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Contribution of Gln9 and Phe80 to Substrate Binding in Ribonuclease MC1 from Bitter Gourd Seeds

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Ribonuclease MC1 (RNase MC1) isolated from bitter gourd (*Momordica charantia*) seeds specifically cleaves phosphodiester bonds on the 5′-side of uridine. The crystal structures of RNase MC1 in complex with 2′-UMP or 3′-UMP reveal that Gln9, Asn71, Leu73, and Phe80 are involved in uridine binding by hydrogen bonding and hydrophobic interactions [Suzuki *et al.* (2000) *Biochem. Biophys. Res. Commun.* 275, 572–576]. To evaluate the contribution of Gln9 and Phe80 to uridine binding, Gln9 was replaced with Ala, Phe, Glu, or His, and Phe80 with Ala by site-directed mutagenesis. The kinetic properties of the resulting mutant enzymes were characterized using cytidylyl-3′,5′-uridine (CpU) as a substrate. The mutant Q9A exhibited a 3.7-fold increased K_m and 27.6-fold decreased k_{cat}, while three other mutations, Q9F, Q9E, and Q9H, predominantly affected the k_{cat} value. Replacing Phe80 with Ala drastically reduced the catalytic efficiency (k_{cat}/K_m) with a minimum K_m value equal to 8 mM. It was further found that the hydrolytic activities of the mutants toward cytidine-2′,3′-cyclic monophosphate (cCMP) were reduced. These results demonstrate that Gln9 and Phe80 play essential roles not only in uridine binding but also in hydrolytic activity. Moreover, we produced double Ala substituted mutants at Gln9, Asn71, Leu73, and Phe80, and compared their kinetic properties with those of the corresponding single mutants. The results suggest that these four residues may contribute to uridine binding in a mutually independent manner.

Key words: additive, *Momordica charantia*, RNase MC1, site-directed mutagenesis, substrate binding.

Ribonuclease MC1 (RNase MC1; EC 3.1.27.1) isolated from bitter gourd (*Momordica charantia*) seeds consists of 190 amino acids (*1*) and belongs to the RNase T2 family typified by fungal RNases, such as RNase T2, RNase M, and RNase Rh (*2*). The distinct feature (*3*) that RNase MC1 specifically cleaves the P-O5′ ester bond of NpU (where N is either A, C, G, or U) distinguishes it from other RNases in the RNase T2 family, which cleave almost all 16 dinucleoside monophosphates at a comparable rate (*2*). In general, RNases have two distinct base binding sites, the primary site (B1 site) and subsite (B2 site), for bases located at the 5′- and 3′-terminal ends of a scissile bond, which is cleaved by the action of a P1 site formed by catalytic residues (*4*). 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

The three-dimensional structures of RNase MC1 complexed with 2′-UMP or 3′-UMP reveal that the uracil moiety forms extensive hydrogen bonds with two N-H donors [Nε2 of Gln9 and the main-chain amide N-H of Val72 with uracil O(4)] and one oxygen acceptor [Oδ1 of Asn71 with uracil N(3)H] (Fig. 1) (*6*). In addition, the uracil base is sandwiched between the aliphatic side chain of Leu73 and the aromatic side chain of Phe80, which form a hydrophobic pocket at the B2 site (*6*). This finding suggests that Gln9, Asn71, Leu73, and Phe80 are strongly involved in uridine binding by RNase MC1. Since Asn71 and Leu73 are variable among RNase T2 family enzymes, it was expected that the two residues are responsible for the uridine specificity of RNase MC1. Our foregoing study by site-directed mutagenesis demonstrated the importance of Asn71 and Leu73 for the uridine specificity for RNase MC1 (*7*).

In contrast to Asn71 and Leu73, Gln9 and Phe80 in RNase MC1 are invariant among plant RNases belonged into the RNase T2 family. In particular, Phe80 is highly conserved in RNase T2 family proteins, although Gln9 is replaced by Phe, Glu, or His in animal or fungal RNases (*2*). This observation suggests that besides a specific interaction with the uracil base, Gln9 and Phe80 in RNase MC1 may play an essential role in enzyme activity. Therefore, a detailed study of Gln9 and Phe80 in RNase MC1 may provide general insight into understanding molecular mechanism of RNase T2 family enzymes. In this study, we produced mutant enzymes at Gln9 and Phe80, and the resulting mutants were characterized in terms of their transphosphorylation and hydrolysis activities toward CpU and

