Amino Acid Residues in Ribonuclease MC1 from Bitter Gourd Seeds Which Are Essential for Uridine Specificity

Tomoyuki Numata,[‡] Akio Suzuki,[§] Min Yao,[§] Isao Tanaka,[§] and Makoto Kimura*[,‡]

Laboratory of Biochemistry, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan, and Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan

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ABSTRACT: The ribonuclease MC1 (RNase MC1), isolated from seeds of bitter gourd (*Momordica charantia*), consists of 190 amino acids and is characterized by specific cleavage at the 5′-side of uridine. Site-directed mutagenesis was used to evaluate the contribution of four amino acids, Asn71, Val72, Leu73, and Arg74, at the α4−α5 loop between α4 and α5 helices for recognition of uracil base by RNase MC1. Four mutants, N71T, V72L, L73A, and R74S, in which Asn71, Val72, Leu73, and Arg74 in RNase MC1 were substituted for the corresponding amino acids, Thr, Leu, Ala, and Ser, respectively, in a guanylic acid preferential RNase NW from *Nicotiana glutinosa*, were prepared and characterized with respect to enzymatic activity. Kinetic analysis with a dinucleoside monophosphate, CpU, showed that the mutant N71T exhibited 7.0-fold increased K_m and 2.3-fold decreased k_{cat}, while the mutant L73A had 14.4-fold increased K_m, although it did retain the k_{cat} value comparable to that of the wild-type. In contrast, replacements of Val72 and Arg74 by the corresponding amino acids Leu and Ser, respectively, had little effect on the enzymatic activity. This observation is consistent with findings in the crystal structure analysis that Asn71 and Leu73 are responsible for a uridine specificity for RNase MC1. The role of Asn71 in enzymatic reaction of RNase MC1 was further investigated by substituting amino acids Ala, Ser, Gln, and Asp. Our observations suggest that Asn71 has at least two roles: one is base recognition by hydrogen bonding, and the other is to stabilize the conformation of the α4−α5 loop by hydrogen bonding to the peptide backbone, events which possibly result in an appropriate orientation of the α-helix (α5) containing active site residues. Mutants N71T and N71S showed a remarkable shift from uracil to guanine specificity, as evaluated by cleavage of CpG, although they did exhibit uridine specificity against yeast RNA and homopolynucleotides.

The 2′,3′-cyclizing ribonucleases (RNases),[1] which cleave phosphodiester bonds of RNA to 3′-nucleotides reaction via 2′,3′-cyclic nucleotides, have been studied in plant cells for many years and classified into the RNase T2 family, according to their molecular weights and amino acid sequences (*1*). The ribonuclease MC1 (RNase MC1), isolated from bitter gourd seeds, consists of a single polypeptide chain of 190 amino acids with a relative molecular mass of 21 222 Da, and it belongs to the RNase T2 family (*2*). RNase MC1 specifically cleaves a phosphodiester bond of NpU (where N is either A, C, or U); the most favorable substrate is CpU (*3*). This absolute uracil specificity distinguishes RNase MC1 from other RNases grouped into the RNase T2 family which cleave almost all of the 16 dinucleoside monophosphates, at a comparable rate, although all do show unique base preferences (*4*). The specificity of RNase MC1 set by uracil at the 3′-terminal side of dinucleoside monophosphates is distinct from other RNases, including those in the RNase A and RNase T1 families. Generally, RNases have two distinct base binding sites: the primary site (B1 site) and subsite (B2 site), for bases located at 5′- and 3′-terminal ends of the scissile bond, respectively. Base specificity usually relates to the nature of the interaction of the B1 site with the base located at the 5′-terminal end (*5*). Thus, a strong interaction between the B2 site and the uridine at the 3′-terminal end is also a characteristic feature found in RNase MC1.

We determined the three-dimensional structure of RNase MC1 in complex with 2′-UMP and 3′-UMP at 1.48 and 1.77 Å resolution, respectively (*6*). In the complexes, the side chains of Gln9 and Asn71 interact with O4 and N3, respectively, of the uracil base by hydrogen bonding (Figure 1). Additionally, the side chains of Leu73 and Phe80 form a hydrophobic pocket, which binds the uracil base by sandwich-like stacking interactions enhancing hydrogen

* To whom correspondence should be addressed at the Laboratory of Biochemistry, Faculty of Agriculture, Kyushu University, Hakozaki 6-10-1, Fukuoka 812-8581, Japan. Tel (Fax): +81-92-642-2854. E-mail: mkimura@agr.kyushu-u.ac.jp.

‡ Kyushu University.

§ Hokkaido University.

[1] Abbreviations: AMP, adenosine monophosphate; CD, circular dichroism; CMP, cytidine monophosphate; CpA, cytidylyl-3′,5′-adenosine; CpC, cytidylyl-3′,5′-cytidine; CpG, cytidylyl-3′,5′-guanosine; CpN, cytidylyl-3′,5′-nucleoside; CpU, cytidylyl-3′,5′-uridine; GMP, guanosine monophosphate; HPLC, high-performance liquid chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; NpU, nucleoside-3′,5′-uridine; RNase, ribonuclease; RNase LE, ribonuclease from cultured tomato (*Lycopersicon esculentum*); RNase MC1, ribonuclease from bitter gourd (*Momordica charantia*); RNase NW, a wound-inducible ribonuclease from leaves of *Nicotiana glutinosa*; RNase Rh, ribonuclease from *Rhizopus niveus*; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; UMP, uridine monophosphate.

