Volume 306, number 2,3, 189–192 FEBS 11310 July 1992

Crystal and molecular structure of RNase Rh, a new class of microbial ribonuclease from *Rhizopus niveus*

Hiroyuki Kurihara[a], Yukio Mitsui[a], Kazuko Ohgi[b], Masachika Irie[b], Hiroshi Mizuno[c] and Kazuo T. Nakamura[a]

[a]*Department of BioEngineering, Nagaoka University of Technology, Nagaoka, Niigata 940-21, Japan,* [b]*Department of Microbiology, Hoshi College of Pharmacy, Ebara, Tokyo 142, Japan* and [c]*National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305, Japan*

Received 12 May 1992

The crystal structure of RNase Rh, a new class of microbial ribonuclease from *Rhizopus niveus*, has been determined at 2.5 Å resolution by the multiple isomorphous replacement method. The crystal structure was refined by simulated annealing with molecular dynamics. The current crystallographic R-factor is 0.200 in the 10–2.5 Å resolution range. The molecular structure which is completely different from the known structures of RNase A and RNase T_1 consists of six α-helices and seven β-strands, belonging to the $\alpha+\beta$ type structure. Two histidine and one glutamic acid residues which were predicted as the most probably functional residues by chemical modification studies are found to be clustered. The steric nature of the active site taken together with the relevant site-directed mutagenesis experiments (Irie et al.) indicates that: (i) the two histidine residues are the general acid and base; and (ii) an aspartic acid residue plays a role of recognizing adenine moiety of the substrate.

Ribonuclease Rh; Crystal structure; *Rhizopus niveus*; Multiple isomorphous replacement method; Enzyme structure

1. INTRODUCTION

Ribonuclease Rh (RNase Rh) was isolated from *Rhizopus niveus* by Tomoyeda et al. [1] and purified by Komiyama and Irie [2]. RNase Rh splits the 3′,5′-phosphodiester linkage of RNA (through 2′,3′-cyclic phosphodiester as an intermediate) without absolute base specificity. Its base specificity estimated from the time-course of release of nucleotides from RNA is in the order A>G>C>U and is very similar to that of RNase T_2 [3] or RNase M [4]. The enzyme consists of a single polypeptide chain (M_r 24,000) of 222 amino acid residues, the sequence of which has been determined by Horiuchi et al. [5].

As shown in Fig. 1, the locations of the ten half-cystines of RNase Rh are almost the same as in RNase T_2 and RNase M, and the sequences around the two histidine and one glutamic acid residues that are suggested to be involved in the active site by Irie [6] are fairly well conserved. The lowest line of Fig. 1 is the sequence of S_2-allelic glycoprotein of *Nicotiana alata* [7]. It is well known that this kind of glycoprotein is involved in self-incompatibility in flowering plants. Although overall homology between S_2-glycoprotein and the family of RNase Rh, T_2 and M is rather poor (about 10%), local homology around the essential residues is fairly high. It was predicted that the glycoprotein must have the RNase specific activity [8], and in fact McClure et al. [9] reported that glycoproteins isolated from style extracts of *Nicotiana alata* exhibited ribonuclease activities. Therefore, it seems that this new class of RNase involving RNase Rh. T_2, M and *S*-glycoproteins shares a common backbone folding, as in the case of the other class of microbial RNases involving a group of much smaller (M_r approx. 11,000) RNases, such as RNase St [10] and RNase T_1 [11].

Here, we present the three-dimensional structure of a microbial ribonuclease, RNase Rh, determined by the multiple isomorphous replacement method at 2.5 Å resolution.

Abbreviations: RNase, ribonuclease; r.m.s., root-mean-square.

Correspondence address: K.T. Nakamura, Department of BioEngineering, Nagaoka University of Technology, Nagaoka, Niigata 940-21, Japan.

2. MATERIALS AND METHODS

2.1. *Crystallization and data collection*

After we had reported crystallization and crystallographic characterization of type I and type II crystals of RNase Rh [12], we found that a large number of crystals gave rise to both types of diffraction patterns and it was very difficult to obtain pure type I crystals suitable for X-ray diffraction experiments. However, on adding divalent cations such as Mg^{2+}, Ca^{2+} and Ba^{2+} at a concentration of 10 mM, crystals with distinct morphology appeared. These crystals showed the same diffraction pattern as type II crystals but the Bragg reflections were much sharper (type II′). Therefore, we decided to use type II′ crystals for crystal structure determination.

