase activity or DNA-binding activity [7]. In this report, we present the chemical synthesis of SSR(1–62), SSR(1–22) and SSR(10–62) and examination of their RNase activity and DNA-binding activity.

2. Materials and methods

2.1. Peptide synthesis and purification

SSR(1–62), SSR(1–22) and SSR(10–62) were synthesized by Boc solid-phase synthesis using Boc-Lys(2-Cl-Z)-Pam-resin or Boc-Val-Pam-resin as the solid support on a Vega automated peptide synthesizer according to the procedure described elsewhere [7]. Cleavage from the resin and deblocking of the protecting groups were achieved with the low-high HF procedure [8]. Briefly, the protected peptide-resins were treated with *p*-thiocresol/*p*-cresol/dimethyl sulfide (DMS)/HF (0.8:3.2:26:10) at −4°C for 2 h. After removal of DMS, HF was again condensed into the reactor. The reaction mixture was kept at −4°C for 1 h. After cleavage and deblocking, the crude peptides were filtered, dried, dissolved in dilute acetic acid and lyophilized. The peptides were purified by preparative high performance liquid chromatography (HPLC) on a C18 column (3.9×150 mm, Waters). The structure of the synthetic peptides was characterized by amino acid analysis and mass spectrometric methods. In the case of SSR(1–62), protein sequencing was performed on an ABI477A protein sequencer.

2.2. Enzyme activity assay

The RNase activity was determined according to the procedure

*Corresponding author. Fax: (81)-78-974 5689.
E-mail: okada@pharm.kobegakuin.ac.jp

0014-5793/00/$20.00 © 2000 Federation of European Biochemical Societies. All rights reserved.
PII: S0014-5793(00)01185-6

described by Fusi et al. [2], with minor modification. The reaction mixture, which consisted of 50 μg of peptides and 400 μg of yeast RNA in 40 mM sodium phosphate buffer, pH 7.8, in a final volume of 0.25 ml, was incubated for 10 min at 60°C. The reaction was stopped by addition of 0.25 ml of 2 mM lantanum nitrite/15% $HClO_4$. After standing on an ice bath for 20 min, the reaction mixture was centrifuged for 10 min at 3000 rpm, then 400 μl aliquot of the supernatant was withdrawn and diluted to 2 ml with water. The absorbance of the solution was measured at 260 nm.

The hydrolysis activity against *t*RNA was examined according to the method described by Fusi et al. [2] with slight modification. $tRNA^{fMet}$ (20 μg) was incubated at 60°C for 60 min with or without 10 μg of synthetic SSR(1–62) in 40 mM sodium phosphate buffer, pH 7.8. A 3 μg of protein kinase K was added to the samples, and they were further incubated at 37°C for 30 min. The reaction mixtures were precipitated with two volumes of ethanol and the cleavage products were then resolved in 19% polyacrylamide gel electrophoresis (PAGE) in the presence of 7 M urea as reported by Maniatis et al. [9]. The gel was stained by the silver staining method according to Morrissey [10].

2.3. DNA-binding activity assay

The DNA-binding activity of peptides was determined on 1% agarose gels [11]. The peptides were incubated with pUCRh2-4 plasmid DNA (ca. 3.9 kbp, 48 μg) at 50°C for 30 min. After incubation of the peptide-DNA complex with restriction endonuclease *Eco*RI (8 U) at 37°C for 2 h, the products were analyzed on 1% agarose gels.

2.4. Circular dichroism (CD) spectroscopy

CD spectra were measured with JASCO J-600 at room temperature in a 1.0 cm cell at wavelengths from 250 to 200 nm. A solution of the peptides was prepared in H_2O, pH ca. 6.0 ~ 6.5.

3. Results

3.1. Synthesis of SSR and SSR-related peptides

Final products were obtained as an amorphous powder with 95% or higher purity estimated by analytical HPLC. In order to prevent racemization during peptide synthesis, the stepwise elongation from the C-terminal by one amino acid at a time using urethane-protected amino acids such as *tert*-butyloxycarbonyl amino acid was employed, because this procedure was established as a racemization-free strategy [7]. The molecular mass of synthetic SSR(1–62) determined by electrospray mass spectra was 7020.5 (*m*/*z*), which was in good agreement with the calculated theoretical mass of 7020.27 $[M+H]^+$ for SSR(1–62). In addition, protein sequencing of synthetic SSR(1–62) led to identification of all 62 amino acid residues of SSR(1–62). The strategy applied in the sequence determination of SSR(1–62) is shown (Fig. 1). The N-terminal sequence of synthetic SSR(1–62) was determined as far as 44 residues by subjecting the intact SSR(1–62) to Edman degradation. The complete sequence was determined as follows: the