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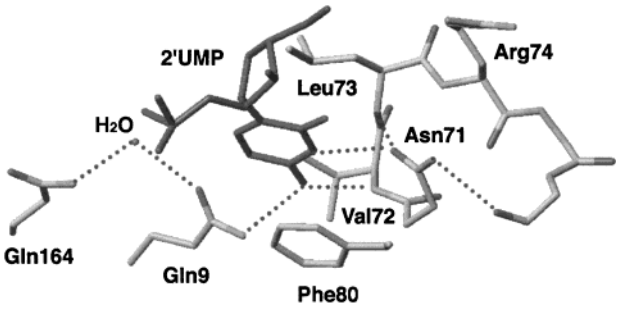

FIGURE 1: Tertiary structure of the 2′-UMP binding site in RNase MC1. The hydrogen bonding network is drawn by dashed lines. Side chains are shown for residues where the contribution to 2′-UMP binding was suggested. In addition, side chains of amino acids examined in this study are also shown.

bonding between the enzyme and base. When these amino acids are compared with corresponding residues in RNases in the RNase T2 family, Gln9 and Phe80 are conserved in RNases in the family, while Asn71 and Leu73 in RNase MC1 are variant in sequences (Figure 2). It is thus suggested that interactions of side chains of Asn71 and Leu73 with the uracil base are responsible for the absolute uridine specificity of RNase MC1.

To evaluate the contribution of Asn71 and Leu73 to the enzymatic activity of RNase MC1, we made use of site-directed mutagenesis of the RNase MC1 cDNA. We report here effects on the enzymatic activity of RNase MC1 when we replaced Asn71, Val72, Leu73, and Arg74, at the α4−α5 loop between α4 and α5 helices (Figure 1), by equivalent amino acid residues in a guanylic acid preferential RNase NW in *Nicotiana glutinosa* leaves (*7*). The role of Asn71 was also investigated after we substituted amino acids, Ala, Ser, Gln, and Asp.

MATERIALS AND METHODS

Materials. The 3′,5′-dinucleoside monophosphates and yeast RNA were purchased from Sigma Chemicals. Restriction endonucleases and DNA-modifying enzymes were obtained from either Toyobo or MBI Fermentas. Plasmid pGEM-T vector and the expression plasmid pET-22b were from Promega and Novagen, respectively. A Chameleon double-stranded site-directed mutagenesis kit was obtained from Stratagene. The oligonucleotides used in this study and Thermo Sequenase fluorescent-labeled primer cycle sequencing kit containing 7-deaza-dGTP were from Amersham Pharmacia Biotech. All other reagents and chemicals were of analytical grade.

Preparation of the Recombinant RNase MC1 and Its Mutants. Site-directed mutagenesis was done using the unique site elimination method (*8*). Mutations were identified by DNA sequence determination; the entire sequence of each mutant gene was determined to rule out that no additional mutations had arisen during the mutagenesis reaction steps. For DNA sequencing, we used a DNA sequencer, DSQ-1000 (Shimadzu), and a Thermo Sequenase fluorescent-labeled primer cycle sequencing kit containing 7-deaza-dGTP (Amersham Pharmacia Biotech). The recombinant RNase MC1 and the mutant proteins were purified by SP−Sepharose Fast Flow followed by butyl - Toyopearl 650M from *Escherichia coli* BL21(DE3) pLysS, as follows: after sonication of *E. coli* cells, the supernatant was first put on a SP−Sepharose column (16 × 80 mm) that had been equilibrated with 10 mM sodium phosphate buffer (pH 6.5). The proteins were eluted with a linear gradient of 0−0.5 M NaCl, in the same buffer, using a gradient volume of 150 mL. Samples of the fractions were analyzed by SDS−PAGE (*9*) followed by activity-staining (*10*). The recombinant RNase MC1 and its mutant enzymes were further purified by hydrophobic chromatography on butyl-Toyopearl 650M (16 × 50 mm) in 10 mM sodium phosphate buffer (pH 6.5). The elution process followed a linear gradient of 1.5−0 M $(NH_4)_2SO_4$ using a gradient volume of 150 mL. The purities of enzymes thus obtained were checked by SDS−PAGE, using a 15% polyacrylamide slab gel (*9*). Protein concentrations were determined by the bicinchoninic acid methods (*11*), using BSA as a standard protein.

Far-Ultraviolet CD. CD spectra in the far-ultraviolet range, 200−250 nm, were recorded at room temperature on a Jasco J-720 spectropolarimeter. Proteins were dissolved to a final concentration of 100 μg/mL in distilled water. Signal averaging during the accumulation of four scans was done automatically.

