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ARTICLE *in* BIOCHEMISTRY · APRIL 2001

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Probing Functional Perfection in Substructures of Ribonuclease T_1: Double Combinatorial Random Mutagenesis Involving Asn43, Asn44, and Glu46 in the Guanine Binding Loop[†]

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Received December 14, 2000; Revised Manuscript Received January 30, 2001

ABSTRACT: Combinatorial random mutageneses involving either Asn43 with Asn44 (set 1) or Glu46 with an adjacent insertion (set 2) were undertaken to explore the functional perfection of the guanine recognition loop of ribonuclease T_1 (RNase T_1). Four hundred unique recombinants were screened in each set for their ability to enhance enzyme catalysis of RNA cleavage. After a thorough selection procedure, only six variants were found that were either as active or more active than wild type which included substitutions of Asn43 by Gly, His, Leu, or Thr, an unplanned Tyr45Ser substitution and Glu46Pro with an adjacent Glu47 insertion. Asn43His-RNase T_1 has the same loop sequence as that for RNases Pb_1 and Fl_2. None of the most active mutants were single substitutions at Asn44 or double substitutions at Asn43 and Asn44. A total of 13 variants were purified, and these were subjected to kinetic analysis using RNA, GpC, and ApC as substrates. Modestly enhanced activities with GpC and RNA involved both k_{cat} and K_M effects. Mutants having low activity with GpC had proportionately even lower relative activity with RNA. Asn43Gly-RNase T_1 and all five of the purified mutants in set 2 exhibited similar values of k_{cat}/K_M for ApC which were the highest observed and about 10-fold that for wild type. The specificity ratio $[(k_{cat}/K_M)_{GpC}/(k_{cat}/K_M)_{ApC}]$ varied over 30 000-fold including a 10-fold increase [Asn43His variant; mainly due to a low $(k_{cat}/K_M)_{ApC}$] and a 3000-fold decrease (Glu46Ser/(insert)Gly47 variant; mainly due to a low $(k_{cat}/K_M)_{GpC}$) as compared with wild type. It is interesting that k_{cat} (GpC) for the Tyr45Ser variant was almost 4-fold greater than for wild type and that Pro46/(insert)Glu47 RNase T_1 is 70-fold more active than the permuted variant (insert)Pro47-RNase T_1 which has a conserved Glu46. In any event, the observation that only 6 out of 800 variants surveyed had wild-type activity supports the view that functional perfection of the guanine recognition loop of RNase T_1 has been achieved.

Ribonuclease T_1 (RNase T_1; EC 3.1.27.3)[1] is a small (104 amino acids), extremely well-characterized extracellular fungal enzyme from *Aspergillus oryzae* that catalyzes the overall hydrolysis of single-stranded RNA at guanylyl residues yielding new guanosine 3′-phosphate and 5′-OH ends (*1*, *2*). This occurs in a two-step process with cleavage of the RNA chain by transesterification of a 5′-phosphoester bond to form a guanosine 2′,3′-cyclic phosphate terminus in the first step followed by its hydrolysis to a 3′-phosphate product in a second, independent step. RNase T_1 is the leading representative of a ∼25-member superfamily of related fungal/bacterial enzymes/toxins, that share sequence and tertiary structural similarities (*2*).

The biological significance of the enzyme's base group specificity (G ≫ A > C,U) is unknown but guanine [in minimal RNA (GpN) substrates; N = A, C, G or U] is preferred over adenine (in ApN substrates) by almost a million-fold (*3*). The guanine binding site is referred to as the primary recognition site (PRS), and it exhibits the greatest affinity by far among other subsites involved in substrate binding (*4*). A number of crystal structures of RNase T_1 complexes with nucleotides or substrate analogues revealed that the guanine moiety is involved in extensive hydrogen bonding with three main chain amide N-H donors [Asn43, Asn44, and Tyr45 with the guanine N(7), O(6) and O(6), respectively) and three oxygen acceptors [$O^{\epsilon 1}$ and $O^{\epsilon 2}$ of Glu46 and the main-chain amide C═O of Asn98 with the guanine N(1)H, N(2)H, and N(2)H, respectively] (*5*). In addition, the guanine base group is sandwiched between the phenolic groups of Tyr42 (buried "floor") and Tyr45 (surface "lid"). A unique aspect of the guanine binding site on RNase T_1 is that, with the sole exception of Asn98, the residues in contact with base compose a contiguous segment ([42]-TyrAsnAsnTyrGlu[46]) of an extensive surface loop from Tyr42 to Pro55 which includes a reverse turn from Asn44

[†] This work was supported by a Research Challenge Grant from the Ohio Board of Regents.

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[1] Abbreviations: RNase, ribonuclease; ApC, adenylyl-3′,5′-cytidine; Bis-Tris, 2-Bis[2-Hydroxyethyl]amino-2-[Hydroxymethyl]1,3-propanediol; DEAE, diethylaminoethyl; dNTP, deoxynucleotide-5′-triphosphate with either adenine, cytosine, guanine, or thymine; EDTA, ethylenediaminetetraacetic acid; ESMS, electrospray mass spectrometry; GpC, guanylyl-3′,5′-cytidine; IPTG, isopropylthiogalactoside; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PRS, primary recognition site (guanine binding loop); SDS, sodium dodecyl sulfate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

10.1021/bi002837c CCC: $20.00
Published on Web 03/02/2001

to Gly47 that is stabilized by side chain (Asn44) to main chain (Phe48) hydrogen bonds. In crystal structures of the enzyme with an empty PRS, the loop can accommodate an alternate conformation (observed in five of 10 crystal structures) that is mainly characterized by a 140° "flip" of the peptide linkage between Asn43 and Asn44 (with Asn44 to Phe48 hydrogen bonds maintained; see above) (*6*). This conformation approximately exchanges the positions of the mainchain Asn44 N-H with the mainchain Asn43 C═O so that the former can no longer act as a hydrogen bond donor with guanine O(6); however, the flipped C═O of Asn43 could act as a hydrogen bond acceptor with an adenine N(6)H (in place of the guanine) which might explain, in part, why low grade catalysis of ApN substrates can occur. About 50% of RNase T_1 in solution exists as a conformer that does not bind guanosine, which could represent this mainchain flipping (*7*). Therefore, mutations in the PRS could indirectly affect catalysis by shifting this conformational equilibrium.