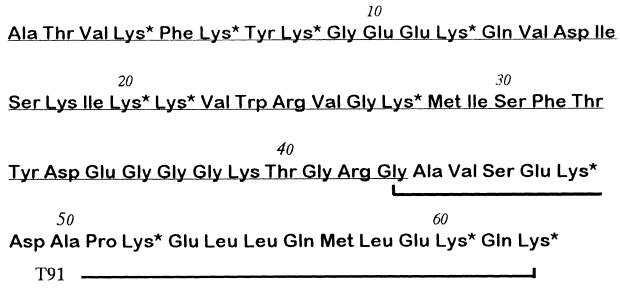

Fig. 1. Determination of primary structure of synthetic SSR(1–62). T indicates peptide from trypsin digestion after maleinylation at the Lys* residues. The Edman degradation was performed on the entire SSR(1–62) and on T91.

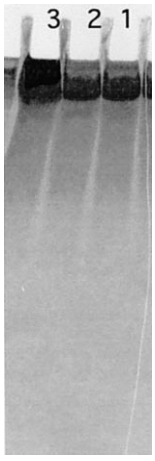

Fig. 2. Electrophoretic profile of $tRNA^{fMet}$ incubated with synthetic SSR(1–62). The reaction mixtures were separated using urea PAGE according to Maniatus et al. [9]. Bands were silver-stained according to Morrissey [10]. Lane 1, $tRNA^{fMet}$-incubated; lane 2, $tRNA^{fMet}$-incubated without synthetic SSR(1–62); lane 3, $tRNA^{fMet}$-incubated with synthetic SSR(1–62).

N^{ε}-maleinyl SSR(1–62) was digested with trypsin, and the resulting peptide fragments were separated by reversed-phase HPLC. The sequence determination of the fragment (T91), corresponding to SSR(44–62), allowed the identification of all amino acid residues. The purified peptides, SSR(10–62) and SSR(1–22), were also identified by amino acid analysis and molecular mass analysis.

3.2. RNase activity of peptides

In order to examine the RNase activity of the synthetic peptides SSR(1–62), SSR(1–22) and SSR(10–62), we measured the RNase activity for all peptides using yeast RNA as substrate according to the method of Fusi et al. [2] with slight modification. Synthetic SSR(1–62) exhibited less than 0.05%

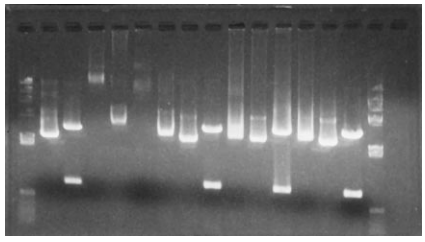

Fig. 3. Protection of pUCRh2-4 plasmid DNA from *Eco*RI digestion by addition of synthetic SSR(1–62) and SSR-related peptides. Lane 1, marker: Lambda DNA digested with *Hin*dIII; lane 2, native pUCRh2-4 plasmid DNA; lane 3, native pUCRh2-4 plasmid DNA incubated with *Eco*RI; lane 4, DNA-SSR(1–62) complex; lane 5, regained plasmid DNA after phenol extraction of DNA-SSR(1–62) complex; lane 6, DNA-SSR(1–62) complex incubated with *Eco*RI; lane 7, DNA incubated with SSR(10–62); lane 8, phenol extraction of the reaction mixture DNA and SSR(10–62); lane 9, the mixture of DNA and SSR(10–62) incubated with *Eco*RI; lane 10, DNA incubated with SSR(17–62); lane 11, phenol extraction of the reaction mixture DNA and SSR(17–62); lane 12, the mixture of DNA and SSR(17–62) incubated with *Eco*RI; lane 13, DNA incubated with SSR(1–22); lane 14, phenol extraction of the reaction mixture DNA and SSR(1–22); lane 15, the mixture of DNA and SSR(1–22) incubated with *Eco*RI; lane 16, marker: the same as lane 1. All samples were separated on 1% agarose gel and stained with ethidium bromide.