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Abbreviations: CD, circular dichroism; cCMP, cytidine-2′,3′-cyclic monophosphate; CpU, cytidylyl-3′,5′-uridine, RNase MC1, ribonuclease MC1 from bitter gourd seeds, RP-HPLC, reverse-phase HPLC, SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

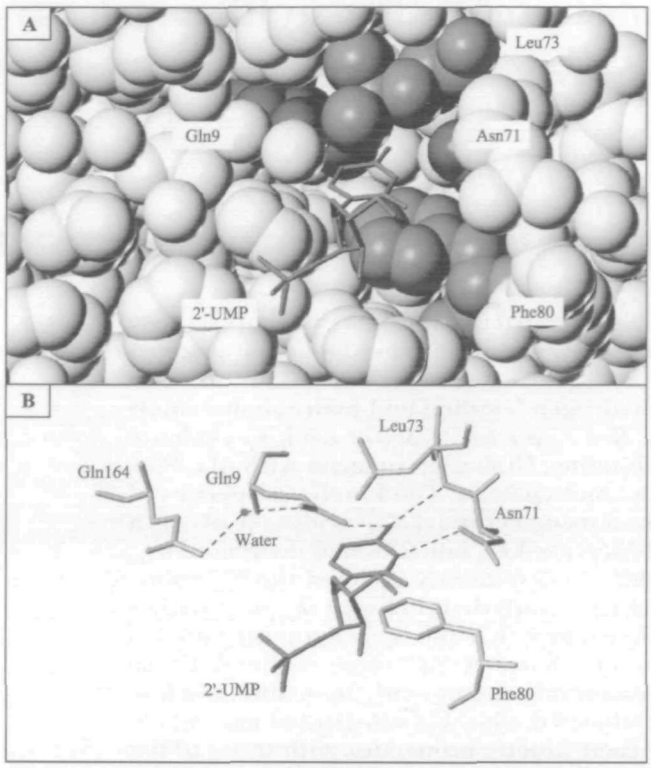

Fig 1 **Intermolecular interactions observed in the crystal structure of the RNase MC1-2′-UMP complex.** (A) A space-filling representation of the complex 2′-UMP, shown in capped sticks, is bound at the cavity formed by the amino acid residues at the B2 site (B) Main interactions found in the B2 site. The backbone atoms of Gln9, Asn71, Val72, Leu73, and Phe80, and the side-chain of Gln9, Asn71, Leu73, and Phe80 are shown Dashed lines represent hydrogen bonds

cCMP, respectively. Moreover, to investigate the mutual interplay of Gln9, Asn71, Leu73, and Phe80 involved in substrate binding, we generated double Ala mutants, in which two of the four residues were simultaneously replaced with Ala, and compared their kinetic properties with those of the corresponding single mutants.

MATERIALS AND METHODS

Materials—Restriction enzymes and DNA modifying enzymes were purchased from MBI Fermentas and Toyobo, respectively. The oligonucleotide primers and thermo sequenase fluorescent labeled primer cycle sequencing kit containing 7-deaza-dGTP were obtained from Amersham Pharmacia Biotech. A Chameleon double stranded site-directed mutagenesis kit was from Stratagene. Plasmid pGEM T-vector and expression plasmid pET-22b were obtained from Promega and Novagen, respectively. *Escherichia coli* strains JM109 and XL*mut*S were used as host cells for cloning and mutagenesis, and strain BL21(DE3)-pLysS was used as host cells for producing mutant enzymes. The cytidylyl-3′,5′-uridine (CpU) and cytidine-2′,3′-cyclic monophosphate (cCMP) were purchased from Sigma Chemicals. All other common chemicals and reagents were purchased at the highest purity available.

Site-Directed Mutagenesis and DNA Sequencing—For mutagenesis experiments, the cDNA coding for RNase MC1 in the plasmid pGEM T-vector was used Site-directed mutagenesis was performed by the unique site elimination method developed by Deng and Nickoloff (*8*). All the mutations introduced were confirmed by DNA sequencing with a DNA sequencer DSQ-1000 (Shimadzu) using the dideoxy terminator sequencing method (*9*).