The results of many site-directed mutagenesis studies of residues 42 through 46 in the PRS have been reported (*8*−*13*). In general, it was found that substitutions at all of these positions were either neutral or deleterious to enzyme function. Substitution of Ala, Phe, and Trp for Tyr45 yielded proteins with the same or slightly enhanced (Tyr45Trp) catalytic activity which suggested that a hydrophobic and/or an aromatic stacking interaction was required at this position (*11*−*13*). Substitutions at Asn43 and Asn44 yielded enzymes with slightly lower or markedly lower catalytic activities, respectively (*8*); also, substitution of Glu46 with Asp, Gln, and Ala substantially decreased activity as did non-aromatic substitutions at Tyr42. Last, a random mutagenesis study of residues 41 through 46 did not reveal any transformants with activity greater than wild type (*14*). All of these findings suggest that guanine loop may have achieved optimization in RNase T_1 even though the enzyme has not achieved catalytic perfection since the maximal value of k_{cat}/K_M for small oligonucleotide substrates [about $(2-5) \times 10^6$ M^{-1} s^{-1} (*3*, *15*)] is considerably less than the theoretical diffusion control limit (10^9-10^{10} M^{-1} s^{-1}), which has essentially been achieved by RNase Ba (Barnase; a bacterial member of the RNase T_1 superfamily) (*16*). However, the conclusion that the PRS of RNase T_1 has achieved functional perfection is premature since systematic studies on combinatorial effects of multiple substitutions and/or insertions/deletions of this substructure have not yet been reported.

The evolution of an extracellular ribonuclease at least involves selection for catalytic efficiency and thermodynamic stability. It is possible that this could result in proteins having less than optimal substructures (subfunctions). The present work was initiated to more rigorously test whether localized perfection has been achieved at the PRS by testing the results of double random combinatorial mutagenesis involving Asn43 and Asn44 which could conceivably result in a different loop structure(s) by replacements at Asn44 which stabilizes the β-turn in the PRS (see above). Also, attempts to find active variants without highly conserved Glu46 employed double random mutagenesis at this position with an adjacent random insertion [a larger PRS loop is precedented in structurally related RNase U_2 which preferentially binds an adenine moiety (*17*)]. A preliminary account of this work has appeared (*18*).

MATERIALS AND METHODS

The plasmid pMcRT1 (*19*; a gift from N. Pace) was used for mutation and expression of wild-type and mutant versions of RNase T_1. Oligodeoxynucleotides were from NBI. All substrates including *Torula* yeast RNA (type VI), GpC, and ApC were from Sigma. Except where indicated, all enzymes were from New England Biolabs. General methods for recombinant DNA manipulations used published protocols (*20*).

Plasmid Engineering. Removal of an unwanted Sac1 site from pMcRT1 was accomplished by PCR amplification of a 253-bp *Eco*RI/*Eag*I fragment with a mutant primer as follows. The PCR reaction used pMcRT1 as the template with a forward primer including the *Eco*RI site, 5′-GGG-GAATTCGACCTCGAGCTTA-3′ (the underlined C substitutes for G to eliminate *Sac*I recognition) and a reverse primer, 5′-GTTTATATCCGGCCGCTTGAGC-3′, that contained the *Eag*I site plus seven downstream nucleotides. The PCR cocktail contained 10 μL of 10× amplification buffer (Promega), 10 μL of 2.5 mM dNTP mix, 6 μL of 25 mM $MgCl_2$, 2 μL (100 pmol) of primer I, 2 μL (100 pmol) of primer II, 100 ng of plasmid pMcRT1, 68.5 μL of deionized water, and 0.5 μL (1 unit) of Taq DNA polymerase (Promega). A GeneAmp PCR system 9600 (Perkin-Elmer) was used as follows: 95 °C for 5 min, 94 °C for 1 min, and 65 °C for 1 min (35 cycles), 72 °C for 10 min, and 4 °C until finished. PCR DNA was purified using the Qiagen PCR purification kit and then digested with *Eco*RI and *Eag*I to generate cohesive ends. After purification this product was ligated (T4 DNA ligase) to the isolated larger product of pMcRT1 after digestion with *Eco*RI then *Eag*I. The ligated product was used to transform competent *E. coli* Wk6λ cells by heat shock, and transformants were selected on a medium containing chloramphenicol. The success of this procedure was verified by digesting the modified pMcRT1 (now called pMcT1) with *Sac*I which now revealed a single site after agarose gel electrophoresis. DNA sequencing (detailed below) was also performed to verify this mutation.

Preparation of a "dummy" vector that does not express active RNase T_1 (pMcdumbT1) was achieved by substituting the 1.3-kbp *Sac*I/*Eag*I fragment from the plasmid pALTER-1 (Promega) for the 101-bp *Sac*I/*Eag*I fragment of pMcT1 coding for RNase T_1 between residues Ala22 to Ser53. Plasmid pMcT1 was digested with *Sac*I then *Eag*I, and the 4.18-kbp fragment was retrieved after agarose gel electrophoresis using a Qiagen gel extraction kit. This fragment was then ligated to the 1.3-kbp product isolated after *Sac*I/*Eag*I digestion of 5.68-kbp pALTER-1. The insert from pALTER-1 contains a unique *Kpn*I site and was selected because its substitution into pMcT1 greatly enhances the electrophoretic resolution of singly cleaved (not desired) and doubly cleaved pMcdumbT1 plasmid after *Sac*I/*Eag*I digestion. Competent *E. coli* strain Wk6λ were transfected with pcMcdumbT1 and cultured on RNase-test plates (see below). Unlike cells transformed with pMcT1 and expressing RNase T_1, clones of cells carrying pMcdumbT1 evidenced no RNase activity (even after 7 days). The identity of the dummy vector was also confirmed by linearization of the plasmid by *Kpn*I digestion.

Random Mutagenesis of Asparagine 43 and 44 in RNaseT1. Equal amounts (50 pmol) of oligodeoxynucleotide A, 5′-

TGCTCAAGCGGCC/GGATATcAACTTCACGAAGACGG-TGAAACTGTTGGATCCAATTCTTACCCACACA-AA3′, and partially complementary oligodeoxynucleotide B, 3′-CCTAGGTTAAGAATGGGTGTGTTTATGNNNN-NNATGCTTCAAAACTAAAGAGACAC\TCGAGAGCGC-GC-5′, (complementary portions underlined; N represents 25% each of A, C, G and T; lower case c represents a substitution of C for A which converts Lys25 to Gln25; / and \ represent the cleavage points for *Eag*I and *Sac*I, respectively) were annealed in 20 μL of 1× annealing buffer [10× annealing buffer is 70 mM Tris-HCl (pH 7.5), 60 mM $MgCl_2$ and 200 mM NaCl] for 5 min at 95 °C then 20 min at 65 °C followed by slow cooling to room temperature for at least 40 min (*21*). To this solution containing the double primed template was added 2 μL of 10× annealing buffer, 4 μL of 25 mM dNTPs, 0.8 μL of 100 mM dithiothreitol, 2.4 μL of DNA polymerase 1 Klenow fragment (5 units/μL), and water to bring the volume to 40 μL. To complete bidirectional DNA synthesis, this mixture was incubated at 37 °C for 30 min and then at 65 °C for 10 min. Amplification of the resulting double-stranded DNA was performed using PCR with Vent polymerase: the 100 μL reactions contained 5 pmol of the just described double-stranded DNA, 100 pmol of oligodeoxynucleotide primers (5′-TGCTCAAGCGGCCGGATAT-CAAC-3′ and 3′-GAGACACTCGAGAGCGCG-5′), 4 μL of 2.5 mM dNTPs, 10 μL of 10× thermopol buffer (New England Biolabs), 5 units of Vent polymerase, and the volume was brought to 100 μL with sterilized deionized water. PCR was carried out as described above and the purified DNA product was digested with *Sac*I then *Eag*I. After electrophoresis and isolation, this fragment was ligated to the 4.18-kbp product of the *Sac*I/*Eag*I digested pMc-dumbT1 which was then used to transform competent *E. coli* XL1-Blue MRF′ Kan strain (Stratagene) by the heat shock method. All of the transformation mix (700 μL) was spread on seven 15-cm RNase-test plates (see below). Colonies that showed a pink halo (after 3 days growth at most) were picked and subcultured to isolate clones which were then maintained on slants as well as stored as glycerol stocks for further use.