The crystals belong to an orthorhombic space group $P2_12_12_1$, with unit cell dimensions a=67.7 Å, b=72.5 Å, c=44.3 Å. The intensity data were collected using either one of the following two devices: a Rigaku

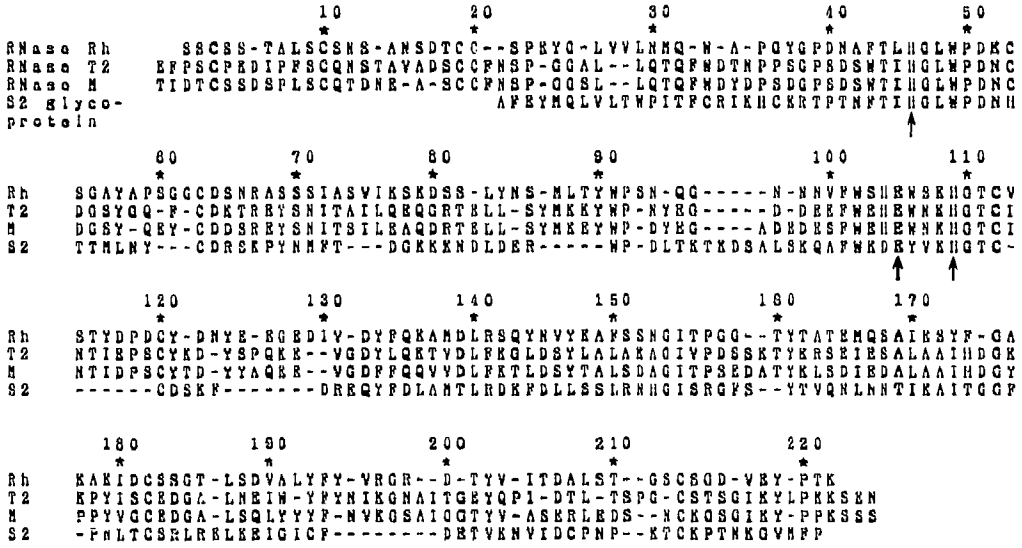

Fig. 1. Primary sequences of microbial RNases. Arrows indicate the residues which were predicted as the most probably functional residues by chemical modification studies.

four-circle diffractometer AFC-5R mounted on an RU-200 rotating anode generator operated at 50 kV, 200 mA and the macromolecule-oriented Weissenberg camera, devised by Sakabe [13], installed at the Beam Line 6A of the Synchrotron Radiation Source at the National Laboratory for High Energy Physics, Tsukuba. Total number of independent reflections in the resolution range of 10–2.5 Å was 7830.

2.2. *Structure determination and refinement*

The crystal structure was solved at 2.5 Å resolution by the multiple isomorphous replacement method using HgO, 3-chloromercuri-2-methoxy propyl urea (CMMPU) (2 data sets), $Pb(OAc)_2$ (2 data sets) and $(NH_4)_2Pt(NO_2)_4$ derivatives for which the ratios (r.m.s.-heavy atom structure factor/r.m.s. lack-of-closure error) were (40.8/28.2), (42.6/23.7, 22.5/12.8), (54.4/28.5, 55.5/35.9) and (27.6/20.3), respectively. The overall figure of merit was 0.721.

The starting model for the refinement was constructed using a stack of mini-maps and program package TOM running on a silicon Graphics IRIS 4D/25TG workstation [14]. The structure was refined by the stimulated annealing procedure employing the program X-PLOR followed by manual refitting of the model to new difference Fourier and fragment-deleted ('omit') difference Fourier maps using TOM. The current crystallographic *R*-factor is 0.200 in the 6.0–2.5 Å range with the r.m.s. deviation from ideality being 0.020 Å for bond lengths.

3. RESULTS AND DISCUSSION

The overall three-dimensional structure of RNase Rh is shown in Fig. 2. The molecule has a dimension of 50 × 40 × 25 Å. The structure consists of six α-helices (αA: 72–79, αB: 82–90, αC: 98–108, αD: 128–143, αE: 146–151, αF: 163–174) and seven β-strands (β1: 25–33, β2: 44–52, β3: 158–162, β4: 179–183, β5: 186–197, β6: 201–