RNase activity of bovine pancreatic RNase A under the conditions described in Section 2. To investigate the cleavage condition of yeast RNA better, synthetic peptides were incubated for 5–60 min at 60°C in 50 mM acetate buffer, pH 5.0, or in 50 mM Tris–HCl buffer, pH 7.5. No effect was exerted by reaction time or pH. Also, varying ratios of synthetic peptides to yeast RNA were employed; peptides (10 μg, 20 μg and 50 μg) to yeast RNA (400 μg). The RNase activity of synthetic peptides was unaffected by the ratio to the substrate. Finally, we were not able to find any RNase activity of synthetic peptides under the conditions we used (see Section 2), although the ratio of synthetic peptides (50 μg) to yeast RNA (400 μg) was much higher than when the usual method is employed.

Furthermore, we tried to examine hydrolysis activity of synthetic SSR(1–62) on tRNA^{fMet} as mentioned by Fusi et al. [2]. Upon incubation of tRNA^{fMet} with synthetic SSR(1–62), no fragment was generated (Fig. 2). We did not observe RNase activity of synthetic peptide(1–62) against yeast RNA or tRNA^{fMet}. These results clearly demonstrated that the property of the synthetic SSR(1–62) was enzymatically distinguishable from that of native P2 [2].

3.3. DNA-binding activity of peptides

The DNA-binding activity of SSR(1–62), SSR(1–22) and SSR(10–62) was investigated. SSR(1–62) bound to plasmid DNA pUCRh2-4 causing a significant mobility change in the SSR(1–62)-plasmid DNA complex compared to plasmid DNA alone on 1% agarose gel (Fig. 3, lines 4 and 2, respectively). Dissociation of the SSR(1–62)-plasmid DNA complex by phenol-10 mM Tris–HCl buffer, pH 8.0, containing 1 mM EDTA extraction, released native plasmid DNA which was digested by restriction endonuclease *Eco*RI leading to a fragment DNA with ca. 800 bp (Fig. 3, lines 5 and 3, respectively). Incubation of the SSR(1–62)-plasmid DNA complex with restriction endonuclease *Eco*RI did not lead to the fragment DNA with ca. 800 bp (Fig. 3, line 6). These results showed that SSR(1–62) bound to DNA as the DNA-binding protein P2. In a previous report [7], we demonstrated that SSR(17–62) did not bind to plasmid DNA. In addition to SSR(17–62), SSR(1–22) with deletion of the C-terminal region (23–62) and SSR(10–62) lacking 9 amino acid residues at the N-terminus did not bind to plasmid DNA. When each mixture of peptide and plasmid DNA was incubated with *Eco*RI, it did release the DNA fragment with ca. 800 bp (Fig. 3, lines 15 and 9, respectively). From these results, only SSR(1–62) with a full sequence of P2 had the ability to bind DNA (Table 1).

3.4. Conformational properties of synthetic peptides

The CD spectra of the synthetic peptides were measured (Fig. 4). Synthetic SSR(1–62), the only peptide with DNA-binding ability, showed a conformation rich in β-sheet characterized by a peak at 217 nm. The CD spectrum of SSR(1–22) did not exhibit either double minima at 222 nm and 208 nm or a minimum at 217 nm, which characterized the α-helix structure and β-sheet structure, respectively [12]. The dominant structure of SSR(1–22) would be a random coil. The CD spectra of SSR(10–62) are also shown in Fig. 4. Comparison of those spectra indicated that deletion of the N- or C-terminal region produced a conformational change.

Table 1
DNA-binding activity of synthetic SSR(1–62) and SSR-related peptides

	−*Eco*RI	+*Eco*RI	DNA-binding activity[a]
SSR(1–62)	Increased M.w.	Not cleaved	+
SSR(1–22)	Unchanged M.w.	Cleaved	−
SSR(10–62)	Unchanged M.w.	Cleaved	−

[a]Plus sign (+) indicates no inhibition of *Eco*RI-binding to DNA. Minus sign (−) indicates inhibition of *Eco*RI-binding to DNA.

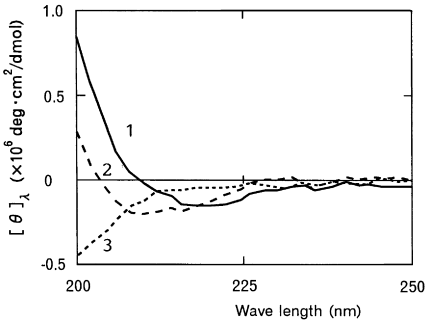

Fig. 4. CD spectra of synthetic peptides SSR(1–62) (line 1), SSR(10–62) (line 2) and SSR(1–22) (line 3).