Random mutagenesis of Glutamate 46 and an adjacent insertion at position 47 used the following oligonucleotides to produce the mutated *Sac*I/*Eag*I insert: 5′-TGCTCAAGC/GGCCGGATATcAACTTCACGAAGACTGAAACT-GTTGGATCCAATTCTTACCCACACAAA-3′ and the partially complementary 3′-CCTAGGTTAAGAATGGGTGT-GTTTATGTTGTTGATGNNNNNNCCAAAACTAA-AGAGACAC\TCGAGAGCGCG-5′ where the complementary portions are underlined; N represents 25% each of A, C, G and T; lower case "c" represents a substitution of C for A which converts Lys25 into Gln25; / and \ represent the bonds cleaved (in double-stranded form) by *Eag*I and *Sac*I, respectively. Subsequent procedures were as described above for random mutagenesis at Asn43 and Asn44.

DNA Sequencing. Plasmids were isolated using a plasmid isolation kit (Boehringer Mannheim). The Cy-5 tagged forward and reverse fluorescent primers were custom synthesized (Pharmacia) as (Cy)5′-CGGTTCTAACTGCTACTC-3′ (sequencing starting at codon for Ser14) and (Cy)5′-TTGAAGACGACACGGTCAGC-3′ (sequencing starting at codon for Gly74), respectively. An ALF sequencing kit was used in conjunction with the ALF-express DNA sequencer (Pharmacia). Both primers provided sequence information that included both protein termini.

Enzyme Purification and Characterization. Cells, conditions for their growth and periplasmic preparations by osmotic shock followed the procedures in ref *22* except that 4 L cultures were used and the first periplasmic fraction (0.6 L) prepared in 20% sucrose, 50 mM Tris-HCl, 10 mM EDTA at pH 7.5 was not pooled with the second fraction (1 L) prepared in the same buffer without sucrose. Both solutions had a final pH of 7.2 and conductance of 2.3 mS/cm.

DEAE-52 (Whatman) chromatography was conducted at room temperature using a 5 cm (id) × 15 cm column equilibrated with 0.10 M sodium phosphate, pH 6.9 (equilibration buffer). The second periplasm extract was loaded first followed by the first extract (both at 0.5 mL cm^{-2} min^{-1}). After the sample was applied, the column was washed with equilibration buffer (0.25 mL cm^{-2} min^{-1}) until the absorbance at 280 nm of the eluant was zero. Isocratic chromatography employed 0.19 mM sodium phosphate, pH 6.9 (elution buffer), at the same flow rate. Fractions (20 mL) were checked for enzyme activity using the RNase-test plate assay (see below). Fractions containing activity were pooled (0.5 to 0.8 L), dialyzed against deionized water (3.5 kDa cutoff) at 4 °C, and lyophilized.

Sephadex G-50 (pharmacia) gel permeation chromatography used a column 2.8 cm (id) × 87 cm which was equilibrated with 50 mM ammonium bicarbonate. The lyophilized powder after DEAE-52 chromatography was dissolved in about 6 mL of deionized water, and insoluble material was removed by centrifugation and/or filtration (10 micron filter). The sample was loaded on the Sephadex G-50 column at a flow rate of 0.16 mL cm^{-2} min^{-1}. Elution used the same buffer and flow rate. Fractions (3 mL) containing activity were pooled, dialyzed, and lyophilized as described above.

Mutant and wild-type RNase T_1 were characterized by UV absorbance spectra (330 to 240 nm) and the ratio of maximum (around 278 nm) and minimum (around 251 nm) absorbancies. Enzyme concentrations were determined by measuring their absorbance at 278 nm using $\epsilon_{278nm} = 19\,290$ M^{-1} cm^{-1} for wild type and all mutants except Asn43Tyr ($\epsilon_{278nm} = 20\,910$ M^{-1} cm^{-1}) and Tyr45Ser ($\epsilon_{278nm} = 17\,670$ M^{-1} cm^{-1}) (*23*). Molecular purity was tested using polyacrylamide electrophoresis under native and SDS/reducing conditions with a Novex XCell II cassette system with 1.5-mm-thick, 14% Tris-glycine gels for native conditions and 1.0-mm-thick NuPAGE Bis-Tris gels for SDS−PAGE. SDS−PAGE gels were fixed in methanol/water/acetic acid [50:40:10 (v:v:v)] and stained with colloidal Coomassie Blue G-250 (Novex). Native gels were fixed and stained by placing the gel into a 65 °C solution containing 0.25 mg of Coomassie Blue R-250 (BioRad), 45 mL of methanol, 45 mL of water, and 10 mL of acetic acid which then was left standing at room temperature for 2 h: destaining at room temperature used the same solution minus the dye. Electrospray mass spectrometry with a Brucker Esquire LC-MS was used to determine the molecular masses of all mutants as cations. Two volumes of protein samples (1 μM) were mixed with 8 vol of methanol/water/acetic acid [50:49:1 (v:v:v)] for application. Three major cation peaks and excellent agreement with theoretical masses (based on DNA sequencing) were observed in all cases except for Asn43Tyr (see below).

Enzyme Assays. RNase-test plates contained LB agar (37 mg/mL), 25 μg/mL Chloramphenicol, 0.5% Torula yeast RNA (type VI), 50 μg/mL toluidine blue O dye and 0.1 mM IPTG (*24*). 1% sodium azide was also added if the plates were used to analyze extracts. For the latter, wells (5 mm diameter) were bored into the gel and 50 μL of the extract solution was added after which the plates were incubated at room temperature. Pink halos developed around wells containing RNase and their diameters were measured after 12 h. These were rated 0 (no activity) and from 1+ through 4+ for increasing activity.