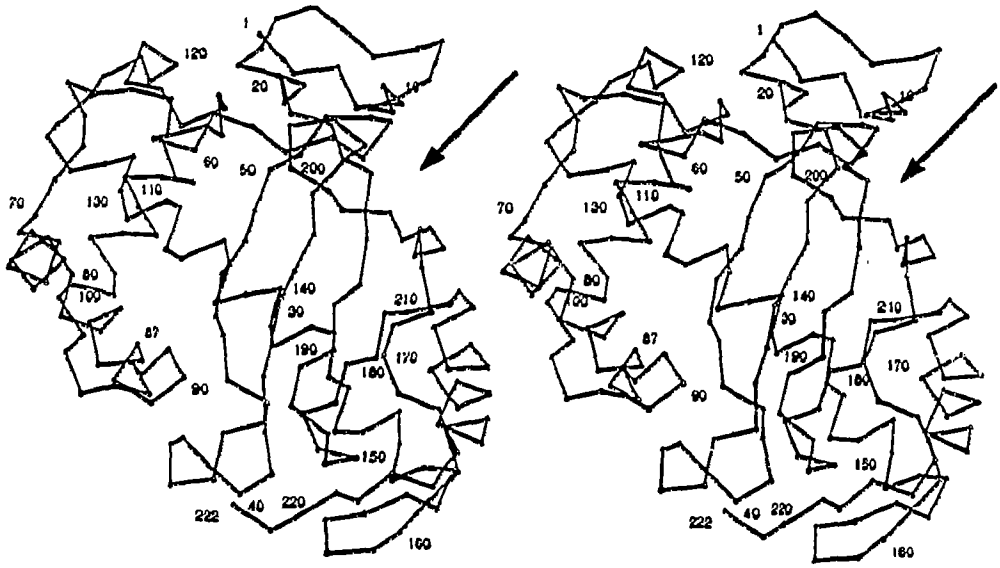

Fig. 2. α-Carbon chain of the RNase Rh molecule (stereo pair).

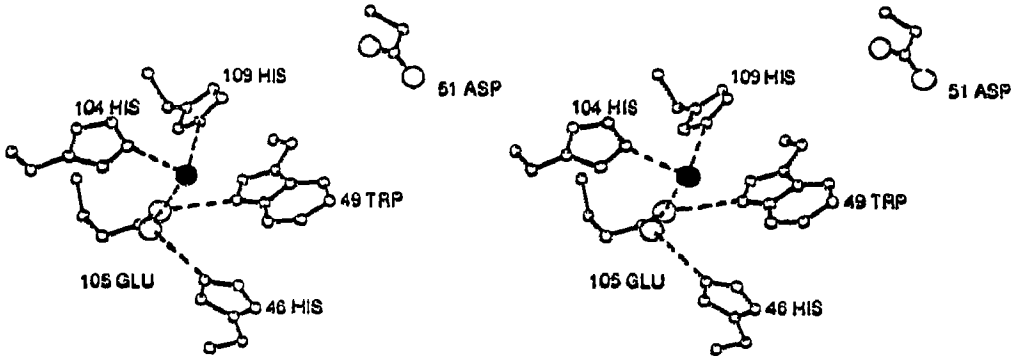

Fig. 3. Stereo view of the active site of RNase Rh. Hydrogen bonds are drawn as broken lines. A filled circle indicates a water molecule.

204, β7: 217–220), belonging to the $\alpha+\beta$ type structure [15]. On the surface of the molecule, a cleft runs along the direction indicated by an arrow in Fig. 1. Two histidine (His^{46} and His^{109}) and one glutamic acid (Glu^{105}) residues which were predicted as the most probably functional residues by chemical modification studies [16–19] are located on the central β-sheet consisting of four β-strands and an αC-helix running parallel to these strands. The connection scheme of five disulfide bonds which had not been conclusively determined by chemical studies was determined as Cys^{3}–Cys^{20}, Cys^{10}–Cys^{53}, Cys^{19}–Cys^{120}, Cys^{63}–Cys^{112} and Cys^{182}–Cys^{213}. The structure of RNase Rh is quite different from the known structures of RNase A [20] and RNase T_1. However, they have a common structural feature that the active site is located on a β-sheet.

Fig. 3 shows the active site of RNase Rh viewed along the same direction as in Fig. 2. As expected, the possible functional residues predicted by chemical modification studies [6] are clustered forming the active site. The carboxylate group of Glu^{105} is directly hydrogen-bonded to imidazole ring of His^{46} while it is indirectly hydrogen-bonded to the imidazole ring of His^{109} via a water molecule. The distance between the imidazole ring of His^{46} and that of His^{109} is about 7 Å. This distance is roughly the same as the corresponding distance between the two catalytic imidazole rings of His^{12} and His^{119} observed in the absence of inhibitor ligands [21], favoring the notion that the two imidazole rings of His^{46} and His^{109} are the catalytically important groups.

