Structural Determinants of the Uridine-Preferring Specificity of RNase PL3

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ABSTRACT: RNase PL3 is a structurally highly conserved, pyrimidine-specific RNase, which strongly prefers to cleave at the 3′-side of uridine. Here, the question of which residues are involved in determining substrate specificity is addressed. The difference in the rate of cleavage of UpA and CpA was found to result from a 375-fold larger k_{cat} for the former substrate, whereas the values of K_m were essentially the same. The pyrimidine specificity of this class of RNases is thought to result from hydrogen bonds between the base and a threonine residue in the B_1 subsite. Mutation of this residue (Thr-44) in RNase PL3 resulted in a strongly reduced activity with UpA and poly(U). However, the activity with CpA and poly(C) had increased. Comparison with the effect of the same mutation in RNase A [delCardayre, S. B., & Raines, R. T. (1994) *Biochemistry 33*, 6031−6037] and angiogenin [Curran et al. (1993) *Biochemistry 32*, 2307−2313] showed that the function of this threonine in substrate recognition is different in three RNase subfamilies. Previous studies have shown that the 36−42 region contains one or more residues that are involved in substrate recognition [Vicentini et al. (1994) *Protein Sci. 3*, 459−466]. Site-directed mutagenesis of amino acids in this region identified Phe-42 as the only single residue that affected the cytidine/uridine specificity ratio. The mutation F42V resulted in a 10-fold increase in k_{cat} and a 1.9-fold decrease in K_m for CpA. The properties of the double mutant F42V/T44A suggested that a suboptimal binding of cytidine is caused by Phe-42, partially through an effect on Thr-44.

Mammalian ribonucleases (RNases) that are homologous to bovine pancreatic RNase A (Blackburn & Moore, 1982) form a superfamily that can be divided into four subfamilies on the basis of amino acid sequence homology (Vicentini et al., 1994). The primary structures of well over 60 enzymes have been determined, providing a large data base for studies on structure−function relationships. RNase PL3 is a member of a structurally highly conserved subfamily of RNases, which have a strong preference for cleaving the P−$O^{5'}$ bond at the 3′-side of uridine in both polymeric and dinucleotide substrates (Shapiro et al., 1986; Vicentini et al., 1994). In contrast, most other pancreatic-type RNases either prefer cytidine or do not discriminate between the two pyrimidines [cf. Richards and Wyckoff (1971), and Blackburn and Moore (1982)]. The pyrimidine specificity of RNase A, the prototypic enzyme of this superfamily, is reasonably well-understood from studies on the three-dimensional structure of enzyme−inhibitor complexes (Richards & Wyckoff, 1971; Wlodawer, 1985). The pyrimidine base binds in a narrow pocket on the surface of the enzyme, the so-called B_1 site (Richards & Wyckoff, 1971), formed by His-12, Val-43, Asn-44, Thr-45, and Phe-120. The main interactions are through hydrogen bonding to Thr-45 and van der Waals contacts with Phe-120. The ═NH of Thr-45 donates a hydrogen to O2 of either pyrimidine base, whereas the $^{\gamma}$OH acts as a hydrogen bond acceptor in the case of uridine but as a donor in the case of cytidine (Richards & Wyckoff, 1971) (Figure 1). The importance of these hydrogen bonds in RNase A has been demonstrated by site-directed mutagenesis; the mutant T45A has a reduced catalytic efficiency with both poly(C) and poly(U) (delCardayre & Raines, 1994, 1995). Qualitatively similar results have been obtained with angiogenin (Curran et al., 1993). Because Thr-45 has been conserved in all RNases, it is likely that it is important for substrate binding in the other subfamilies as well.

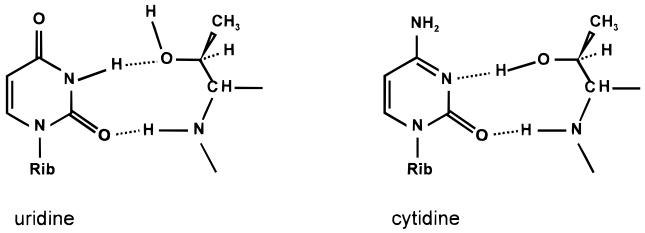

FIGURE 1: Schematic representation of the binding of the two pyrimidine bases to Thr-45 in RNase A. Hydrogen bonds have been indicated as broken lines.