Steady-state kinetic experiments with RNA used a continuous spectrophotometric method at 298.5 nm to determine initial velocities (*25*). Molecular velocities were calculated using $\Delta\epsilon_{298.5\ nm} = -45\ M^{-1}\ cm^{-1}$ (calculated by dividing the value of $\Delta A_{298.5nm}$ after the reaction was complete by the concentration of guanylate residues assuming that the RNA was cleaved at all guanylate residues which were 25% of the total). A stock substrate solution was prepared from *Torula* type VI RNA as follows: 15 g of RNA was dissolved in 60 mL of 0.05 M Tris-HCl and 0.05 M NaCl 0.002 M EDTA, pH 7.5, with the pH maintained at 7.5 by adding NaOH. The resulting yellow solution was dialyzed (12 kDa cutoff) at 4 °C for 2 days against 2 L of the same buffer with one buffer change after the first day. The concentration of RNA (in mononucleotide units) was determined by measuring A_{260nm} and using a molar absorptivity at 260 nm of 8000 $M^{-1}\ cm^{-1}$ per nucleotide. Ten- and 2-mm path length cuvettes thermostated at 25 °C were used with an Olis (Cary 118) UV/VIS spectrophotometer. The substrate concentration range was 1.01×10^{-3} to 2.46×10^{-2} M with enzyme concentrations from 5.4×10^{-9} to 1.3×10^{-7} M. Experiments were conducted at pH 7.5 in 0.002 M EDTA, 0.05 M Tris-HCl, 0.05 M NaCl. Data were analyzed using EnzFitter (BioSoft).

Kinetic studies with dinucleoside monophosphate substrates used a continuous spectrophotometric method at 280 nm with GpC and 268 nm with ApC to determine initial velocities (*3*); the concentration range for GpC was 1.27×10^{-5} M to 1.50×10^{-4} M. Direct determination of k_{cat}/K_M at substrate concentrations at least 20-fold lower than K_M was accomplished by observing first-order progress curves for at least three half-lives and for GpC these values of k_{cat}/K_M agreed with those determined from initial velocity measurements (*26*). When this method was used with ApC at 4.16×10^{-5} M, the data were excellently fit by a single-exponential term using the Olis software. Experiments were conducted at pH 6.0 in 0.05 M MES, 0.002 M EDTA at 25 °C.

RESULTS AND DISCUSSION

Sequence variations in the guanine binding loop of orthologues of RNase T_1 in fungi and bacteria are listed in Table 1. An aromatic residue is always found at position 42, and Glu46 is conserved in all cases. An aromatic residue at position 45 is conserved in fungal but not bacterial forms of the enzyme. Only one conservative variation (Asp) occurs with Asn44, presumably because of its involvement in loop stability (see above) whereas, more substitutions are tolerated at Asn43 whose side chain does not interact with the protein. It was interesting that a Asn43His/Asn44Asp mutation of RNase T_1, which converts this loop to that of RNase Ms, resulted in an enzyme with only 5% the wild-type activity (*8*), even though the activity of RNase Ms with GpC is about the same as RNase T_1 (*27*). A similar loss of activity was observed when the PRS of RNase Bi was converted to that for RNase Sa (involving three substitutions) (*28*). Therefore, it is likely that the functioning of the PRS in some cases is dependent on the structural context of a given orthologous form of the enzyme. Paralogous RNase U_2 (28% sequence identity with RNase T_1) cleaves RNA preferentially at adenosyl residues and has the sequence, TyrAspGluAlaSer, in place of AsnAsnTyr, between equivalent Tyr42 and Glu46 residues (RNase T_1 numbering) in its PRS (*17*).

Table 1: Overall Sequence Similarity and Variation at the Primary Recognition Site of Guanine-specific RNase T_1 Orthologues (*2*, *30*−*33*)

ribonuclease	% identity (# residues)	loop 42 to 50 (T_1 numbering)[a]
T_1	100 (104)	Y N N Y E G F D F
N_1	65 (104)	Y N N Y E G F D F
F_1	61 (105)	Y N N Y E G F D F
Fl_1	59 (105)	Y N N Y E G F D F
U_1	48 (105)	Y N N Y E G F D F
Th_1	56 (107)	Y N N Y E G F R F
Ap_1	71 (104)	Y R N Y E G F N F
Pch_1	72 (102)	Y R N Y E G F D F
Pb_1	68 (102)	Y H N Y E G F D F
Fl_2	56 (105)	Y H N Y E G F D F
C_2	69 (102)	Y R N Y E G F N F
Ms[b]	64 (105)	Y H D Y E G F D F
Po	41 (101)	Y N N F E G F S F
fungal consensus		Y 3 2 2 E G F 4 F
bacterial consensus[c]		F 3 N R E 3 5 L P

[a] Underlined residues are not common with RNase T_1; numbers in the consensus sequences represent the different amino acids at a given position. [b] Specificity: G ≫ U ≥ A = C. [c] For RNases Ba, Bci, Bi, Bpo, Sa, Sa2, Sa3, and St (ranging from 96 to 111 amino acid residues with 18 to 22% sequence identity with RNase T_1).

Strategy for the Screening Protocol. Unlike global mutational methods for enzymatic enhancements (*29*), the present work investigated the possibility of functional improvement by amino acid substitutions in a local, functionally defined portion of enzyme structure. A major purpose was to test the range of combinatorial mutations that yield mutant enzymes with the same or greater activity as wild type. Therefore, first efforts were made to identify *all* clones with the highest activity on RNase-test plates. The number of transformant colonies that need to be screened to explore all recombinants can be approximated as, $n\ln(n)$, where n is the number of recombinants ($20^2 = 400$ for both cases in the present study). For a 90% probability that all recombinants are examined, about 3100 transformants need to be assayed.

Screening for Active Combinatorial Mutants at Asn43 and Asn44 (Set 1). About 3150 colonies were originally screened on RNase-test plates and about 40% of these had detectable activity. Some colonies evidenced a halo after 16 h which was sooner than wild type (~20 h). Twenty one of the most active clones (and one inactive clone) were selected for DNA sequencing and enzyme activity measurement of corresponding periplasmic fractions using the RNase-test plate assay. The results of these experiments are shown in Table 2.

