THE AMINO ACID SEQUENCE OF RIBONUCLEASE St

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Received 26 January 1976

1. Introduction

Ribonuclease St (RNase St), the extracellular ribonuclease from *Streptomyces erythreus,* has a substrate specificity similar to ribonuclease T_1 (RNase T_1) which catalyzes the splitting of the phosphodiester bond of guanylic acid [1]. When detailed comparisons were made, a marked difference in the kinetic and thermodynamic patterns was found [2]. To gain information on structure-function relationships, we examined the interaction of RNase St with a competitive inhibitor for the enzyme by means of circular dichroism spectra [3]. In developing these investigations we undertook a study of the amino acid sequence of the enzyme. This paper summarizes the determination of the structural formula of RNase St.

2. Materials and methods

RNase St was isolated from the culture medium of *Streptomyces erythreus* as described earlier [4]. Oxidized RNase St and *S*-carboxylmethylcysteinyl RNase St were prepared by the method of Hirs [5] and Crestfield et al. [6], respectively. The amino acid composition was determined with a Hitachi amino acid analyzer, KLA-5. Most of the amino acid sequence of the isolated peptides was determined by manual Edman degradation [7] using reagents for sequence analysis from Wako Pure Chemical Industries, Japan. The phenylthiohydantoin (Pth) amino acids were identified by thin-layer chromatography on silica gel plates and amino acid analysis after conversion to free amino acid by 6 N HCl hydrolysis [8]. Pth cystenic acid, histidine and agrinine were analyzed only

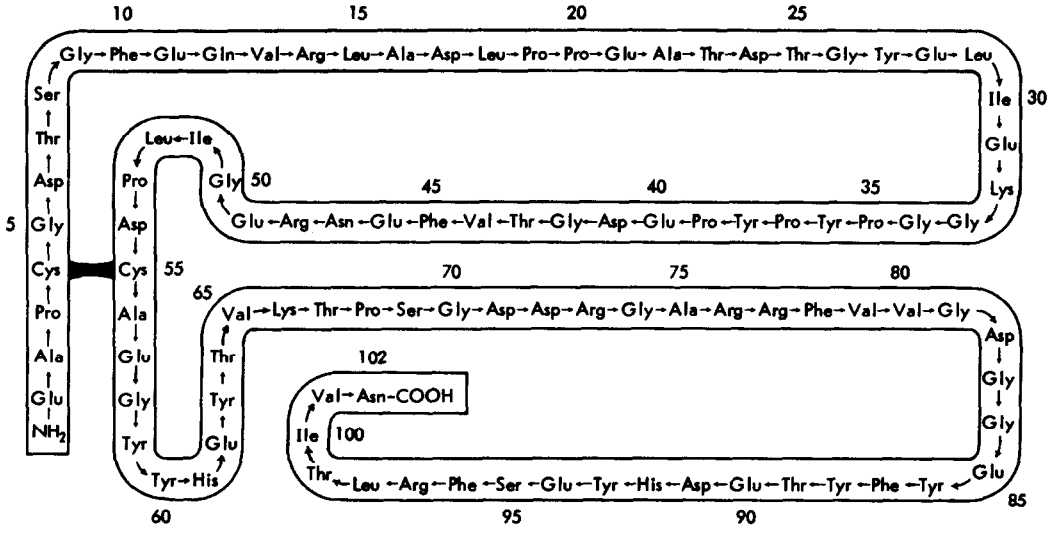

Fig.1. Covalent structure of ribonuclease St.

qualitatively. With this method, 20 consecutive amino acid residues of tryptic peptides and 10 residues of chymotryptic peptides usually could be identified. Some of the peptides were determined by subtractive Edman degradation method [9].

3. Results

Amino terminal analysis with both 1-fluoro-2,4-dinitrobenzene [10] and 1-dimethylaminonaphthalene-5-sulfonyl chloride methods [11] gave glutamic acid. The amino terminal sequence of the oxidized protein was determined by manual Edman degradation procedure to be Glu–Ala–Pro–Cys–Gly–Asp–Thr. The carboxyl terminal sequence was determined by the carboxypeptidase A and carboxypeptidase A plus B procedures to be Phe–Arg–(Leu, Thr, Ile)–Val–Asn. The tryptic hydrolyzate products of the oxidized protein were separated by chromatography on a column of Dowex 50W x 2 [12] with subsequent purification by high voltage electrophoresis on paper or paper chromatography. Acid-insoluble tryptic peptides were purified by DEAE-Sephadex chromatography [13]. Thus nine tryptic peptides including free arginine were isolated and their sequences were determined (OT- in fig.2). On the basis of terminal analyses, OT-1 and OT-9 must represent the amino- and carboxyl-terminal peptides of the original protein, respectively. OT-8 was assumed to be at the terminus contiguous to OT-9, because of the carboxyl-terminal sequence.