RNase PL3, and its human orthologue RNase 4 (Zhou & Strydom, 1993), cleave dinucleotides of the structure NpA approximately 400-fold better with uridine than with cytidine in the N position (Shapiro et al., 1986; Vicentini et al., 1994). Since both Thr-45 and Phe-120 have been conserved in these enzymes (Thr-44 and Phe-117, respectively), their uridine-preferring specificity raises the question as to which structural features determine substrate recognition. Previously, we have found that replacement of residues 36−42 of RNase PL3 by the corresponding ones from RNase A yielded a hybrid protein (RNase PLRA1) with a more "RNase A-like" behavior, i.e. an increased catalytic efficiency (k_{cat}/K_m) for cytidine-containing substrates (Vicentini et al., 1994). The catalytic efficiency for CpA[1] increased 82-fold, but that for UpA only increased 9-fold. Such a preferential increase in activity was also observed with the polymeric substrate poly(C) compared to poly(U). In RNase A, Val-43 is in van der Waals contact with the pyrimidine ring [cf. Wlodaw-

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er et al. (1983)]. Since RNase PL3 contains a much larger residue at this position (Phe-42) than RNase A, it was proposed that it is involved in discriminating between uridine and cytidine (Vicentini et al., 1994).

In this paper, we have examined the function of Thr-44 in substrate recognition by RNase PL3 by determining the properties of a T44A mutant. Furthermore, we have studied the role of residues in the 36−42 region of RNase PL3 by mutating them one by one into their RNase A counterpart.

MATERIALS AND METHODS

Materials. Restriction enzymes used for recombinant DNA manipulations were obtained from Boehringer-Mannheim. The dinucleotides UpA and CpA were supplied by Dr. W. Pfleiderer (University of Konstanz, Germany) and Sigma (St. Louis, MO), respectively. Poly(U) and poly(C) were obtained from Pharmacia (Uppsala, Sweden).

Site-Directed Mutagenesis and DNA Sequencing. All oligonucleotides were prepared using standard phosphoramidite chemistry on an Applied Biosystems Model 380A DNA synthesizer. For mutagenesis experiments, the cDNA coding for RNase PL3 in Bluescript M13 was used (Vicentini et al., 1994). Overlap extension using PCR was employed to introduce the mutations (Ho et al., 1989), which were verified by sequencing both strands of DNA by the dideoxy method (Sanger et al., 1977).

Expression and Purification of the Recombinant Proteins. Wild-type and mutant RNase PL3 were expressed in *Escherichia coli* strain Topp-2 (Stratagene, La Jolla, CA) using the vector ptrp ss PL3 described previously (Vicentini et al., 1994). Proteins were purified from total extracts of the cells essentially as described (Vicentini et al., 1994). For the wild-type enzyme, a slightly modified protocol was used. The final purification step by reversed phase HPLC was replaced by ion exchange chromatography on an FPLC mono S column. The column was equilibrated in 15 mM KH_2PO_4 (pH 6.0) and eluted with a linear gradient of 15 to 350 mM of the same buffer in 60 min, at a flow rate of 1 mL/min. Aliquots of the purified RNases were dialyzed against 50 mM NH_4HCO_3 (pH 7.8) and used for protein chemical characterization and kinetic analysis.

In order to verify the presence of the desired mutation, and the correctness of the rest of the sequence, the expression plasmid was purified from the same cells as the protein and sequenced.

Protein Chemistry. The purity of the RNases was established by reversed phase HPLC, SDS−PAGE, Edman degradation, and amino acid analysis as described (Vicentini et al., 1994). From the latter, the exact concentration of the protein and, in most cases, additional evidence for the mutation were obtained.

Kinetic Studies and Data Analysis. Activity toward the dinucleotides UpA and CpA was measured in 50 mM MES−NaOH (pH 6.9) containing 125 mM NaCl, 1 mM EDTA, 0.2 mM DTT, 0.1% poly(ethylene glycol) ($M_r = 6000$), and 0.2 mg of bovine serum albumin per milliliter. The reactions and the quantitation of products were performed as described previously (Vicentini et al., 1994). The specificity constants for cleavage, k_{cat}/K_m, were determined at [S] $\ll K_m$. Data were fitted to the equation describing first-order kinetics using the program Enzfitter (Leatherbarrow, 1987). The individual kinetic parameters, k_{cat} and K_m, were obtained from initial rates of cleavage at substrate concentrations ranging from $0.3K_m$ to $3K_m$ (five points). Enzyme concentrations and reaction times were chosen so that less than 10% of the substrate was consumed. The data were fitted to the Lineweaver−Burk equation using previously described procedures (Hofsteenge et al., 1986). All kinetic parameters were determined at least twice in independent experiments. Activities toward the homopolynucleotide substrates were determined as described (Vicentini et al., 1994).