Fifteen transformant clones evidenced wild type or greater activity (3+ or 4+ ratings, respectively) and all had Asn at

Table 2: Catalytic Activity of Mutants after Random Mutagenesis of Asn43 and Asn44 at the Primary Recognition Site of RNase T_1

genetic group	# clones	residues 42−46[a]	activity[b]
wild type	5	Y N N Y E	+++
Asn43 substitutions	1	Y G N Y E	++++
	2	Y H N Y E	++++
	2	Y I N Y E	++++
	2	Y T N Y E	++++
	1	Y Y N Y E	++
Asn44 substitutions	2	Y N H Y E	+
	1	Y N K Y E	+
	1	Y N T Y E	+
Asn43/Asn44 double substitutions	2	Y Q G Y E	++
	1	Y T G Y E	++
	1	Y C H Y E	+
	1	Y I G Y E	0[c]
Tyr45 substitution	1	Y N N S E	++++

[a] Substitutions are underlined. [b] Activity determined for periplasmic extracts in wells on RNase-test plates. [c] No activity observed for this clone on RNase-test plates after 7 days culture.

position 44. Five of these also had Asn at position 43 (i.e., were wild type) which were more than expected considering the greater codon frequencies for Gly, Ile, and Thr. This latter result cannot be explained by possible bias in the original random (25% each) nucleotides in view of the codon sequences for Asn, Gly, His, and Thr; contamination of the original transformation mixture with pMcT1 is also ruled out since DNA sequencing revealed that all wild-type transformants had Gln at position 25 and not Lys as coded in pMcT1. The fact that eight (out of 15) of the high activity clones were duplicates or a quintuplicate of the same DNA sequences, strongly suggests that most of the mutants expressing this level of activity have been identified. The origin of the unplanned Tyr45Ser mutant is unknown. In contrast with single substitution mutants at Asn43, only four active clones revealed single substitutions at Asn44 and all of these had relatively low activity. Although each case would have to be tested, it is statistically reasonable to assume that recombinants not listed in Table 1 have low or no activity with RNA.

It was hoped that several double substitution mutants would express wild-type activity but this was not the case. Nevertheless, two of the four clones for this class were Asn43Gln/Asn44Gly which had moderately high activity. Two of the three active double substitution mutants had Gly at position 44 but this, by itself, does not ensure significant activity as indicated by the completely inactive Asn43Ile/Asn44Gly variant which was picked by chance (this point is emphasized by the fact that the Asn43Ile single mutant was quite active). It is possible that the Gln43 side chain of the Asn43Gln/Asn44Gly mutant acts as a structural surrogate for the Asn44 side chain hydrogen bonding in wild-type RNase T_1 (see above) which, in turn, could be augmented by increased conformational flexibility due to Gly44.

Screening for Active Combinatorial Mutants at Glu46 and an Insert at Position 47 (Set 2). Approximately 3100 transformant colonies were screened for ribonuclease activity using RNase-test plates. About 20% showed activity and all of the most active, several less active, and one inactive clones were cultured to provide plasmid DNA for sequencing and periplasmic extracts to assay ribonuclease activity. The results of these experiments are shown in Table 3. The fact that the six most active clones (RNase-test plate assay: 4+ activity) expressed the same mutant suggests that it is the only variant enzyme with greater or near wild-type activity since there is a 90% probability that all recombinants were represented in the initial screen considering the number of colonies observed. Likewise, it is also likely that variants with 2+ activity represent nearly all members of this category. On the other hand, the four variants evidencing 1+ activity in Table 3 are representatives of a larger class. It is curious that the Glu46Ser/(insert)47Ser mutant is completely inactive considering that the active mutants Glu46Thr/(insert)47Ser and Glu46Ser/(insert)47Gly differ by a methyl group and a hydroxymethyl group, respectively. It is also interesting that (insert)47Pro mutant is less active than Glu46Pro/(insert)-47Glu mutant considering that the former variant has the conserved Glu residue at position 46. The finding that proline represented 50% of the mutant residues in the five most active variants from set 2 was unexpected and might reflect, in part, a favorable effect of these residues on protein folding involving an expanded PRS.

Table 3: Catalytically Active Mutants after Random Mutagenesis of Glu46 and an Adjacent Insertion in the Guanine Binding Loop of RNase T_1

substitution−insertion[a]	# clones	residues 42−48	activity
none (wild type)	0	Y N N Y E G F	+++
Pro-Glu	6	Y N N Y P E G	++++
Glu-Pro	1	Y N N Y E P G	++
Pro-Pro	1	Y N N Y P P G	++
Gly-Leu	1	Y N N Y G L G	++
Pro-Tyr	1	Y N N Y P Y G	++
Ser-Gly	1	Y N N Y S G G	+
Arg-Arg	1	Y N N Y R R G	+
Thr-Ser	1	Y N N Y T S G	+
Asn-Glu	1	Y N N Y A G G	+
Ser-Ser	1	Y N N Y S S G	0

[a] Residues at positions 46−47.

Purification and Properties of the Recombinant Enzymes. RNase activity of mutant enzymes in periplasmic extracts reflects not only their intrinsic catalytic power but also their stability and concentration. Therefore, selected mutants were purified for discrete kinetic analysis. As shown in Figure 1, panel B, an acceptable purity for all purified variants from set 1 was indicated by single bands after SDS−PAGE; however, the Asn43Tyr varant had a considerably higher mobility as compared with wild type and all of the other mutants in this set. This particular mutant also exhibited peculiar properties after electrophoresis under nondenaturing conditions where several bands were observed in addition to the one that comigrates with wild type and all of the other mutants (Figure 1, panel A).[2] The lack of mobility differences for mutants Asn43His and Asn44His in nondenaturing electrophoresis is most likely due to the pH employed (pH = 8.6) where the additional imidazole group should be mostly deprotonated.

The most active variant, Glu46Pro/(insert)47Glu-RNase T_1, three of the four 2+ activity variants and one of the 1+ activity variants (Glu46Ser/(insert)47Gly) from set 2 were purified. In contrast with the results for wild type and the variants from set 1, the yields of the mutant proteins in set 2 were relatively low with the exception of Pro46/(insert)-47Glu-RNase T_1. However, all of the isolated variants from

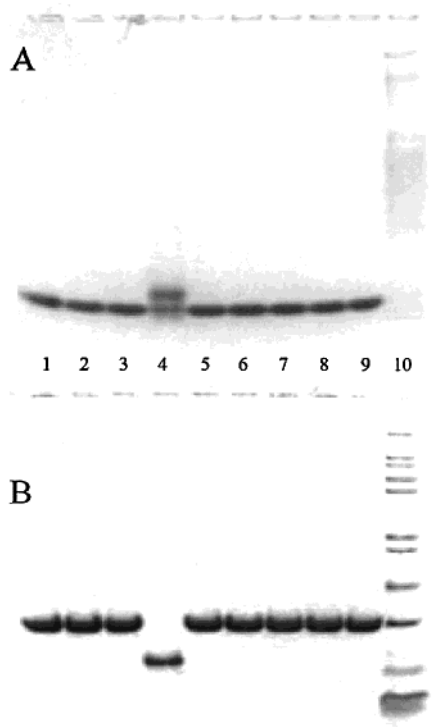

FIGURE 1: Electrophoresis of purified recombinant wild-type RNase T_1 and eight double substitution mutants. (A) Native PAGE at pH 8.6 with 12 μg of protein in lanes 1−9: lane 1, Tyr45Ser; lane 2, Asn43Gln/Asn44Gly; lane 3, Asn44His; lane 4, Asn43Tyr; lane 5, Asn43Thr; lane 6, Asn43Ile; lane 7, Asn43His; lane 8, Asn43Gly; lane 9, wild type; lane 10, multimark standards (Novex). (B) SDS−PAGE at pH 8.6 with 10 μg of proteins in lanes 1−9 which are arranged as in panel A. Lane 10, Mark 12 standards (Novex) which are 200, 116.3, 97.4, 66.3, 55.4, 36.5, 31, 21.5, 14.4, 6, 3.5, and 2.5 kDa from top to bottom, respectively. Experiments were performed as described under Materials and Methods.