Expression and Purification of the Recombinant Proteins—The wild-type and mutant RNases were expressed in *E. coli* strain BL21(DE3)pLysS using the expression plasmid pET-22b (*10*), as described previously (*11*). Proteins were purified from total cell extracts as previously described The purity of the wild-type and mutant enzymes was confirmed by reverse-phase HPLC (RP-HPLC) on a Cosmosil $5C_4$-300 (4.6 × 150 mm) column and SDS-PAGE using a 15% polyacrylamide gel (*12*). Mutations at the Gln9 residue were verified by N-terminal amino acid sequencing using a gas-phase protein sequencer PSQ-1 (Shimadzu). Protein concentrations were determined by the bicinchoninic acid methods (*13*), using BSA as a standard protein.

Circular Dichroism (CD) Spectroscopy—The CD spectra from 200 to 250 nm were recorded at room temperature with a Jasco J-720 spectropolarimeter at the enzyme concentration of 14.5 μM in distilled water in a cell with a 0.1-cm long optical path.

Kinetics of CpU Transphosphorylation—The enzymatic activity toward dinucleoside monophosphate CpU was measured at 37°C in 50 μl of 50 mM sodium-acetate buffer (pH 5.5). The reactions and quantification of product were performed as described previously (*7*). Enzyme concentrations and reaction times were chosen so that less than 20% of the substrate was consumed. The CpU concentrations varied between 40 μM and 8 mM. Kinetic parameters were calculated from the Hanes-Woolf plot, except for the F80A and six double Ala mutants. For these mutants, the second-order rate constant (k_{cat}/K_m) for the transphosphorylation of CpU was determined from the initial velocities. All kinetic parameters were determined at least twice in independent experiments. The quantitative effects of mutations on kinetics are expressed as changes in free energy compared to that of the wild-type enzymes. Differences in the free energy change ($\Delta\Delta G$) caused by mutation were calculated using the equation:

$$\Delta\Delta G = -RT \ln \frac{(k_{cat}/K_m)_{mutant}}{(k_{cat}/K_m)_{wild\text{-}type}}$$

where R is the gas constant and T is the absolute temperature. In order to analyze the mutual interplay of amino acids involved in uridine binding, the free energy barriers to substrate specificity introduced by single and double mutations were analyzed by double mutation cycles (*14*). In this analysis, two mutations, X and Y, which affect the parameters of the wild-type enzyme by $\Delta\Delta G$(X) and $\Delta\Delta G$(Y), respectively, and by $\Delta\Delta G$(X+Y) in a double mutant, were considered. When the sum of the $\Delta\Delta G$ values for single mutants is equal to that of the double mutant [$\Delta\Delta G$(X+Y) = $\Delta\Delta G$(X) + $\Delta\Delta G$(Y)], the sites function independently.

Hydrolysis of cCMP—Hydrolytic activity was measured in 100 μl of 50 mM sodium-acetate buffer (pH 4.5). The incubation was carried out at 37°C for 10–40 min and the reaction was terminated by injecting a 10 μl portion onto RP-HPLC on a TSK gel ODS 80TM column (4.6 × 250 mm) equilibrated with 0.1% TFA. The reaction product, 3′-CMP,

was eluted with a linear gradient of 0 to 1% acetonitrile in 0.1% TFA during 10 min. The flow rate was 1.0 ml/min, and the eluate was monitored by measuring the absorption at 280 nm. The cCMP concentration used was 200 μM and the enzyme concentrations were varied between 145 and 400 nM depending on the enzyme used.

RESULTS AND DISCUSSION

Production and Characterization of Mutant Enzymes—The crystal structures of the RNase MC1-uridylic acids complexes show that Gln9 hydrogen bonds with uracil O(4), and Phe80 stacks with the aromatic ring of uracil bases (*6*). To evaluate the contribution of Gln9 and Phe80 to uridine binding by RNase MC1, four Gln9 mutant enzymes, Q9A, Q9F, Q9E, and Q9H, and one Phe80 mutant, F80A, were prepared as described in "MATERIALS AND METHODS." The yields of the mutant enzymes were comparable to that of the wild-type enzyme, approximately 0.5 mg/liter. The purity of the mutant enzymes was confirmed by SDS-PAGE and RP-HPLC (data not shown). Mutations at the Gln9 residue were also verified by amino acid sequencing using a gas-phase protein sequencer PSQ-1. The CD spectra of all mutant enzymes measured in the short wavelength region (200–250 nm) were essentially the same as that of the wild-type RNase MC1 (Fig. 2), indicating that the backbone conformation of the mutant enzymes is practically the same as that of RNase MC1.