To obtain overlapping peptides, three kinds of cleavage of the protein were performed; tryptic hydrolysis of the protein modified at the lysine residues by maleic anhydride [14], and chymotryptic and thermolytic hydrolyses. The tryptic peptides from the oxidized-maleylated protein were demaleylated [15] and subjected to gel filtration on Sephadex G-25 (1.9 X 200 cm, 5 mM NH_4OH). The fraction which eluted fastest from the G-25 column was run through Sephadex G-50 and divided into two parts, the earlier fraction being further purified by chromatography on a column of Dowex 50W X 2 (OMT- in fig.2). Two lysine-containing peptides, OMT-IA1 and -IA2, could account for the connection of OT-2 → OT-3 and OT-4 → OT-5, respectively.

Chymotryptic peptides were purified by chromatography on Sephadex G-25 and DEAE-Sephadex [13] and high voltage paper electrophoresis. When the

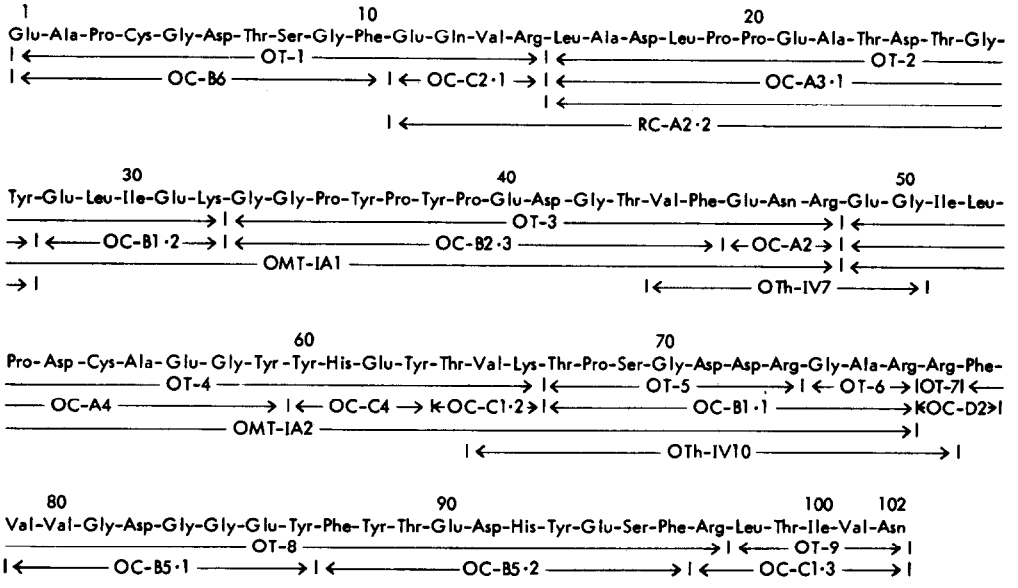

Fig.2. Derivation of the sequence of amino acid residues in ribonuclease St from knowledge of the peptides derived by tryptic, chymotryptic and thermolytic cleavage. OT, trypsin peptide; OC and RC, chymotrypsin peptide; OTh, thermolysin peptide; OMT, trypsin peptide on oxidized and maleylated protein.

oxidized protein was digested with 2% by weight of chymotrypsin at 37°C, pH 8.5, for 18 h, 14 major peptides were obtained (OC- in fig.2). Three could be used for connecting the tryptic peptides; OC-B1-1, -D2, -C1-3. The connection between OT-1 and OT-2 was elucidated by the peptide RC-A2-2 being obtained from the digestion of *S*-carboxymethylcysteinyl RNase St with 1% chymotrypsin for 4 h. These chymotryptic hydrolyses led to some cleavages of lysyl and arginyl bonds.

Hydrolysis of the oxidized RNase St by thermolysin at an enzyme to substrate ratio of 1 : 200 by weight at 40°C and pH 8.5 for 2.5 h helped to solve the remaining connections of the tryptic peptides. Seven arginine-containing thermolytic peptides were purified by high voltage paper electrophoresis at pH 3.6 after separation by Sephadex G-50 (2.2 × 200 cm, 5 mM NH_4OH). Analyses of the peptides OTh-IV-7 and -IV-10 confirmed the alignment of OT-3 → OT-4 and OT-6 → OT-7.

To determine whether OT-7 was followed by OT-8 or not, the oxidized protein modified with 1,2-cyclohexanedione by the method of Patthy and Smith [16] was hydrolyzed with trypsin which ought to have cleavaged two peptide bonds at the lysine loci for RNase St. Two lysine-containing peptides, which corresponded to OT-1 → OT-2 and OT-3 → OT-4, were found in the acid-soluble fraction of the tryptic digest. These peptides were regenerated with hydroxylamine treatment [16], then separated from each other by high voltage paper electrophoresis at pH 1.9. The amino acid composition of the peptide isolated from the acid-insoluble fraction equaled the sum of those of OT-5, -6, -7, -8 and -9. The sequence of thirteen residues from the N-terminus of the insoluble peptide was determined to be Thr–Pro–Ser–Gly–Asp–Asp–Arg–Gly–Ala–Arg–Arg–Phe–Val. This corresponds to the sequence from position 67, which is an amino terminal residue of OT-5, to valine at position 79. With this information the complete amino acid sequence of RNase St was established as shown in fig.1.