RESULTS

Isolation and Characterization of Mutants of RNase PL3. All mutant proteins could be purified by the standard procedure described in Materials and Methods with a yield comparable to that of the wild-type enzyme (approximately 1.5 mg/L; Vicentini et al., 1994). For those mutations that resulted in a change in charge, the chromatographic behavior on the ion exchange column changed accordingly. The proteins were judged to be pure, from analysis by SDS−PAGE and amino acid composition (data not shown). Moreover, the identity of each of the mutants was confirmed by isolating the expression plasmid from the same transformed bacterial culture as the protein and sequencing again the RNase-coding region.

Effect of Mutation of Thr-44 on the Specificity. Given the results obtained with RNase A (delCardayre & Raines, 1994) and angiogenin (Curran et al., 1993), removal of the side chain of Thr-44 in RNase PL3 was expected to decrease the catalytic efficiency with both cytidine- and uridine-containing substrates. The RNase PL3 mutant T44A had a 19-fold-reduced activity with poly(U) and a 313-fold-decreased k_{cat}/K_m for UpA. With cytidine-containing substrates, however, an increase in activity was observed, 2.6- and 5.2-fold for poly(C) and CpA, respectively (Tables 1 and 2). The reduction in activity of the mutant enzyme with UpA resulted from a 160-fold decrease in k_{cat}, whereas K_m increased only 1.8-fold. Likewise, the improved activity with CpA was caused by an increase in k_{cat} (6-fold). The mutant had RNase A-like properties (Table 1), cleaving preferentially after cytidine.

Effect of Mutation of Residues in the 36−42 Region on the Cytidine/Uridine Specificity Ratio. To test whether the large preferential increase in catalytic activity for cytidine-containing substrates of mutant RNase PLRA1 (RNase PL3 with residues 36−42 replaced by the corresponding ones from bovine pancreatic RNase A; see introductory section) was caused by the mutation F42V, this residue was mutated back to phenylalanine, yielding RNase PLRA1 V42F. The initial rate of cleavage of poly(C) by this mutant enzyme and the k_{cat}/K_m for CpA were reduced 44- and 54-fold, respectively, compared to those for RNase PLRA1. In contrast, the enzymatic activity with poly(U) had not changed (Table 2), whereas with UpA, only a small decrease (3-fold) was observed (Table 1). Consequently, the specificity ratios of RNase PLRA1 V42F, $(k_{cat}/K_m)_{CpA}/(k_{cat}/K_m)_{UpA}$ and $(v_0)_{poly(C)}/(v_0)_{poly(U)}$, decreased in comparison with those of RNase PLRA1 to values that were nearly the same as those of the wild-type enzyme (Figure 2). These results suggested that of the residues in the 36−42 region only Phe-42 affected

[1] Abbreviations: CpA, cytidylyl-3′,5′-adenosine; MES, 2-(*N*-morpholino)ethanesulfonic acid; PCR, polymerase chain reaction; SDS−PAGE, polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate; UpA, uridylyl-3′,5′-adenosine; $(v_0)_{poly(N)}$, initial rate of cleavage of poly(C) or poly(U).

Table 1: Kinetic Parameters for the Cleavage of UpA and CpA by Wild-Type and Mutant RNase PL3[a]