Apart from the three 'functional' residues, a tryptophan residue was predicted to be located near the active site through chemical modification studies [22]. In fact the indole ring of Trp^{49} is found to be located in the active site, hydrogen-bonded to the carboxyl group of Glu^{105}, and partially stacked with the imidazole ring of His^{109}. The function of the indole ring of the Trp^{49} seems to be to fix the orientations of the two side groups. Assuming that the locus occupied by a bound water molecule appearing in the center of Fig. 2 is the site for binding the phosphate group of substrates, the side chains of the following residues are within hydrogen-bond distance of the phosphate group: His^{46}, His^{104}, His^{109} and Glu^{105}.

Site-directed mutagenesis experiments carried out by Irie and his colleagues (unpublished) show that substitution of Asp^{51} with Asn remarkably reduces only the specific activity toward adenine, indicating that Asp^{51} is involved in the binding of adenine base of a substrate to RNase Rh. As shown in Fig. 2, Asp^{51} is located 7 Å apart from His^{109} in the active site cleft. When RNA binds to RNase Rh along the cleft, the carboxylate group of Asp^{51} may well interact with the adenine base. Evolutionarily all the residues but His^{104} shown in Fig. 2 are completely conserved among RNase Rh, T_2, M and S-glycoproteins. This fact strongly indicates that all of these residues are functionally important and that the sterically similar active site may be found in RNase M, T_2 and S-glycoproteins as well.

Acknowledgements: This work was partly supported by a grant-in-aid from the Ministry of Education, Science and Culture of Japan given to K.T.N.

REFERENCES

[1] Tomoyeda, M., Eto, Y. and Yoshino, T. (1969) Arch. Biochem. Biophys. 131, 191–202.
[2] Komiyama, T. and Irie, M. (1971) J. Biochem. (Tokyo) 70, 765–773.
[3] Sato, K. and Egami, F. (1957) J. Biochem. (Tokyo) 44, 753–767.
[4] Imasawa, M., Irie, M. and Ukita, T. (1968) J. Biochem. (Tokyo) 64, 595–602.
[5] Horiuchi, H., Yanai, K., Takagi, M., Yano, K., Wakabayashi, E., Sanda, A., Mine, S., Ohgi, K. and Irie, M. (1988) J. Biochem. (Tokyo) 103, 408–418.
[6] Omitted.
[7] Anderson, M.A., Cornish, Mau, S.-L., Williams, E.G., Hogart, R., Atkinson, A., Bonig, I., Grego, B., Simpson, R., Roche, P.J., Haley, J.D., Penschow, J.D., Niall, H.D., Tregear, G.W., Coghlan, J.P., Crawford, R.J. and Clarke, A.E. (1986) Nature 321, 38–44.
[8] Sakiyama, F. (1990) Kagaku (in Japanese) 45, 662–663.
[9] McClure, B.A., Haring, V., Ebert, P.R., Anderson, M.A., Simpson, R.J., Sakiyama, F. and Clarke, A.E. (1989) Nature 342, 955–957.
[10] Nakamura, K.T., Iwahashi, K., Yamamoto, Y., Iitaka, Y., Yoshida, N. and Mitsui, Y. (1982) Nature (London) 299, 564–566.

[11] Arni, R., Heinemann, U., Tokuoka, R. and Saenger, W. (1988) J. Biol. Chem. 263, 15358–15368.
[12] Kurihara, H., Mitsui, Y., Nakamura, K.T., Wakabayashi, E., Ohgi, K. and Irie, M. (1989) J. Mol. Biol. 206, 791–792.
[13] Sakabe, N. (1983) J. Appl. Crystallogr. 16, 542–545.
[14] Cambillau, C. and Horjales, E. (1987) J. Mol. Graphics 5, 174–177.
[15] Levitt, M. and Chothia, C. (1976) Nature 261, 552–558.
[16] Sanda, A. and Irie, M. (1974) J. Biochem. (Tokyo) 75, 419–428.
[17] Sanda, A. and Irie, M. (1979) Chem. Pharm. Bull. 27, 2310–2315.
[18] Sanda, A., Takizawa, Y. and Irie, M. (1985) Chem. Pharm. Bull. 33, 4515–4521.
[19] Sanda, A., Takizawa, Y., Iwama, M. and Irie, M. (1986) J. Biochem. (Tokyo) 98, 125–132.
[20] Kartha, G., Bello, J. and Harker, D. (1967) Nature 213, 862–865.
[21] Wlodawer, A., Bott, R. and Sjolin, L. (1982) J. Biol. Chem. 257, 1325.
[22] Sanda, A. and Irie, M. (1980) J. Biochem. 87, 1079–1087.