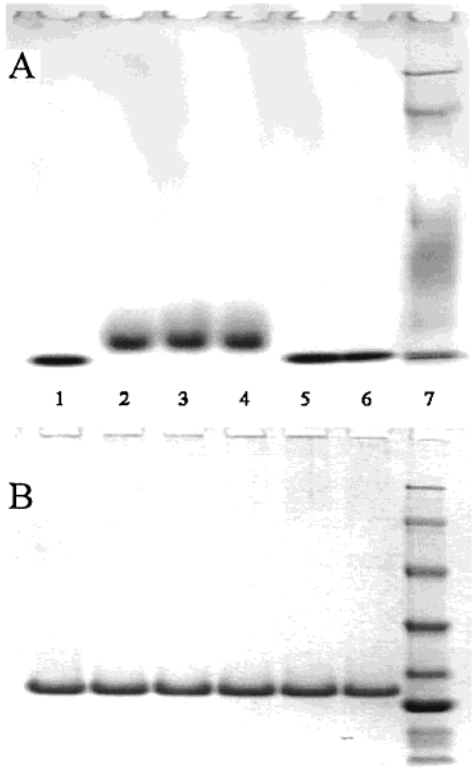

FIGURE 2: Electrophoresis of purified recombinant wild-type RNase T_1 and five substitution/insertion mutants. (A) Native PAGE at pH 8.6 with 12 μg of protein in lanes 1−6: lane 1, wild-type RNase T_1; lane 2, Glu46Ser/(insert)47Gly mutant; lane 3, Glu46Pro/(insert)47Tyr mutant; lane 4, Glu46Pro/(insert)47Pro mutant; lane 5, (insert)47Pro mutant; lane 6, Glu46Pro/(insert)47Glu mutant. (B) SDS−PAGE at pH 8.6 with 10 μg of proteins in lanes 1−6 which are arranged as in panel A. Lane 7 in both gels are Multimark (Novex) standards which are 185, 98, 52, 31, 17/19, 11, 6, and 3 kDa from top to bottom, respectively in panel B. Experiments were performed as described (*17*).

this set were judged to be highly purified by electrophoretic analysis as shown in Figure 2. The lower mobilities for the Glu46Pro/(insert)47Pro, Glu46Pro/(insert)47Tyr, and Glu46Ser/(insert)47Gly mutants in electrophoretograms obtained under nondenaturing (native) conditions is the expected result considering that they have one less carboxyl group as compared with wild type and the other variants.

For all purifed variants, the measured molecular masses of the purified proteins from both sets were determined by ESMS, and these exactly agreed with those calculated from the DNA sequencing results. They also had UV absorption spectra (230 to 330 nm) that were essentially the same as that for wild type except, as expected, for Asn43Tyr (one additional Tyr) and Tyr45Ser (one less Tyr) (data not shown).

[2] The chromatographic behavior (i.e., on DEAE-52 and Sephadex G-50 columns) and yield of the Asn43Tyr mutant were indistinguishable from wild type. It had an unusual ESMS spectrum but the expected UV spectrum (maximum at 277.2 nm versus 278.5 nm for wild type). The multiple bands for this mutant in native PAGE might reflect alternate disulfide formation since only one major band was observed after disulfide reduction in SDS−PAGE. The much higher mobility for this mutant protein in SDS−PAGE might be due to peptide cleavage or an unusual structure that does not unfold in the presence of SDS; these possibilities are currently being investigated. It is interesting that the RNase U_2 (a paralogue of RNase U_1 having 114 amino acids and 31% sequence homology with RNase T_1) has [43]TyrTyrAspGlu[46] (as compared with [42]TyrTyrAsnTyr[45] for the Asn43Tyr mutant of RNase T_1) at its PRS and that Asp45 spontaneously undergoes a β-isomerization that extends the loop mainchain by one (C^{β}) atom (*17*).

Kinetic Studies of Variants from Set 1. Steady-state kinetic parameters for the recombinant enzymes from set 1 with ApC, GpC, and RNA are listed in Table 4. The kinetic results with RNA generally agree with those using a single time point and single RNA concentration in the semiquantitative RNase-test plate assay (Table 2). The slightly enhanced values of k_{cat}/K_M for variants Asn43Gly and Asn43His vis à vis wild type appear to be significant with GpC whereas, no such differences were observed with RNA as a substrate; on the other hand, the results for the Asn43Thr variant indicated no significant differences with wild type using both substrates. A significant substrate-dependent difference for Asn43Leu-RNase T_1 as compared to wild type was noted where activity was about the same as wild type with GpC but significantly lower with RNA. The cause for this difference is not known but it is mainly reflected in K_M. Also, the different activities with RNA as a substrate for Asn43Ile in the periplasmic RNase-plate assay (4+), and the results in Table 3 (k_{cat}/K_M is 20% of that for wild type) is currently unexplained but could reflect a preferential loss of activity with RNA on purification.

The observation that the Asn43Gly, Asn43His, and Asn43Thr mutants have wild-type values of k_{cat}/K_M with RNA, makes it difficult to understand why Asn is dominant at this position among orthologous forms of the enzyme [i.e., in 7 out of 13 cases (54%); see Table 1] and mutants in the

Table 4: Kinetic Parameters for Random Substitution Mutants at Asn43 and Asn44 of RNase T_1 with GpC and ApC (pH 6.0 in 0.05 M MES, 0.002 M EDTA) and RNA (pH 7.5 in 0.002 M EDTA, 0.05 M Tris-HCl, 0.05 M NaCl) at 25 °C