	UpA			CpA		
RNase	k_{cat} (s^{-1})	K_m ($\times 10^3$ M)	k_{cat}/K_m[b] ($\times 10^5$ M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m ($\times 10^3$ M)	k_{cat}/K_m[b] ($\times 10^5$ M^{-1} s^{-1})
wild-type	675	2.7	2.5 (1)	1.8 ± 0.2	2.8 ± 0.7	6.4 ± 1.6 (1)
T44A	4.2 ± 0.1	4.9 ± 0.2	0.008 ± 0.0002 (3×10^{-3})	11.1 ± 2	3.3 ± 1.0	33 ± 5 (5.2)
PLRA1	2103 ± 171	0.9 ± 0.1	23.6 ± 1.9 (9.4)	78.5 ± 2.6	1.4 ± 0.1	526 ± 20 (82)
PLRA1 V42F	706 ± 36	0.9 ± 0.1	7.7 ± 0.5 (3.1)	3.9 ± 0.3	4.6 ± 0.5	9.8 ± 0.6 (1.5)
S36K	nd	nd	2.2 ± 0.01 (0.9)	nd	nd	5.5 ± 0.01 (0.8)
H37D	nd	nd	3.0 ± 0.01 (1.2)	nd	nd	7.5 ± 0.02 (1.1)
Y38R	nd	nd	2.8 ± 0.01 (1.1)	nd	nd	5.0 ± 0.02 (0.7)
R41P	nd	nd	3.5 ± 0.01 (1.4)	nd	nd	6.1 ± 0.02 (0.9)
F42V	1026 ± 104	1.4 ± 0.3	7.4 ± 0.8 (3.0)	18.3 ± 0.8	1.5 ± 0.1	119 ± 12 (19)
F42V/T44A	11.9 ± 1.1	9.3 ± 1.1	0.013 ± 0.001 (5×10^{-3})	29.9 ± 4.5	2.3 ± 0.6	130 ± 15 (20)
RNase A[c]			35.3			46000

[a] The reported values are the weighted means of at least two experiments together with their standard error. The values in parentheses give the value of k_{cat}/K_m relative to that of the wild-type enzyme. The data for the wild-type enzyme with UpA are from Vicentini et al. (1994). [b] Values for k_{cat}/K_m were also determined under pseudo first-order conditions ([S] $\ll K_m$), and were within 15% of those listed here. [c] These values have been taken from Vicentini et al. (1994).

Table 2: Initial Rate of Cleavage of Poly(U) and Poly(C) by Wild-Type and Mutant RNase PL3[a]

RNase	poly(U) [nM (ng of RNase)$^{-1}$ mL^{-1} min^{-1}]	poly(C) [nM (ng of RNase)$^{-1}$ mL^{-1} min^{-1}]
wild-type	580 (1)	8.8 (1)
T44A	30 (0.05)	23.1 (2.6)
PLRA1	4200 (7.2)	2000 (227)
PLRA1 V42F	4410 (7.6)	45 (5.1)
S36K	1360 (2.3)	4.9 (0.6)
H37D	860 (1.5)	8.3 (0.9)
Y38R	1140 (2.0)	2.9 (1.2)
R41P	800 (1.4)	3.62 (1.4)
F42V	680 (1.2)	260 (30)
F42V/T44A	100 (0.2)	436 (50)

[a] The numbers represent the rate of production of acid-soluble nucleotides and are the average of duplicate determinations. The numbers in parentheses give the activity of the mutant relative to that of the wild-type enzyme.

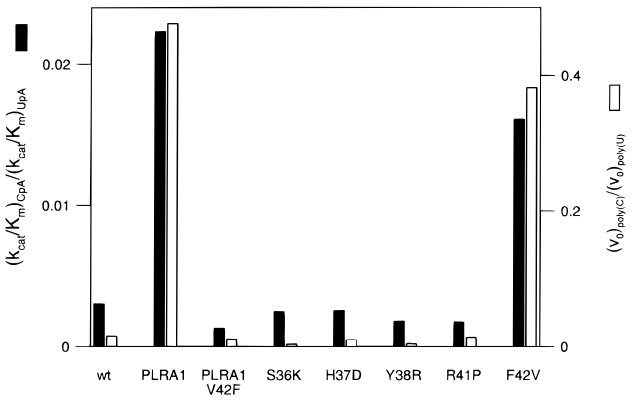

FIGURE 2: Effect of mutation of RNase PL3 on substrate specificity. The effect of the mutation of residues in the 36−42 region of RNase PL3 is expressed as the ratio of the specificity constants for the dinucleotide substrates (shaded bars) or as the ratio of the initial rate of cleavage for homopolymeric substrates (open bars). The absolute values of these parameters have been given in Tables 1 and 2.