Mutations at Gln9—The kinetic parameters of the Gln9 mutants for transphosphorylation of CpU were measured and the results are shown in Table I. All mutant enzymes exhibited reduced catalytic efficiency (k_{cat}/K_m). The mutation of Gln9 to Ala, in which the interaction between the side-chain of Gln9 and substrate is truncated, generated an enzyme with lower activity, showing 103-fold decreased catalytic efficiency compared with that of the wild-type enzyme. The K_m and k_{cat} values of the Q9A mutant increased 3.7-fold and decreased 27.6-fold, respectively (Table I). The results suggest that Gln9 may play an important role in uridine binding that results in the acceleration of turnover of RNase MC1. Alternatively, it was speculated that Gln9 may be somehow involved in catalysis, because Gln9 is located close to the active site residues of RNase MC1, including His34, His83, Glu84, Lys87, and His88 (*11*).

To address these possibilities, Gln9 was replaced by Phe, Glu, and His, as in the cases of animal and fungal RNases (*2*), and the resulting mutants were analyzed in terms of the transphosphorylation activity toward CpU. Substitutions of Gln9 with Phe, Glu, and His reduced catalytic efficiency (k_{cat}/K_m) by 57.9-, 14.9-, and 9.4-fold, respectively, as compared with the wild-type enzyme (Table I). These mutations had little effect on the K_m value; the reduced catalytic efficiency was mainly caused by the decrease in the k_{cat} value. The results indicate that the simple binding of Gln9 to the uracil base would not enhance catalysis, but rather that Gln9 may be directly or indirectly involved in catalysis by RNase MC1.

These three mutant enzymes, Q9F, Q9E, and Q9H, retained significant residual activity compared with the Q9A mutant, indicating that the amino acid residues at the 9th position of these mutants function in enzymatic activity. Moreover, it was shown that polar or charged amino acid residues, which might be able to hydrogen bond with uracil, are suitable at this position because the mutants Q9E and Q9H are more active than the mutant Q9F. RNase T2 and RNase M are members of the RNase T2 family from *Aspergillus oryzae* and *A. saitoi*, respectively, and have Phe at the position equivalent to Gln9 in RNase MC1. It is likely that the phenyl ring of the Phe residue in RNase T2 and RNase M may recognize the base by hydrophobic interaction, which is a different base recognition mode than in the case

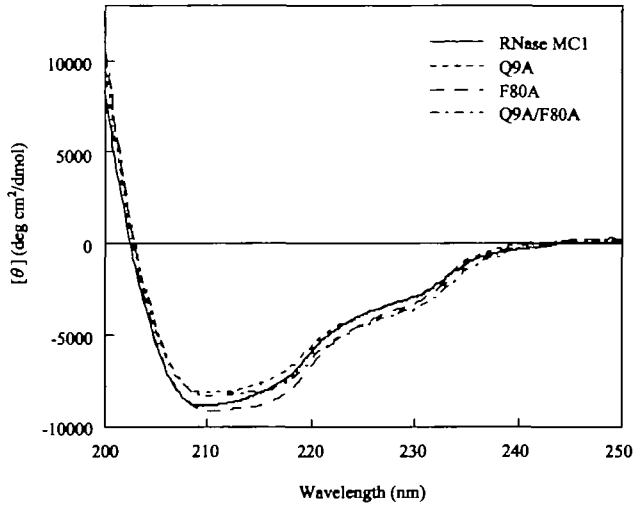

Fig 2 **CD spectra of RNase MC1 and its mutant enzymes in the far ultraviolet region.** The spectra were recorded in the far-ultraviolet region (200–250 nm) at room temperature. Data were averaged over four scans. The protein concentration was 14.5 μM. The spectra of the other Gln9 mutants (Q9F, Q9E, and Q9H) and five double Ala mutants (Q9A/N71A, Q9A/L73A, N71A/L73A, N71A/F80A, and L73A/F80A), which exhibited similar CD spectra to that of the wild-type RNase MC1, are not shown

TABLE I **Kinetic parameters for the cleavage of a dinucleoside monophosphate CpU by wild-type and mutant RNase MC1.**