RNA Hydrolysis Activity. RNase activity toward high molecular weight RNA was determined by the method of Irie et al. (*3*). Briefly, a total volume of 450 μL, containing 100 μg of yeast RNA in 50 mM sodium phosphate buffer (pH 6.5), 20 mM EDTA or 100 μg of homopolyribonucleotides in 50 mM sodium phosphate buffer (pH 6.5), was incubated with the enzymes (0.2−10 nM) for 4−30 min at 37 °C. After adding 200 μL of precipitation solution (20 mM lanthane nitrate in 15% perchloric acid), the nonhydrolyzed RNA was precipitated on ice for 20 min. After centrifugation, the supernatant (100 μL) was diluted with 900 μL of distilled water. The absorbency of the diluted solution was measured at 260 nm for poly A, poly U, and yeast RNA, at 250 nm for poly I, or at 280 nm for poly C.

Dinucleoside Monophosphate Cleavage. The enzymatic activity toward dinucleoside monophosphates was measured in 50 μL of 50 mM sodium phosphate buffer (pH 6.5), consisting of 10−1000 nM enzyme and appropriate amounts of substrate (8.0×10^{-5} to 1.1×10^{-2} M). The incubation was carried out at 37 °C for 5−60 min, and the reaction was terminated by adding a 50 μL portion of 30% acetic acid. Ten microliters of the reaction mixture was loaded onto reverse-phase HPLC on a Wakosil $5C_{18}$ column (4.6 × 150 mm) equilibrated with 50 mM KH_2PO_4. The reaction product, nucleosides, was eluted with an increasing 80% aqueous acetonitrile concentration at the flow rate of 1.0 mL/min. Kinetic parameters, K_m and k_{cat}, were calculated from a Lineweaver−Burk plot.

Release of Four Nucleotides from RNA. A small volume of the enzyme was added to a reaction mixture (1 mL) comprised of 5 mg of yeast RNA in 50 mM sodium phosphate buffer (pH 6.5) containing 20 mM EDTA. The reactions were at 37 °C, and 100 μL samples were withdrawn at appropriate intervals. Ten volumes of ice-cold ethanol were added to the samples, which were then kept overnight at −80 °C. The precipitate formed was removed by centrifugation, and the supernatants were dried in vacuo. The dried samples were dissolved in distilled water. The nucleotides were separated by HPLC on a column of TSK gel ODS 80TM (4.6 × 250 mm) equilibrated with 0.1% TFA. The column was eluted with a linear gradient of 0−1% acetonitrile in

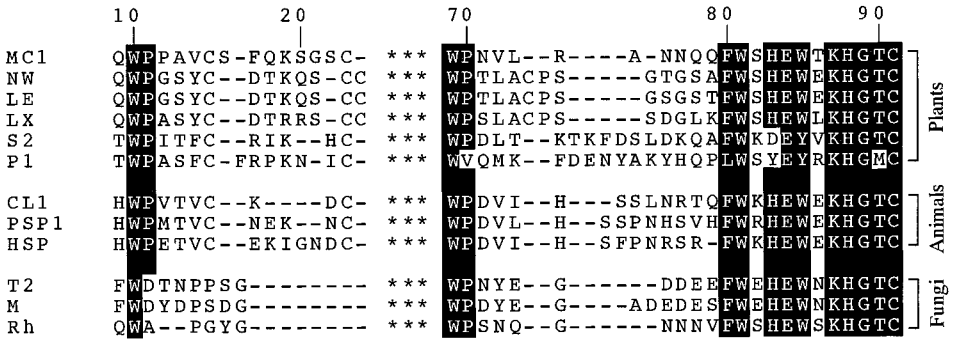

FIGURE 2: Comparison of amino acid residues involved in uracil binding of RNase MC1 with the corresponding residues in RNase T2 family RNases. MC1, RNase MC1 from *Momordica charantia* (*2*); NW, RNase NW from *Nicotiana glutinosa* (*7*); LE, RNase LE from *Lycopersicon esculentum* (*13*); LX, RNase LX from *Lycopersicon esculentum* (*18*); S2, a self-incompatibility RNase from *N. alata* (*19*); P1, RNase from *Petunia inflata* (*20*); CL1, RNase from chicken liver (*21*); PSP1, RNase from porcine spleen (*22*); HSP, human spleen RNase (*23*); T2, RNase T2 from *Aspergillus oryzae* (*24*); M, RNase M from *Aspergillus saitoi* (*25*); Rh, RNase Rh from *Rhizopus niveus* (*26*). The amino acid residues conserved in RNase T2 family RNases are indicated in white letters. The numbers at the top represent RNase MC1 numbering.

0.1% TFA during 30 min. The flow rate was 1 mL/min, and the elute was monitored by measuring the absorption at 254 nm.

RESULTS

Production and Characterization of Mutant Enzymes. To evaluate the contribution of Asn71 and Leu73 to a specific recognition of uridine by RNase MC1, Asn71 and Leu73 as well as their flanking residues, Val72 and Arg74, located at the α4−α5 loop between α4 and α5 helices were in turn replaced by the corresponding amino acids in a guanylic acid preferential RNase NW from *N. glutinosa* leaves. RNase NW is a wound-inducible RNase in *N. glutinosa* leaves, and its base specificity was considered, based on the relative rates of hydrolysis of homopolyribonucleotides to be a preference for guanine (*7*). The four mutant enzymes (N71T, V72L, L73A, R74S) were synthesized in *E. coli* BL21(DE3) pLysS cells, using the expression plasmid pET-22b, and then purified to be homogeneous by SDS−PAGE, as described under Materials and Methods (data not shown). All four mutants were produced in an amount similar to that for the wild-type RNase MC1 recombinant protein; the yields of the enzymes from 1 L of cultured broth were about 0.5 mg for all mutant enzymes. The secondary structure of each mutant enzyme was checked by examining the circular dichroic (CD) spectrum in the 200−250 nm region (Figure 3). The CD spectra of mutants in the short-wavelength region (200−250 nm), reflecting backbone polypeptide chain conformations, were essentially the same as that of the wild-type RNase MC1. Therefore, the backbone conformation of the mutant proteins seemed to be practically the same as that of RNase MC1.