the cytidine/uridine specificity ratio. To confirm this, mutant enzymes in which Ser-36, His-37, Tyr-38, Arg-41, or Phe-42 was replaced individually by its RNase A counterpart were examined for their substrate specificity. With the exception of F42V, none of the single mutations caused a significant change in the values for k_{cat}/K_m with the dinucleotide substrates UpA and CpA (Figure 2, Table 1) or in the initial rate of cleavage of poly(U) or poly(C) (Table 2). Furthermore, the cytidine/uridine specificity ratio for these substrates had increased only in the F42V mutant (Figure 2). The improved catalytic activity with CpA in mutant RNase PL3 containing the F42V substitution was mainly due to an increase in k_{cat}. The values for this parameter increased 44- and 10-fold in RNase PLRA1 and RNase PL3 F42V, respectively, but the value of K_m decreased only approximately 2-fold (Table 1). In agreement with these observations, also, the reduction in catalytic activity of RNase PLRA1 V42F with CpA, compared to that of RNase PLRA1, was largely caused by an effect on k_{cat} (Table 1).

On the basis of the three-dimensional structure of RNase A, it seemed likely that Phe-42 and Thr-44 are close to each other in space (Figure 3). It was, therefore, of interest to examine the effect of adding the mutation T44A to the mutant F42V. Compared to that of RNase PL3 F42V, the catalytic efficiency of the double mutant RNase PL3 F42V/T44A had increased only 1.1- and 1.7-fold with CpA and poly(C), respectively. With UpA and poly(U), however, the catalytic activity was reduced (570- and 6-fold; Tables 1 and 2), compared to that of RNase PL3 F42V. The properties of the double mutant resembled more closely those of the F42V than those of the T44A mutant when cytidine-containing substrates were used, whereas with UpA and poly(U), the reverse was true.

DISCUSSION

Role of Thr-44. The pyrimidine specificity of RNase A is mediated by Thr-45, which forms hydrogen bonds with the pyrimidine base on the 5′-side of the scissile bond (Figure 1). Since both uridine and cytidine make two hydrogen bonds with the threonine, this interaction explains the approximately equal catalytic efficiency for cleavage after either CpA or UpA (Richards & Wyckoff, 1971). However, also angiogenin, which cleaves preferentially after cytidine (Harper & Vallee, 1989), and RNase PL3 contain a threonine at this position (Thr-44). This raised the question about the exact function of this residue in substrate recognition, and the involvement of other amino acids.

Determination of the steady state kinetic parameters for the cleavage of UpA and CpA by wild-type RNase PL3 showed that the uridine-preferring specificity of this enzyme resulted from a 375-fold higher catalytic constant, k_{cat}, for UpA. On the other hand, the values of K_m for the two

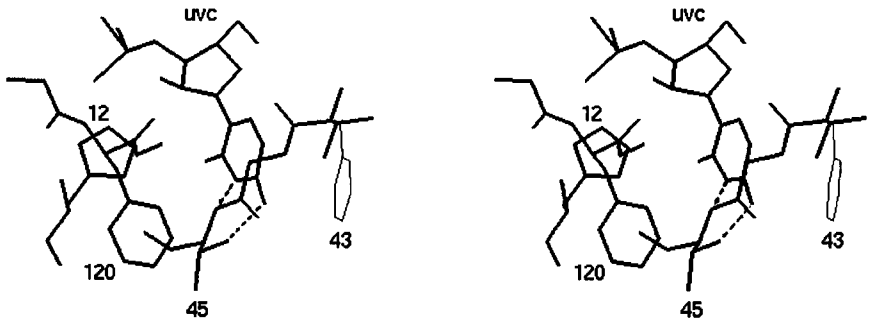

FIGURE 3: Stereoview of the pyrimidine pocket in RNase A. The structure shown was taken from the RNase A−uridine vanadate complex (6RSA; Wlodawer et al., 1983). At the position of Val-43 (homologous to Phe-42 in RNase PL3), a phenylalanine residue has been built in (thin lines), to illustrate its possibly close proximity to the pyrimidine base and Thr-45. Hydrogen bonds have been indicated by broken lines.