	GpC[a]			RNA[a]			ApC[a,b]	specificity ratio
enzyme	$10^4 \cdot K_M$ (M)	k_{cat} (s^{-1})	$10^{-6} \cdot k_{cat}/K_M$ (M^{-1} s^{-1})	$10^4 \cdot K_M$ (M)	k_{cat} (s^{-1})	$10^{-6} \cdot k_{cat}/K_M$ (M^{-1} s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	$(K_{cat}/K_M)_{GpC}$ $(K_{cat}/K_M)_{ApC}$
wild type	1.35 ± 0.13 (100)	170 ± 10 (100)	1.25 ± 0.23 (100)	3.21 ± 0.56 (100)	492 ± 46 (100)	1.53 ± 0.50 (100)	4.4 ± 0.2 (100)	2.84×10^5
N43G	0.581 ± 0.05 (43)	100 ± 3 (59)	1.72 ± 0.21 (138)	7.30 ± 0.48 (227)	1222 ± 50 (248)	1.67 ± 0.20 (109)	40 ± 4 (909)	4.30×10^4
N43H	1.73 ± 0.01 (128)	290 ± 1 (171)	1.69 ± 0.02 (135)	6.98 ± 0.58 (217)	1347 ± 50 (274)	1.93 ± 0.25 (126)	0.52 ± 0.06 (12)	3.25×10^6
N43I	0.758 ± 0.06 (56)	81 ± 3 (48)	1.07 ± 0.13 (86)	15.5 ± 0.35 (483)	484 ± 8 (98)	.312 ± 0.013 (20)	6.1 ± 0.6 (139)	1.75×10^5
N43T	0.772 ± 0.03 (57)	116 ± 2 (68)	1.50 ± 0.09 (120)	26.4 ± 1.1 (822)	3485 ± 13 (708)	1.32 ± 0.06 (86)	0.92 ± 0.02 (21)	1.63×10^6
N43Y	2.59 ± 0.31 (192)	76 ± 6 (45)	0.293 ± 0.67 (23)	9.61 ± 0.93 (299)	54.2 ± 2.5 (11)	.0564 ± 0.009 (4)	nd[c]	
N44H	1.56 ± 0.04 (115)	3.22 ± 0.05 (1.9)	0.02 ± 0.008 (1.7)	196 ± 19 (6100)	30.8 ± 1.8 (6)	.0016 ± 0.0003 (0.1)	1.9 ± 0.1 (43)	1.05×10^4
N43Q/N44G	5.49 ± 0.68 (407)	134 ± 13 (79)	0.244 ± 0.06 (20)	45.6 ± 0.8 (1420)	324 ± 7 (66)	.0711 ± 0.018 (5)	0.81 ± 0.03 (18)	3.01×10^5
Y45S	6.06 ± 0.93 (449)	614 ± 7 (361)	1.01 ± 0.20 (81)	4.33 ± 0.30 (134)	336 ± 16 (68)	0.775 ± 0.077 (51)	1.7 ± 0.2 (39)	5.94×10^5

[a] Percent of wild-type values are in parentheses. [b] Only k_{cat}/K_M could be determined. [c] Not determined.

Table 5: Kinetic Parameters for Random Substitution/Insertion Mutants of RNase T_1 at Glu46 with GpC and ApC (pH 6.0 in 0.05 M MES, 0.002 M EDTA) and RNA (pH 7.5 in 0.002 M EDTA, 0.05 M Tris-HCl, 0.05 M NaCl) at 25 °C

	GpC[b]			RNA[b]			ApC[b,c]	specificity ratio
mutant[a]	$10^4 \cdot K_M$ (M)	k_{cat} (s^{-1})	$10^{-6} \cdot k_{cat}/K_M$ (M^{-1} s^{-1})	$10^4 \cdot K_M$ (M)	k_{cat} (s^{-1})	$10^{-6} \cdot k_{cat}/K_M$ (M^{-1} s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	$(k_{cat}/K_M)_{GpC}$ $(k_{cat}/K_M)_{ApC}$
wild type	1.35 ± 0.13 (100)	170 ± 10 (100)	1.25 ± 0.23 (100)	3.21 ± 0.56 (100)	492 ± 46 (100)	1.53 ± 0.50 (100)	4.4 ± 0.2 (100)	2.84×10^5
Pro-Glu	0.757 ± 0.07 (56)	124 ± 1 (73)	1.64 ± 0.03 (131)	69.4 ± 2.6 (216)	511 ± 8 (104)	0.736 ± 0.041 (48)	42 ± 2 (954)	3.90×10^4
Pro-Pro	5.06 ± 0.10 (375)	14.7 ± 0.2 (8.6)	0.029 ± 0.001 (2.3)	14700 (458000)	83000 (16900)	0.0567 ± 0.008 (3.7)	45 ± 3 (1023)	6.44×10^2
Pro-Tyr	nd[d]	nd	0.040 ± 0.002 (3.2)	*c*	*c*	.0055 ± 0.0007 (0.36)	33 ± 2 (750)	1.21×10^3
Glu-Pro	3.61 ± 0.27 (267)	6.4 ± 0.3 (3.8)	0.018 ± 0.002 (1.4)	*c*	*c*	.0419 ± 0.004 (2.7)	37 ± 3 (841)	4.86×10^2
Ser-Gly	nd	nd	0.0037 ± 0.0002 (0.29)	*c*	*c*	.0083 ± 0.0006 (0.54)	38 ± 2 (841)	97

[a] Residues at positions 46−47. [b] Percent of wild-type values are in parentheses. [c] Only k_{cat}/K_M could be determined. [d] Not determined.

present study (i.e., represented in 41% of the clones as compared with 59% for the structurally important Asn44; see Table 2). It might be argued that Asn43 plays some other important role (e.g., protein folding or binding with an intracellular inhibitor) but such possibilities do not seem likely since the Asn43His variant has high activity is produced in good yield and has a PRS sequence that also characterizes RNase Pb_1 and RNase Fl_2.

The most active double mutant (Asn43Gln/Asn44Gly) is less active than wild type mainly due to K_M differences that are further accentuated with the RNA substrate. Until the molecular complexity of Asn43Tyr-RNase T_1 is sorted out, the kinetic results for this mutant must be tentative; however, it is possible that not all native electrophoretic forms of this mutant are active that would be reflected in a lower value of k_{cat}.

It was generally thought that a hydrophobic/aromatic residue is needed at position 45 based on site-directed mutagenesis studies where Ala, Phe, and Trp were substituted for Tyr45 (*11*−*13*). In the present study, we serendipitously found an active mutant where Ser was substituted for Tyr45. In the original RNase-test plate assay, this mutant consistently showed higher activity than wild type as indicated in Table 2, but this was not verified for the pure protein with RNA where k_{cat}/K_M was about half of that for wild type (Table 4). On the other hand, this parameter is not significantly different from wild type with GpC as a substrate and the value of k_{cat} for this mutant was the highest by far of all the enzymes tested. Saturation mutagenesis at this position might reveal additional surprises.

Kinetic Studies of Variants from Set 2. Wild type and the five purified mutants were subjected to kinetic analysis using ApC, GpC, and RNA substrates which gave the results in Table 5. The Glu46Pro/(insert)47Glu mutant had a slightly greater value of k_{cat}/K_M with GpC when compared to wild type which was mainly the result of a decreased K_M. Therefore, it is likely that the guanine moiety interacts at least as well with this mutant as it does with wild type. In contrast, the permutated counterpart of this mutant, (insert)-47Pro, had a markedly decreased k_{cat}/K_M mostly because of a decreased value of k_{cat}. The fact that the Glu46Pro/(insert)-47Pro and Glu46Pro/(insert)47Tyr variants have similar

kinetic parameters with GpC as those for the (insert)47Pro-RNase T_1 suggests that Glu46 in the latter is not interacting with the guanine base. On the other hand, it is possible that Glu47 in Glu46Pro/(insert)47Glu-RNase T_1 plays the same role in guanine binding as Glu46 with the wild-type enzyme.[3] A previous study using site-directed mutagenesis showed that Glu46Ala and Glu46Gln mutants were characterized by values of k_{cat}/K_M for GpC that were less than 1% of that for wild type (*10*), which is a more drastic reduction in activity than that found for all of the 2+ activity mutants tested here; these results suggest that the loop insertion per se assists the binding of the guanine (and adenine; see below) moiety in the absence of a carboxylate interaction with N(1)H and N(2)H (*5*; see above).