Enzymatic Properties of Mutant Proteins. When we used yeast RNA as substrate, the enzymatic activities of the mutant enzymes N71T and L73A, in which Asn71 and Leu73 were replaced with the corresponding residues Thr and Ala in RNase NW, were about 47.5 and 70.2% of the wild-type enzyme, respectively (Figure 4A). In contrast, mutations of Val72 and Arg74 by Leu and Ser had little effect on the enzymatic activity: the mutants V72L and R74S were almost as active as the wild-type, exhibiting 96.3 and 82.0% activities, respectively (Figure 4A). pH optima of the four mutant enzymes were between pH 6.0 and 7.0 (data not shown), values similar to that of the wild-type enzyme (pH 6.5). Therefore, the decrease in enzymatic activity found in the mutant enzymes N71T and L73A could not be attributed to a change in the optimal pH.

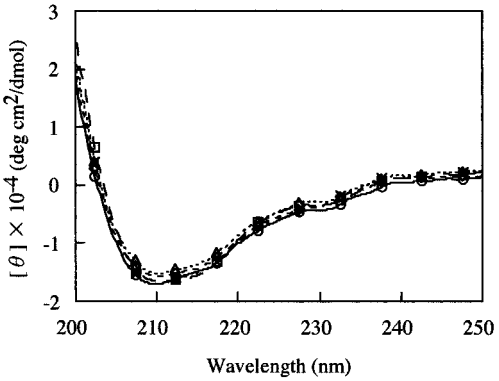

FIGURE 3: Circular dichroic spectra of RNase MC1 and its mutant enzymes in the far-ultraviolet region. The spectra were measured on a JASCO-J 720 spectropolarimeter at 25 °C. The protein concentration was 100 μg/mL, as described under Materials and Methods. ○, □, ◇, ×, and △ indicate the CD spectra of RNase MC1, N71T, V72L, L73A, and R74S, respectively.

Since RNase MC1 preferentially cleaves CpU among four dinucleoside monophosphates, NpU (N denotes A, G, C, or U) (*3*), the kinetic constants of four mutant proteins for CpU were measured. The kinetic parameters thus obtained are summarized in Table 1. Consistent with the result obtained using yeast RNA as a substrate, the mutants V72L and R74S retained the enzymatic activity to a same extent as did the wild-type RNase MC1. Mutation of Asn71 to Thr resulted in a 7.0-fold increase in K_m and a 2.3-fold decrease in k_{cat}, compared with the wild-type enzyme. Furthermore, the Leu73 to Ala mutation resulted in a 14.4-fold increase in K_m, although it had little effect on the value of k_{cat}. Thus, Asn71 plays an important role in the enzymatic activity, contributing to both catalytic efficiency and substrate affinity, while Leu73 predominantly contributes to the substrate affinity of RNase MC1. The resulting specificity constants (k_{cat}/K_m) of N71T and L73A were reduced 16- and 14-fold, respectively, in comparison to that of the wild-type enzyme.

Characterization of the Asn71 Mutants. The present study showed that the Asn71 to Thr mutation reduced both substrate binding and catalysis, suggesting that Asn71 plays

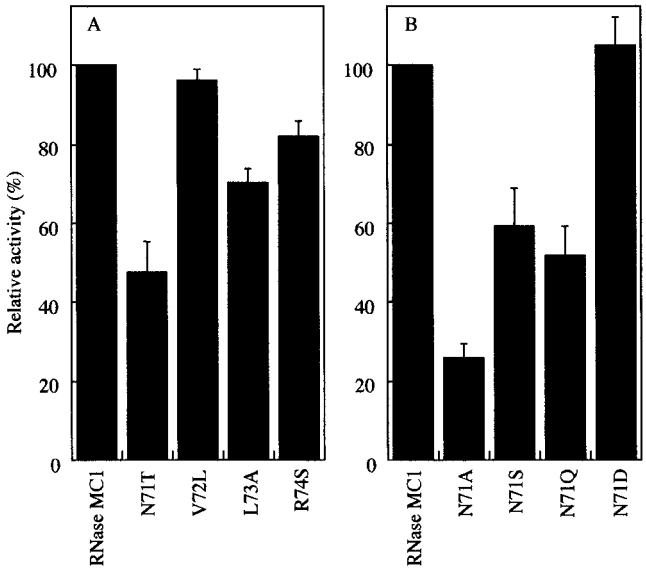

FIGURE 4: RNA degrading activity of RNase MC1 and its mutants. The enzymatic activity of RNase MC1 and its mutants was assessed by following the increase in acid-soluble nucleotides after digestion of yeast RNA at pH 6.5 and 37 °C, as described by Irie et al. (*3*). Enzymatic activity is expressed relative to that of wild-type. Values are expressed as averages with standard deviations.