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (10^{-2} μM^{-1} min^{-1})	$\Delta\Delta G$ (kcal/mol)
RNase MC1	362 ± 91.8 (1)	16,000 ± 2,549 (1)	4,508 ± 517 (1)	0
Q9A	1,325 ± 355 (3.7)	579 ± 142 (0.04)	43.8 ± 1.01 (0.01)	2.85
Q9F	423 ± 7.71 (1.2)	330 ± 76.2 (0.02)	77.8 ± 16.6 (0.02)	
Q9E	535 ± 141 (1.5)	1,596 ± 510 (0.1)	302 ± 94.1 (0.07)	
Q9H	392 ± 134 (1.1)	1,836 ± 404 (0.1)	478 ± 60.9 (0.11)	
F80A	>8,000	ND	0.329 ± 7.78E-4 (0.00007)	5.87
N71A	3,784 ± 332 (10.5)	606 ± 32.9 (0.04)	16.1 ± 0.593 (0.004)	3.47
L73A	1,222 ± 235 (3.4)	2,432 ± 489 (0.15)	200 ± 21.2 (0.04)	1.92

All parameter values are average values for at least two independent experiments. The numbers in parentheses show values relative to that of the wild-type RNase MC1. ND, not determined. Difference in the free energy change ($\Delta\Delta G$) was derived from the equation, $\Delta\Delta G = -RT \ln[(k_{cat}/K_m)_{mutant}/(k_{cat}/K_m)_{wild\text{-}type}]$, as described in "MATERIALS AND METHODS."

of RNase MC1.

Next, we measured the hydrolytic activity of the Gln9 mutants using cCMP as a substrate. Figure 3 shows the initial velocities for the hydrolysis of cCMP by wild-type and mutant enzymes. The mutant Q9A hydrolyzed cCMP at a rate of 16% of the wild-type RNase MC1. Moreover, the mutants Q9E and Q9H also showed reduced catalytic activity, 41 and 49% compared with that of wild-type, respectively. In contrast, the mutant Q9F showed slightly increased hydrolytic activity by 1.2-fold compared with that of wild-type RNase MC1. Although we have no explanation for this phenomenon in Q9F, it appears that mutations of Gln9 affect the nature of the P1 and B1 sites, because cCMP does not interact directly with the B2 site, but rather is hydrolyzed only by the actions of the P1 and B1 sites.

X-ray crystallographic studies showed that the Gln9 side chain is stabilized by hydrogen bonding with a water molecule, which is in turn bound to Gln164 (Fig. 1). Moreover, the side chain of Gln164 interacts with the imidazole ring of His34, a catalytic residue in RNase MC1, by hydrogen bonding. The initial rate of hydrolysis of cCMP for the mutant E164A, in which Glu164 in RNase MC1 was replaced by Ala, was 7.8% compared with that of wild-type RNase MC1 (T. Numata *et al.*, unpublished result). The results suggest that a network of hydrogen bonds around Gln164 may play an essential role in catalysis. The replacement of Gln9 by Ala removes a hydrogen bond, which results in a decrease in catalytic activity. In other words, Gln9 may contribute to maintaining the conformation around the active site by hydrogen binding. This is consistent with the notion that Gln9 takes part in catalysis in the transphosphorylation reaction of CpU described above.

We further analyzed the base specificity of four Gln9 mutants using four dinucleoside monophosphates CpN, in which N denotes A, C, G, or U All mutant enzymes exhibited lower enzymatic activity toward all four dinucleoside monophosphates (data not shown). Although the mutants Q9A and Q9E exhibited absolute uracil specificity, they acquired apparently higher activity for CpA cleavage compared to the wild-type enzyme (data not shown). In the previous study, replacement of Asn71 with Ser or Thr completely changed the base specificity of RNase MC1 (*7*) Therefore, it could be expected that substitutions at Gln9 with amino acids having an adequate size and polarity to hydrogen bond with the base may lead to a specificity for adenine rather than uracil. To address this assumption, kinetic studies of various Gln9 mutants are currently underway.