substrates were essentially the same (Table 1). As for most enzymes, the substrate specificity of RNase PL3 is determined by preferentially recognizing the substrate in the transition state, rather than in the recognition of the ground state. An overly strong binding of the latter would lead to suboptimal catalysis [cf. Fersht (1985)]. Mutation of Thr-44 into an alanine greatly reduced the cleavage of UpA and poly(U), but it increased the cleavage of CpA and poly(C) (Tables 1 and 2). From the kinetic parameters for the dinucleotides, it is evident that mainly catalysis, k_{cat}, is affected but that K_m is altered only slightly. It is not known whether in RNase PL3 Thr-44 actually hydrogen bonds to uridine and cytidine as shown in Figure 1. In any case, its side chain appears to be important for the cleavage of uridine-containing substrates but appears to have a negative effect on that of cytidine-containing ones, possibly by causing suboptimal binding with respect to the catalytic machinery. The magnitude of the effect of the T44A mutation was smaller for poly(U) and poly(C) than for the corresponding dinucleotides (Tables 1 and 2). In particular, the decrease observed with UpA (313-fold) was much larger than with poly(U) (20-fold). This could be explained if this polymeric substrate made energetically important interactions with the enzyme at subsites that are distant from the active site and the pyrimidine-binding pocket. Such subsites have been demonstrated in RNase A (de Llorens et al., 1989) and other RNases, including RNase PL3 (O. Zelenko, U. Neumann, and J. Hofsteenge, manuscript in preparation).

Comparison of the results presented here with those obtained for RNase A and angiogenin revealed that, although Thr-45 has been structurally conserved in all 62 RNases sequenced to date (Beintema, 1987; J. J. Beintema, personal communication), its function seems to differ in at least these three RNase subfamilies. Thus, whereas the Thr → Ala mutation in RNase A decreases the cleavage of uridine- and cytidine-containing substrates to approximately the same extent (delCardayre & Raines, 1994), it reduces k_{cat}/K_m of angiogenin for CpA 10-fold more than that for UpA (Curran et al., 1993). In RNase PL3, the mutation decreased k_{cat}/K_m for UpA but increased that for CpA (Table 1). This suggests that the role of the threonine residue depends on structural features unique to each RNase, probably in particular on those of the B_1 subsite. Indeed, for both RNase A and angiogenin, residues have been identified that interact with the threonine and modulate pyrimidine binding. Asp-83 in RNase A can form a hydrogen bond with Thr-45, which is important for the binding and cleavage of poly(U), but not that of poly(C) (delCardayre & Raines, 1995). The relatively low ribonucleolytic activity of angiogenin has been rationalized by hydrogen bonds between the side chain carboxamide of Gln-117 and =NH and O^{γ} of Thr-44 (Acharya et al., 1994; Russo et al., 1994). In RNase PL3, one of the residues that could be responsible for the way in which Thr-44 functions is Phe-42 (see below).

The Thr → Ala mutation reduced k_{cat}/K_m for UpA 313-fold which corresponds to a loss of 3.3 kcal/mol of binding energy in the transition state. This value is higher than expected for the removal of a single hydrogen bond (1.5−2.0 kcal/mol; Fersht et al., 1985), an observation that was also made for RNase A (delCardayre & Raines, 1994). It seems possible that interactions with Thr-44 are important for correct orientation of the uridine and that, in their absence, other contacts that are essential for transition state stabilization are impaired.

Effect of Mutation of Residues in the 36−42 Region and the Role of Phe-42. From previous studies on RNase PL3, it was concluded that, in addition to the conserved residues in the B_1 pocket, Thr-44 and Phe-117 residues in the 36−42 region must also be involved in pyrimidine recognition (Vicentini et al., 1994). Two mutagenesis experiments, the back-mutation of Val-42 in RNase PLRA1 into Phe and the single-site mutation F42V, showed that Phe-42 is the only single residue that influences the cytidine/uridine specificity (Figure 2). Compared to the wild-type enzyme, the mutant F42V had an increased activity with both uridine- and cytidine-containing substrates. However, the increase with CpA or poly(C) was much larger than with UpA or poly(U) (Tables 1 and 2). In agreement with the finding in the wild-type enzyme that catalysis and not substrate binding determines specificity, the major contribution to the improved cleavage of CpA by RNase PL3 F42V resulted from an increase in k_{cat}, rather than in K_m (Table 1). The much smaller increase in k_{cat}/K_m for UpA was distributed about equally over the two parameters.

The above-mentioned results clearly established Phe-42 as the most important residue in the 36−42 region determining the cytidine/uridine specificity. However, one or more of the remaining residues play a role as well, by affecting Phe-42. The effect of the F42V mutation on the specificity ratio, as calculated from Table 1, was about 2.8-fold greater in the context of the RNase A loop than in that of RNase PL3. This indicates a cooperative interaction between Phe-42 and the other residues of the loop.