essential roles in uracil recognition as well as in catalytic activity. To gain insight into the role of Asn71 in the enzyme reaction, we further substituted Asn71 for Ala, Ser, Gln, and Asp, and the resulting mutants were characterized in terms of enzyme activity. The Ser, Gln, and Asp residues were chosen because they occur at equivalent positions in fungal, plant, and animal RNases belonging to the RNase T2 family (Figure 2), and Ala caused a loss of the side chain at position 71. The mutant enzymes were purified to apparent homogeneity by procedures identical to those used to describe the mutant enzymes described above. The structure and optimum pH of the mutants were first evaluated by CD spectra and by measuring their activity to hydrolyze yeast RNA, respectively. The CD spectra of these mutants were essentially identical to that of wild-type (data not shown), indicating that the secondary structure was not effected by substitutions of the Asn residue. All the mutant enzymes used, except N71Q, have pH optima between pH 6.0 and 7.0, which is similar to findings in the wild-type. The mutant N71Q has an optimum pH around 5.5, for which we have no appropriate explanation. The enzyme activity for N71Q was therefore measured at pH 5.5.

Replacing Asn71 with Ala generated an enzyme of lower activity, with 26.1% activity compared with wild-type, and substitutions of Asn71 for Ser and Gln resulted in slightly less activity enzymes, with 59.3 and 51.8% activities, respectively (Figure 4B). In contrast, the Asn71 to Asp mutation slightly increased the enzyme activity (Figure 4B). Kinetic parameters for the four mutant enzymes toward CpU were measured and are summarized in Table 1. Substitution of Asn71 for Ala drastically reduced both substrate binding and catalysis, findings which further support the notion that Asn71 plays essential roles in both uracil binding and catalysis for RNase MC1. Substitutions of Asn71 for Ser and Asp also reduced the binding affinity by 8.6-fold and 2.1-fold, respectively, but here k_{cat} values increased, as compared with that of wild-type enzyme. Particularly, the Asp substitution increased k_{cat} by 4.7-fold against CpU. The Gln substitution had little effect on substrate binding, but it slightly decreased k_{cat}. Consequently, Ala and Thr at position 71 reduced both binding affinity and catalysis, whereas Ser, Asp, or Gln affected either substrate binding or catalysis, respectively.

Substrate Specificity of Mutant Enzymes. In the present mutagenesis study, the amino acids essential for uridine recognition by RNase MC1 were first replaced by corresponding residues in the guanylic acid preferential RNase NW (*7*). It could be thus anticipated that mutant proteins might have acquired a guanylic acid instead of uridine. Hence, the substrate specificity of the two mutant enzymes N71T and L73A was first analyzed, using four dinucleoside monophosphates (CpN, where N denotes A, G, C, or U). The initial rate of the cleavage reaction of CpU for mutants N71T and L73A decreased 5.9 and 6.6%, respectively, compared with that of the wild-type RNase MC1 (Figure 5). Interestingly, the mutant N71T had a significantly higher activity in CpG cleavage while the other three dinucleoside monophosphate cleavage activities were negligible. In contrast, L73A exhibited lower enzymatic activity toward all four dinucleoside monophosphates.

We further characterized four Asn71 mutants in terms of an initial rate of the cleavage reaction of CpN. As shown in Figure 5, the mutant N71S, as in the case for the mutant enzyme N71T, exhibited a significant catalytic activity toward CpG, although the three other mutant enzymes showed no significant catalytic activity toward CpN, except for CpU (Figure 5). The kinetic constants for N71T and N71S as well as RNase MC1 toward CpG were then measured (Table 1). The K_m and k_{cat} values of N71T decreased 17-fold and increased 6.8-fold, respectively, and N71S showed a decrease in K_m of 14-fold and an 8.1-fold increase in catalysis, compared with wild-type. The resulting specificity constants of N71T and N71S toward CpG increased about 114- and 112-fold, in comparison to the wild-type, showing that the two mutants acquired enzymatic activity toward CpG, although the wild-type enzyme had a little activity toward the substrate.

Next, rates of release of the four nucleotides from yeast RNA by mutants N71T and N71S during the course of hydrolysis were measured using HPLC, as described under Materials and Methods. They released UMP most rapidly; this tendency is similar to that of RNase MC1 (Figure 6). The substrate specificity of the two mutant enzymes was further evaluated by hydrolysis of four homopolynucleotides. They rapidly cleaved poly U, and the rates of hydrolysis of the other three were much lower than that of RNA (data not shown). Thus, base specificities of N71T and N71S toward high molecular weight RNA are similar to that of the wild-type enzyme. This observation suggested that RNase MC1, as well as the B1 and B2 sites, may possess additional base binding site(s) which render uridine specificity for RNase MC1.