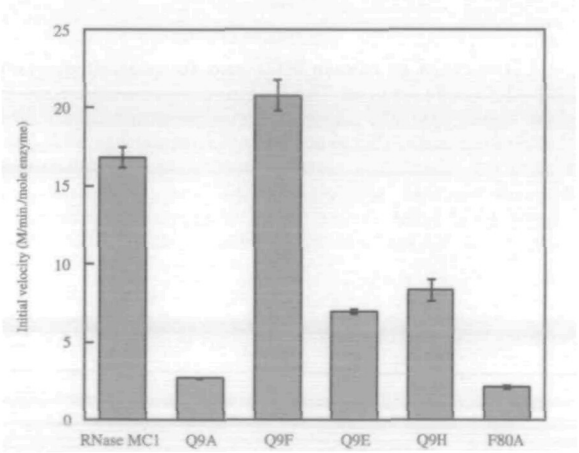

Fig 3 **Hydrolysis of cCMP catalyzed by RNase MC1 and its mutant enzymes.** The enzymatic reaction was performed in 50 mM sodium-acetate buffer (pH 4.5) at 37°C. Substrate concentrations used were 200 μM and enzyme concentrations varied between 145 and 400 nM depending on the enzyme used The product (3'-CMP) concentrations were determined by RP-HPLC on a TSK gel ODS 80TM column (4 6 × 250 mm) equilibrated with 0 1% TFA. Values are means ± SD.

Mutation of Phe80—In contrast to the Gln9 mutants, a linear dependence of the initial velocities v_i on the substrate concentration was observed in the transphosphorylation reaction catalyzed by F80A (data not shown). Because the initial slope of the v_i *versus* substrate concentration plot is identical to V_{max}/K_m, we calculated the second-order rate constant (k_{cat}/K_m) for the transphosphorylation reaction. Furthermore, we defined a minimum value of K_m equal to 8 mM (Table I). Truncation of the Phe80 side chain to Ala resulted in a drastic reduction in enzyme activity, showing a 13,700-fold lower catalytic efficiency compared to the wild-type enzyme. Although we could not define the precise kinetic parameters (K_m, k_{cat}), our data demonstrate that Phe80 does play an essential role in binding the uracil base in the transphosphorylation reaction.

In the crystal structures of RNase MC1 liganded with UMP, the phenyl ring of Phe80 and the uracil base are involved in a parallel face-to-face stacking interaction (*6*). Since Phe80 (RNase MC1 numbering) is a highly conserved amino acid in RNase T2 family members, this interaction may be a common feature in RNase T2 family enzymes. In fact, the cytosine base of the dinucleoside monophosphate d(ApC) was stacked with the side-chain of Phe101 (equivalent to Phe80 in RNase MC1) of RNase Rh from *Rhizopus niveus* (*2*). Ohgi *et al.* reported that a mutation at Phe101 resulted in a reduction in catalytic activity (*15*). In addition, it was shown that Phe89 (the equivalent to Phe80 in RNase MC1) from RNase LE from tomato cultured cells forms a hydrophobic pocket and is thought to be responsible for base recognition (*16*). Hence, the hydrophobic environment provided by Phe80 may be most important for substrate recognition in RNase T2 family RNases Consistent with this notion, the replacement of Phe80 with Ala has a devastating effect ($\Delta\Delta G$ = 5.87 kcal/mol) on the enzymatic activity (Table I). This effect is much larger than those of the other three Ala mutations at the B2 site ($\Delta\Delta G$ = 1.92–3.47 kcal/mol).

The hydrolytic activity of the mutant F80A was examined using cCMP as a substrate. The mutant hydrolyzed cCMP at the rate 12.6% that of the wild-type, as shown in Fig. 3. As described above, Phe80 forms a hydrophobic environment, which is common feature in RNase T2 family enzymes. Therefore, Phe80, along with Gln9, may also contribute to catalysis through the formation of the catalytic center.

Additivity in Uracil Binding—As for RNase T1, a guanine-specific RNase from *A. oryzae*, Tyr42, Tyr45, and Glu46 are considered building components that constitute the B1 site, based on site-directed mutagenesis and X-ray crystallographic studies (*5, 17–20*). The interaction of individual side chains with guanine was analyzed by comparing the kinetics of the wild-type enzyme with those of a mutant in which the side chain is truncated (*20*). Further-

TABLE II **Difference in the free energy change of the transphosphorylation reaction by RNase MC1 resulting from double mutations.**

	k_{cat}/K_m (10^{-3} μM^{-1} min^{-1})	$\Delta\Delta G$ (kcal/mol)
Q9A/N71A	2 24 ± 0 0156	4 69
Q9A/L73A	4 25 ± 0 270	4 29
Q9A/F80A	0 0105 ± 8 49E-5	7 99
N71A/L73A	3.74 ± 0 105	4 37
N71A/F80A	0 0177 ± 4 10E-4	7 67
L73A/F80A	0 0463 ± 2.31E-3	7 08

All parameter values are average values for at least two independent experiments. $\Delta\Delta G$ was calculated from the specificity constant (k_{cat}/K_m), as described in "MATERIALS AND METHODS."