4. Discussion

The single disulfide bond links the middle region of the protein with the amino-terminal region, forming a large loop of 52 residues corresponding to nearly one half of the whole of 102 residues. Interestingly, treatment of the native enzyme with carboxypeptidase A resulted in neither release of amino acid nor decrease of the enzymatic activity, even if the reaction was kept in the presence of sodium dodecyl sulfate at 45°C for a few hours (N. Yoshida & A. Sasaki, unpublished). Thus, the polypeptide chain of the carboxyl-terminus of the native RNase St probably is folded or less exposed due to some hydrophobic interaction, based upon the unique Leu–Thr–Ile–Val sequence at position 98 to 101 together with Tyr–Phe–Tyr (86 to 88) or/and Phe–Val–Val (78 to 80).

According to the results of chemical modification studies (N. Yoshida & A. Sasaki, unpublished), the active site of RNase St is probably composed of two histidines, arginine and glutamic acid which are observed similarly in RNase T_1 and RNase U_2 [17]. Surprisingly, in both RNase St and RNase T_1, arginine and histidine were at positions 77 and 92, respectively, and positions 78 to 80 in the enzymes built up a significantly hydrophobic region; Phe–Val–Val in RNase St and Val–Val–Phe in RNase T_1. On the other hand, no common region could be found in the surroundings about His-92.

Of additional interest is the occurrence of the same sequence of residues 38 and 39, Tyr–Pro, in the two RNases, being followed by Glu-40 in RNase St and His-40 in RNase T_1. Therefore, it appears likely that Glu-40 for RNase St must be the residue modified by iodoacetate as well as Glu-58 for RNase T_1 [18]. Another histidine residue for RNase St was found at position 61 corresponding to the location of Glu-58 in RNase T_1. It is also noteworthy that the sequence of Tyr–Tyr occurs at two residues on the amino-terminal side of His-61 in RNase St and Glu-58 in RNase T_1.

Finally, we point out that in RNase St the structure

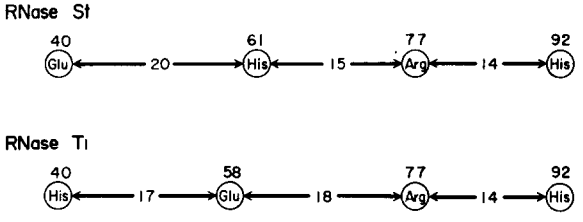

Fig.3. Proposed active-site topographies of ribonuclease St and ribonuclease T_1.

of the active site, including the crucial glutamic acid, the histidine other than His-92 and Arg-77, was analogous to that of RNase T_1, though there was an interconversion between the positions of Glu and His; the sequence of the other sites was quite different from that of RNase T_1. Based on these considerations, fig.3 shows the active-site topographies of the two microbial ribonucleases possessing the same substrate specificity. Other aspects of this investigation will be fully described later.

References

[1] Tanaka, K. (1961) J. Biochem. Tokyo 50, 62–69.
[2] Yoshida, N. and Otsuka, H. (1971) Biochim. Biophys. Acta 228, 648–653.
[3] Yoshida, N., Kuriyama, K., Iwata, T. and Otsuka, H. (1971) Biochem. Biophys. Res. Commun. 43, 954–961.
[4] Yoshida, N., Inoue, H., Sasaki, A. and Otsuka, H. (1971) Biochim. Biophys. Acta 228, 636–647.
[5] Hirs, C. H. W. (1956) J. Biol. Chem. 219, 611–621.
[6] Crestfield, A. M., Moore, S. and Stein, W. H. (1963) J. Biol. Chem. 238, 622–627.
[7] Iwanaga, S., Wallén, P., Gröndahl, N. J., Henschen, A. and Blombäck, B. (1969) Eur. J. Biochem. 8, 189–199.
[8] Van Orden, H. O. and Carpenter, F. H. (1969) Biochem. Biophys. Res. Commun. 14, 399–403.
[9] Dopheide, T. A. A., Moore, S. and Stein, W. H. (1967) J. Biol. Chem. 242, 1833–1837.
[10] Fraenkel-Conrat, H., Harris, J. I. and Levy, A. L. (1956) Method Biochem. Anal. 2, 360–383.
[11] Hartley, B. S. (1970) Biochem. J. 119, 805–822.
[12] Matsubara, H. and Sasaki, R. M. (1968) J. Biol. Chem. 243, 1732–1757.
[13] Salnikow, J., Liao, T.-H., Moore, S. and Stein, W. H. (1973) J. Biol. Chem. 248, 1480–1488.
[14] Delange, R. J., Fambrough, D. M., Smith, E. L. and Bonner, J. (1969) J. Biol. Chem. 244, 319–334.
[15] Butler, P. G., Harris, J. I., Hartley, B. S. and Leberman, R. (1967) Biochem. J. 103, 789.
[16] Patthy, L., and Smith, E. L. (1975) J. Biol. Chem. 250, 557–564.
[17] Sato, S. and Uchida, T. (1975) Biochem. J. 145, 353–360.
[18] Takahashi, K., Stein, W. H., and Moore, S. (1967) J. Biol. Chem. 242, 4682–4690.