DISCUSSION

Our recent crystal structure of the RNase MC1−uridylic acid complexes shows a sandwich-like stacking interaction between the hydrophobic side chains of Leu73 and Phe80, and hydrogen bondings of Asn71 and Gln9 with the uracil

Table 1: Steady-State Kinetic Parameters for Cleavage of Dinucleoside Monophosphates by Wild-Type and Mutant RNase MC1[a]

	CpU			CpG		
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
RNase MC1	1.38 ± 0.2 (1.0)	746 ± 95 (1.0)	542 ± 50 (1.0)	20.2 ± 1.4 (1.0)	30.8 ± 0.6 (1.0)	1.53 ± 0.1 (1.0)
N71T	9.60 ± 1.5 (7.0)	322 ± 16 (0.4)	33.8 ± 3.6 (0.06)	1.18 ± 0.2 (0.06)	209 ± 92 (6.8)	174 ± 48 (114)
V72L	1.57 ± 0.2 (1.1)	738 ± 88 (1.0)	469 ± 4.6 (0.9)	ND	ND	ND
L73A	19.9 ± 3.6 (14.4)	786 ± 120 (1.1)	39.6 ± 1.1 (0.07)	ND	ND	ND
R74S	1.29 ± 0.2 (0.9)	511 ± 82 (0.7)	395 ± 13 (0.7)	ND	ND	ND
N71A	32.2 ± 3.5 (23.3)	73.7 ± 8.6 (0.1)	2.29 ± 0.0 (0.004)	ND	ND	ND
N71S	11.8 ± 0.8 (8.6)	1079 ± 5.7 (1.4)	91.3 ± 5.8 (0.2)	1.46 ± 0.2 (0.07)	249 ± 43 (8.1)	171 ± 12 (112)
N71Q	1.77 ± 0.3 (1.3)	236 ± 45 (0.3)	133 ± 14 (0.2)	ND	ND	ND
N71D	2.93 ± 0.3 (2.1)	3481 ± 412 (4.7)	1187 ± 30 (2.2)	ND	ND	ND

[a] K_m, k_{cat}, and k_{cat}/K_m values are expressed as $\times 10^3$ M min^{-1}, and $\times 10^{-3}$ M^{-1} min^{-1}, respectively. All parameter values are average values for at least two independent experiments. The values in parentheses show the value relative to that of the wild-type RNase MC1. ND was not determined.

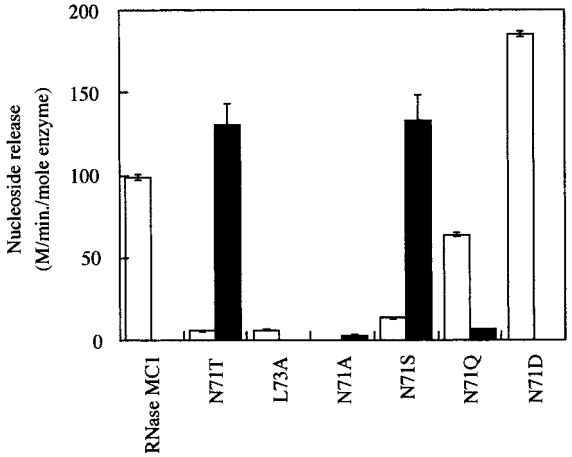

FIGURE 5: Relative rate of cleavage of dinucleoside monophosphates by RNase MC1 and its mutants. The enzymatic activity of RNase MC1 and its mutants was assessed using reverse-phase HPLC on a Wakosil $5C_{18}$ column (4.6 × 150 mm) equilibrated with 50 mM KH_2PO_4, as described under Materials and Methods. Since the cleavage activities toward CpA and CpC were negligible [<0.65 M min^{-1} (mol of enzyme)$^{-1}$], they are not indicated in the figure. Values are means ± SD. □, CpU; ■, CpG.

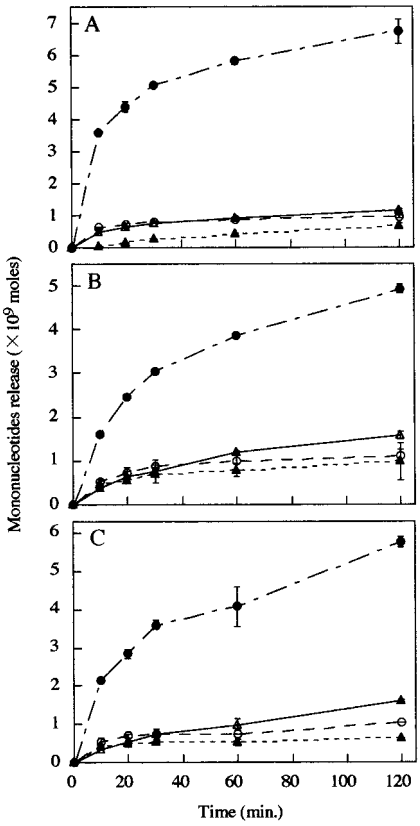

FIGURE 6: Release of four nucleotides during the course of hydrolysis of RNA by RNase MC1 and the mutants. Yeast RNA was digested by RNase MC1 (A), the mutant N71T (B), and the mutant N71S (C) in 50 mM sodium phosphate buffer (pH 6.5) containing 20 mM EDTA at 37 °C, and the release of each nucleotide (2′,3′-cyclic nucleotide + 3′-nucleotide) was monitored, as described under Materials and Methods. △, AMP; ○, CMP; ▲, GMP; ●, UMP. Values are means ± SD.