TABLE III **Comparison of the sums of $\Delta\Delta G$ of component mutants *vs.* the double mutants.**

Position	$\Delta\Delta G$ (kcal/mol)			
	Component mutant		Sum	Double mutant
Gln9, Asn71	Q9A	+ N71A		Q9A/N71A
	2 85	3 47	6.32	4 69
Gln9, Leu73	Q9A	+ L73A		Q9A/L73A
	2 85	1 92	4 77	4 29
Gln9, Phe80	Q9A	+ F80A		Q9A/F80A
	2 85	5 87	8 72	7 99
Asn71, Leu73	N71A	+ L73A		N71A/L73A
	3 47	1 92	5 39	4 37
Asn71, Phe80	N71A	+ F80A		N71A/F80A
	3 47	5 87	9 34	7 67
Leu73, Phe80	L73A	+ F80A		L73A/F80A
	1 92	5 87	7 79	7 08

$\Delta\Delta G$ values of each mutant were taken from the data in Tables I and II

more, the interplay of a pair of these residues in guanine binding was analyzed by measuring the degree to which one mutation affects the contribution of a second mutation (quantified by the coupling term $\Delta\Delta G$). It was concluded that Tyr42 and Glu46 contribute to guanine specificity in a mutually independent way (*20*)

This study, together with the previous study (*7*), identified Gln9, Asn71, Leu73, and Phe80 as essential components in the B2 site of RNase MC1. RNase MC1 is distinct from other RNases in that it preferentially cleaves the phosphodiester bond, NpU, recognizing the uracil at the 3'-terminal side of the scissile bond. It is thus of interest to investigate the interplay of these four residues in substrate binding. For this purpose, we produced six double alanine mutants at Gln9, Asn71, Leu73, or Phe80, in which two of the four residues were simultaneously replaced by Ala, and the apparent free energy changes to the specificity constant introduced by the single and double mutations were analyzed by double mutant cycles (*14*).

All mutant enzymes were purified to homogeneity and their purity was evaluated by SDS-PAGE and RP-HPLC. The secondary structures were found to be the same as that of the wild-type RNase MC1 (Fig. 2), indicating that these mutations caused no structural perturbations.

The kinetic parameters for the transphosphorylation reaction using CpU are summarized in Table II As in the case of the mutant F80A, a linear dependence of the initial velocities v_i on the substrate concentration was observed in the transphosphorylation reaction catalyzed by all double Ala mutants. Therefore, we calculated the second-order rate constant (k_{cat}/K_m) for the transphosphorylation reaction. All double Ala mutants were less active than the component single mutants.

The sums of the change in free energy of the individual single mutations are within 0.11 $\Delta\Delta G$ units of the double mutants, Q9A/L73A, Q9A/F80A, and L73A/F80A (Table III). In the case of three double mutants, Q9A/N71A, N71A/L73A, and N71A/F80A, containing Asn71 mutations, the sums of the change in free energy of a component single mutant are slightly larger than those of the double mutants by 0.35, 0.23, and 0.22 $\Delta\Delta G$ units, respectively. At the moment, we have no appropriate explanation for the slight deviation from additivity observed in the Asn71 mutants. It is, however, reported that a slight deviation from additivity is likely to occur where the sites of mutations are very close together (< 4 Å distant) (*21*). The distance between Asn71 and three other amino acid residues are about 4–7 Å. It is thus likely that the slight deviation might be due to the proximity to the mutation sites to each other, suggesting that the mutation at Asn71 may have a slight effect on other amino acid residues at the B2 site.

In conclusion, the change in free energy for the double mutants relative to the wild-type enzyme is nearly the sum of the component single mutants. These results suggest that Gln9, Asn71, Leu73, and Phe80, components of the B2 site of RNase MC1, play roles in uracil binding in a mutually independent manner.

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