4. Discussion

We have succeeded in preparing peptides SSR(1–62), SSR(10–62) and SSR(1–22), by chemical methods. Synthetic SSR(1–62) displayed less than 0.05% of RNase activity of bovine pancreatic RNase A with the same assay method used by Fusi et al. [2,13] and no ability to hydrolyze tRNA^{fMet}. Fusi et al. reported that protein P2, not only isolated from the thermoacidophilic archaebacterium *S. solfataricus* [2] but also expressed from *Escherichia coli* and yeast cells with use of the synthetic P2 encoding gene [14], was able to hydrolyze tRNA^{fMet}. Thus, our results conflict with those of Fusi et al. [2,13,14]. The wild-type P2 enzyme was partially methylated at Lys^4 and Lys^6, while synthetic P2 was not methylated. However, lysine methylation would not play a role in RNase activity, because recombinant P2 exhibited RNase activity, although the lysine residues of recombinant P2 were probably not methylated. Fusi et al. demonstrated that P2 on gel filtration displayed two peaks, the assessed molecular masses of which were 13.0 and 7.3 kDa, respectively [2]. One possible explanation of the disparity between our results and those of Fusi et al. would be that synthetic SSR(1–62) differed from P2 in quaternary structure so that synthetic SSR(1–62) lacks the properties of RNase. Our results suggest the necessity to investigate further: (i) whether the P2 protein has two stable conformations, only one of which is enzymatically active while the other is inactive, and the chemically synthesized P2 was in an inactive state; (ii) whether the P2 protein isolated from *S. solfataricus* cells and host cells of *E. coli* and *S. cerevisiae* had adsorbed any nucleolytically active protein; (iii) by what mechanism did P2, lacking His residues, hydrolyze RNA when, in fact, His residues are the most common constituents of nucleolytic enzymes.

Although synthetic SSR(1–62) was inactive as an RNase, SSR(1–62) exhibited DNA-binding activity. On the other hand, neither SSR(10–62) nor SSR(1–22) could bind DNA.

This suggests that some part of the N-terminal region might interact with some part of the C-terminal region of SSR(1–62) to form a conformation necessary for DNA-binding.

Acknowledgements: The authors are grateful to Ms. S.D. Bryant of the National Institute of Environmental Health Sciences (NIEHS) for her kind help during the preparation of this manuscript. This work was supported in part by a grant from the Science Research Promotion Fund of the Japanese Private School Promotion Foundation.

References

[1] Darr, S.C., Brown, J.W. and Pace, N.R. (1992) Trends Biochem. Sci. 17, 178–182.
[2] Fusi, P., Tedeschi, G., Aliverti, A., Ronchi, S., Tortora, P. and Guerritore, A. (1993) Eur. J. Biochem. 211, 305–310.
[3] Kimura, M., Kimura, J., Davie, P., Reinhardt, R. and Dijk, J. (1984) FEBS Lett. 176, 176–178.
[4] Grote, M., Dijk, J. and Reinhardt, R. (1986) Biochim. Biophys. Acta 873, 405–413.
[5] Choli, T., Wittman-Liebold, B. and Reihardt, R. (1988) J. Biol. Chem. 263, 7087–7093.
[6] Okada, Y., Joshi, S., Shintomi, N., Kondo, Y., Tsuda, Y., Ohgi, K. and Irie, M. (1999) Chem. Pharm. Bull. 47, 1089–1096.
[7] Tsuda, Y., Shima, J., Shintomi, N., Kondo, Y., Ohgi, K., Irie, M. and Okada, Y. (1999) Chem. Pharm. Bull. 47, 1097–1101.
[8] Tam, J.P., Heath, W.F. and Merrifield, R.B. (1983) J. Am. Chem. Soc. 105, 6442–6455.
[9] Maniatis, T. and Efstraiadis, A. (1980) in: Method in Enzymology (Grossman, L. and Moldave, K., Eds.), Vol. 65, pp. 299–305, Academic Press, New York.
[10] Morrissey, J.H. (1981) Anal. Bochem. 117, 307–310.
[11] Kulms, D., Schaffer, G. and Hahn, U. (1995) Biochem. Biophys. Res. Commun. 214, 646–652.
[12] Greenfield, N. and Fasman, G.D. (1968) Biochemistry 8, 4109–4120.
[13] Fusi, P., Grisa, M., Tedeschi, G., Nergi, A., Guerritore, A. and Tortora, P. (1995) FEBS Lett. 360, 187–190.
[14] Fusi, P., Grisa, M., Mombelli, E., Consonni, R., Tortora, P. and Vanoni, M. (1995) Gene 154, 99–103.