base are involved in recognition of the uracil base (*6*). Since Gln9 and Phe80 are conserved in base-nonspecific RNases, Asn71 and Leu73 may be responsible for the absolute uridine specificity of RNase MC1 (*6*). The present study indicated that replacements of Asn71 and Leu73 reduced the K_m values for CpU, compared with that of the wild-type, demonstrating that they do play an essential role in recognition of the uracil base at the B2 site in the enzyme reaction. When comparing the contribution of Asn71 and Leu73 to uracil binding, since the K_m value of N71A toward CpU is slightly larger than that of L73A, the hydrogen bonding interaction of Asn71 with the uracil base seems to be more essential for uracil recognition than does the stacking interaction of Leu73. A crystal structure of plant RNase LE from tomato cultured cells has been determined at 1.65 Å resolution (*12*). RNase LE, the tomato orthologue of RNase NW from *N. glutinosa*, is a phosphate-starvation-induced RNase and preferentially hydrolyzes guanylic acid, although it does exhibit nonabsolute guanine specificity (*13*). It is postulated from the crystal structure that a hydrophobic pocket containing the side chains of Tyr17 (Val14 in RNase MC1) and Phe89 (Phe80 in RNase MC1) is the B2 site of RNase LE, and their stacking interaction with guanine base may be responsible for the guanylic acid preferential. Since Tyr and Phe residues are highly conserved in sequences among plant RNases, as shown in Figure 2, their interaction with the guanine base at the B2 site seems to be a general recognition mode for plant RNases. This recognition mode is, however, slightly different from that of RNase MC1. The side chain of Val14 in RNase MC1 is evidently too far away to interact with the uracil base moiety bound at the B2 site (*6*). Instead, Leu73 in RNase MC1 that is not conserved in other plant RNases participates in a stacking interaction with the base, together with Phe80. Hence, substitution of Tyr for Val at position 14 and those with Asn and Leu at positions 71 and 73, respectively, are attributed to a unique uridine preference of RNase MC1, as compared with other plant RNases.

The present study showed that Thr and Ala at position 71 reduced both substrate binding and catalysis, whereas Ser, Asp, or Gln influenced either substrate binding or catalysis, respectively. This means that Asn71 has at least two roles: one is base recognition by hydrogen bonding, and the other is to enhance catalysis. In crystal structures of the complexes, in addition to hydrogen bonding to the uracil base, the amide hydrogen and oxygen atoms of the side chain of Asn71 are hydrogen bonded to the main-chain carbonyl oxygen of Ala75 and the main-chain amide hydrogen atom of Leu73, respectively (*6*). A hydrogen bond of the side chain of Asn71 with uracil may contribute to substrate binding, whereas interactions with Leu73 and Ala75 backbones may be involved in catalysis, possibly by stabilizing the conformation of the α-helix (α5) containing active site residues His83, Glu84, Lys87, and His88 (*14*). If this is the case, since the Asn71 to Ser or Asp mutations increased catalysis toward a dinucleoside monophosphate, CpU, the Ser and Asp residues in fungal and animal RNases, as compared with Asn71 in RNase MC1, may more strongly stabilize the loop structure, thereby enhancing catalysis. By contrast, plant RNases, such as RNase NW and RNase LE, have an unfavorable Thr at the position equivalent to Asn71 in RNase MC1. In this context, it is of interest to note that plant RNases with Thr at the position equivalent to Asn71 in RNase MC1, such as RNase NW and RNase LE, have an extra disulfide bond in the neighborhood of the Thr residue. A crystal structure analysis of RNase LE showed that the disulfide bond between the $\beta1-\beta2$ loop and the αB−αC loop stabilizes their conformation (*12*). Hence, the extra disulfide bond in RNase NW and RNase LE probably plays a role equivalent to that of Asn71 in RNase MC1.

The base specificity for RNases grouped into RNase A and RNase T1 families has been extensively studied and well characterized, at an atomic resolution. These studies showed that the base specificity is generally attributable to the nature of the interaction of the B1 site with bases. This is also the case for RNases in the RNase T2 family. Fungal RNases, such as RNase Rh and RNase M, are adenylic acid preferential, and the crystal structure of the complex made by fungal RNase Rh and adenylic acid showed a strong interaction of adenine with amino acids composed of the B1 site (*15*). It is assumed that plant RNases in the RNase T2 family, as for RNase MC1, more strongly recognize bases at the B2 site than at the B1 site. This suggests that amino acid residues with the B2 site play a more essential role in base recognition by plant RNases than do those of the B1 site. In the present study, the Asn71 to Thr or Ser mutations, which occur in guanylic acid preferential *N. glutinosa* RNase NW or tomato RNase LE and RNase LX, showed a shift from uridine to guanosine specificity, a finding consistent with the base preference of these plant enzymes. This finding demonstrates that Thr or Ser in plant RNases at the equivalent position to Asn71 in RNase MC1 serves as one of the determinants for base preference for these enzymes. In contrast, the two mutant enzymes N71S and N71D exhibited no adenylic acid preferential, even when Asn71 was substituted for the corresponding amino acids Ser and Asp in adenylic acid preferential RNase Rh and RNase M, respectively. This is probably because fungal RNases recognize the base by means of amino acid residues composed of the B1 site.