Previously, it has been noted that, compared to RNase PL3, RNase PLRA1 shows improved catalytic efficiency with all substrates tested. It was assumed that this was due to mutation of one or more residues, other than Phe-42, surrounding Lys-40, a residue possibly involved in transition

state stabilization (Vicentini et al., 1994). In particular, the replacement of Arg-41 by a proline residue was thought to be important. The results obtained here for the single-amino acid mutants S36K, H37D, Y38R, and R41P show that no single residue increased the cleavage of both cytidine- and uridine-containing substrates (Tables 1 and 2). Only the combination of the single mutations, as in RNase PLRA1 V42F, caused such an increase. These residues surround Lys-40, which in analogy to Lys-41 in RNase A could be important for stabilization of the pentacoordinated reaction intermediate (Richards & Wyckoff, 1971). It is plausible that the spatial structure of this region, in particular the turn formed by residues 36−38, influences the alignment of the lysine residue with the other catalytic residues. Apparently, the structure of this region in RNase PL3 attenuates the ribonucleolytic activity, compared to that in RNase A. In angiogenin, replacement of the residues adjacent to the lysine by the corresponding ones from RNase A also causes a general increase in ribonucleolytic activity (Harper et al., 1990). In addition, an effect on other nearby residues, like e.g. Thr-44, would also be possible.

Properties of the Double Mutant RNase PL3 T44A/F42V. In the three-dimensional structure of RNase A, the shortest distance between the side chains of the residues corresponding to Thr-44 and Phe-42 (Thr-45 and Val-43) is 5.1 Å. It was, therefore, conceivable that the much larger side chain of Phe-42 contacts Thr-44 and affects its function in substrate binding. Comparison of the kinetic data for UpA and CpA in Table 1 showed that the T44A mutation decreased k_{cat}/K_m for UpA 1.8-fold more strongly in the presence than in the absence of Phe-42, whereas the (improving) effect on CpA cleavage was about 5-fold greater in the presence of Phe-42. There seems to be greater cooperativity between Phe-42 and Thr-44 for CpA than for UpA catalysis. In fact, the T44A mutation hardly influences CpA cleavage when Phe-42 is not present (Table 1).

In the absence of the three-dimensional structure of RNase PL3, hypotheses about the exact role of Phe-42 and Thr-44 can only be deduced from the structure of RNase A and its complexes with substrate analogues (Wlodawer et al., 1983). From the RNase A−uridine vanadate complex, it can be seen that one of the C^{γ} atoms of Val-43 is in van der Waals contact with the base moiety of uridine. The much larger phenylalanine in RNase PL3, together with Phe-117, could provide additional contacts to the bases, e.g. by stacking interactions between the three aromatic rings. How this would lead to discrimination between cytidine and uridine is at present unclear. This steric model would, however, not explain the effect of Phe-42 on Thr-44. Phe-42 either contacts Thr-44 directly or affects the properties of this residue via other amino acids. In this context, it is interesting to note that, in RNase A, Asp-83, situated on an adjacent β-strand, can hydrogen bond to Thr-45 and influence substrate binding (delCardayre & Raines, 1995). Structural studies have revealed that Val-43 can occupy two alternative positions, which correlate with Asp-83 pointing toward Thr-45 or away from it toward Arg-85 (Svensson et al., 1986). The large phenylalanine at this position in RNase PL3 could determine the position of the aspartate (Asp-80) more permanently and thereby the properties of Thr-44.

The two RNase PL3 mutants T44A and F42V/T44A are RNase A-like enzymes, cleaving CpA 4- and 10-fold better than UpA. However, the absolute value of their k_{cat}/K_m for CpA was much lower than that of RNase A (Table 1). This must mean that, in addition to residues 36−44, still other parts of the structure are affecting the activity with this substrate. The C-terminus of RNase PL3 is two residues shorter than that of RNase A (Hofsteenge et al., 1989). This could affect the properties of the B_1 subsite, through the absence of a residue equivalent to Ser-123 in RNase A, and the close proximity of the C-terminal α-carboxylate (Zhou & Strydom, 1993; Vicentini et al., 1994). Studies to test these hypotheses are now in progress.

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