One of the challenging subjects in protein engineering for RNase is to generate a novel base-specific enzyme. To date, efforts have been directed mainly to guanine-specific RNase T1 as a prototype. However, attempts to change the substrate specificity of this enzyme have been unsuccessful. Thus, a number of single amino acid substitutions within the guanine binding site had effects on enzyme activity, whereas the specificity was not altered (*16*, *17*). In the present study, substitution of Asn71 for Thr and Ser led to about a 100-fold increased specificity constant (k_{cat}/K_m) toward CpG, compared to the wild-type enzyme, although they did exclusively exhibit uridine specificity as demonstrated by the hydrolysis of yeast RNA and homopolynucleotides. In addition, the Asn71 to Asp mutation significantly enhanced the enzyme activity toward CpU as well as to poly U. These observations suggest that more extensive studies on the recognition mechanism by which RNase MC1 specifically cleaves the 5′-side of uridine will pave the way for protein engineers studying RNases.

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REFERENCES

1. Green, P. J. (1994) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 45*, 421−445.
2. Ide, H., Kimura, M., Arai, M., and Funatsu, G. (1991) *FEBS Lett. 284*, 161−164.
3. Irie, M., Watanabe, H., Ohgi, K., Minami, Y., Yamada, H., and Funatsu, G. (1993) *Biosci. Biotechnol. Biochem. 131*, 191−202.
4. Horiuchi, H., Yanai, K., Takagi, M., Yano, Y., Wakabayashi, E., Sanda, A., Mine, S., Ohgi, K., and Irie, M. (1988) *J. Biochem. 103*, 408−418.
5. Steyaert, J. (1997) *Eur. J. Biochem. 247*, 1−11.
6. Suzuki, A., Yao, M., Tanaka, I., Numata, T., Kikukawa, S., Yamasaki, N., and Kimura, M. (2000) *Biochem. Biophys. Res. Commun. 275*, 572−576.
7. Kariu, T., Sano, K., Shimokawa, H., Itoh, R., Yamasaki, N., and Kimura, M. (1998) *Biosci. Biotechnol. Biochem. 62*, 1144−1151.
8. Deng, W. P., and Nickoloff, J. A. (1992) *Anal. Biochem. 200*, 81−88.
9. Laemmli, U. K. (1970) *Nature 227*, 680−685.
10. Abel, S., and Glund, K. (1986) *Physiol. Plant 66*, 79−86.
11. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Garther, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem. 150*, 76−85.
12. Tanaka, N., Arai, J., Inokuchi, N., Koyama T., Ohgi, K., Irie, M., and Nakamura, K. (2000) *J. Mol. Biol. 298*, 859−873.
13. Nurnberger, T., Abel, S., Jost, W., and Glund, L. (1990) *Plant Physiol. 92*, 970−976.
14. Numata, T., Kashiba, T., Hino, M., Funatsu, G., Ishiguro, M., Yamasaki, N., and Kimura, M. (2000) *Biosci. Biotechnol. Biochem. 64*, 603−605.
15. Kurihara, H., Nonaka, T., Mitsui, Y., Ohgi, K., Irie, M., and Nakamura, K. T. (1996) *J. Mol. Biol. 255*, 310−320.
16. Grunert, H.-P., Landt, O., Zirpel-Giesebrecht, M., Backmann, J., Heinemann, U., Saenger, W., and Hahn, U. (1993) *Protein Eng. 6*, 739−744.
17. Loverix, S., Doumen, J., and Steyaert, J. (1997) *J. Biol. Chem. 272*, 9635−9639.
18. Loffler, A., Glund, K., and Irie, M. (1993) *Eur. J. Biochem. 214*, 627−633.

19. McClure, B. A., Haring, V., Ebert, P. R., Anderson, M. A., Simpson, R. J., Sakiyama, F., and Clarke, A. E. (1989) *Nature 342*, 955−958.
20. Ai., T., Singh, A., Coleman, C. E., Ioerger, T. R., Kheyr-Pour, A., and Kao, T.-H. (1990) *Sex. Plant Reprod. 3*, 130−138.
21. Uchida, T., Hayano, K., Iwana, M., Watanabe, H., Sanda, A., Ohgi, K., and Irie, M. (1996) *Biosci. Biotechnol. Biochem. 60*, 1982−1988.
22. Kusano, A., Iwama, M., Sanda, A., Suwa, K., Nakaizumi, E., Nakatani, Y., Ohkawa, H., Ohgi, K., and Irie, M. (1997) *Acta Biochim. Pol. 44*, 689−700.
23. Trubia, M., Sessa, L., and Taramelli, R. (1997) *Genomics 42*, 342−344.
24. Kawata, Y., Sakiyama, F., and Tamaoki, H. (1988) *Eur. J. Biochem. 176*, 683−697.
25. Watanabe, H., Naitoh, A., Suyama, Y., Inokuchi, N., Shimada, H., Koyama, T., Ohgi, K., and Irie, M. (1990) *J. Biochem. 108*, 303−310.
26. Horiuchi, H., Yanai, K., Takagi, M., Yano, K., Wakabayashi, E., Sanda, A., Mine, S., Ohgi, K., and Irie, M. (1988) *J. Biochem. 103*, 408−418.

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