With RNA as a substrate, it was surprising to find that k_{cat}/K_M for the Glu46Pro/(insert)47Glu variant was about half of that for wild type since the RNase-test plate assay of clones and periplasmic extracts were consistently greater for this mutant as compared with the original enzyme. It is possible that the catalytic activity of this mutant is lost after purification and/or that its concentration in the periplasm is greater than that for wild type in the expression system used. In any event, the opposite effects on K_M for GpC and RNA for the purified form of this mutant suggests the possibility that improved binding interactions with guanine moiety of GpC are offset with RNA because of unfavorable contacts with other portions of this polymeric substrate; for example, the Glu46Pro substitution and adjacent Glu47 insertion might cause mainchain and/or side chain excursions at the PRS that interferes with productive subsite contacts (*4*). The Glu46Pro/(insert)47Tyr variant also had relatively lower activity with RNA as compared with GpC (Table 5).

Specificity Changes in Enzyme Activity. Modest increases in activity of a mutant enzyme with RNA might reflect a loss in specificity which enables cleavage at other than guanylyl residues. Possible changes in specificity for the mutants was assessed by changes in $\Delta\epsilon_{298.5\ nm}$ for RNA degradation by the different mutant enzymes but none were observed. Nevertheless, some dramatic differences in k_{cat}/K_M for ApC were noted (Tables 4 and 5). Specificity ratios $((k_{cat}/K_M)_{GpC}/(k_{cat}/K_M)_{ApC})$ ranged from 33000-fold from 97 (for the Glu46Ser/(insert)47Gly variant) to 3.3×10^6 (for the Asn43His variant) with 2.8×10^5 for wild type. The 11-fold *increase* in specificity for guanine versus adenine by Asn43His-RNase T_1 as compared to wild type is the first report of this phenomenon.

A previous attempt to alter the specificity of RNase T_1 employed random mutagenesis involving six residues at the PRS from position 41 to 46 (*14*). The activity of 180 variants in periplasmic extracts were studied with GpC and ApC as substrates. This previous work emphasized the finding that the 8/3-variant (K41S/N43W/N44H/Y45A/E46D-RNase T_1) had a greatly altered preference for GpC as compared with ApC.[4] However, A similar change in specificity was found for Ser46/(insert)47Gly-RNase T_1 in the present study where its specificity ratio was about 3000-fold lower than that for wild type (see Table 5). The specificity ratio was lower for all variants from set 2 as compared with the wild-type enzyme as shown in Table 5. It should be noted that values of $(k_{cat}/K_M)_{ApC}$ are unexpectedly similar for a variety of mutants (see Tables 4 and 5 and footnote 3 for the 8/3-variant). Since an 11-fold increase in k_{cat}/K_M for ApC was the maximum observed for any mutant to date, it appears that lowering the above specificity ratio is more a matter of decreasing guanine specificity rather than increasing adenine specificity.

Comparisons with Previous Work. A recent report on random mutagenesis of the PRS of RNase T_1 involved six positions from Lys41 through Glu46 which should theoretically yield 6.4×10^7 combinations (*14*). This large number made it difficult to detect all possible transformants which approximately requires that, $n\ln(n) = 1.2 \times 10^9$, be screened (see above). In this case, 1.6×10^6 colonies ($\leq 0.2\%$) were screened and only 180 of these had activity when cultured on RNase-test plates. However, no colony evidenced activity that exceeded that for wild type and the two variants that were purified [i.e., the 8/3-variant (*14*; see above) and the 9/5-variant (K41E/Y42F/N43R/Y45W/E46Q-RNase T_1) (*35*)] had 1−2% of wild-type activity with RNA. Wild-type and more active variants (e.g., Gly, His, and Thr substitutions at Asn43) might not have been detected in this study because only a small portion of the combinatorial library was assessed. In any event, the aim of this previous study was to identify variants of RNase T_1 that had altered specificity with GpC and ApC (*14*); it is interesting that the present work involving 160000-fold fewer recombinants has elucidated mutants having essentially the same change in specificity (see above).

Previously reported substitution mutants of RNase T_1, Asn43Arg, and Asn43His/Asn44Asp, which converted its PRS to that of RNase Pch_1 and RNase Ms, respectively, resulted in variants having decreased activity (*8*); however, in the present study the Asn43His mutation, which converts the PRS of RNase T_1 into that for RNases Pb_1 and Fl_2, enhances RNase T_1 activity (Tables 2 and 4). The activities of RNase T_1 and RNase Pb_1 have been compared, and it was found that k_{cat}/K_M was at least 4-fold lower for the latter enzyme with GpN and poly(I) (*36*), which indicates that this difference between orthologous enzymes is probably not due to the amino acid residue at position 43.

[3] The prominent UV-difference spectrum that occurs when RNase T_1 binds guanine-containing ligands (*34*) is most likely the result of the interaction of guanine moiety with the ionized γ-carboxylate of Glu46 (Chitester, B. J., and Walz, Jr., F. G., unpublished experiments). To test whether the γ-carboxylate group of Glu47 in Glu46Pro/(insert)-Glu47-RNase T_1 interacts in a like manner, UV difference spectral experiments were performed using 2′-GMP with enzyme and ligand concentrations of 3.4×10^{-5} and 6.4×10^{-5} M, respectively. The shape of the difference spectra with this mutant and wild type were similar in sharing a positive maximum around 290 nm and a larger, negative minimum around 246 nm. However, a larger positive peak at around 263 nm and two new minor peaks (around 278 and 284 nm) were observed with the mutant enzyme. On the basis of these results, and the fact that k_{cat}/K_M for this mutant with GpC is greater than that for wild type (mainly due to a lower K_M; see Table 5), it appears likely that Glu47 interacts with the guanine group of 2′-GMP and GpC.

[4] The values of k_{cat}/K_M for RNase T_1 and the 8/3-variant with ApC were not reported but can be reasonably deduced from the time-course data for ApC cleavage (*14*) which showed first-order kinetics with $k = 1.98 \times 10^{-4}\ s^{-1}$ and $k = 1.67 \times 10^{-4}\ s^{-1}$, respectively. Assuming that the original ApC concentration (6.0×10^{-4} M) is at least 20-fold lower than K_M for both enzymes, these rate constants divided by the enzyme concentrations (2×10^{-4} M and 5×10^{-6} M, respectively) should equal $(k_{cat}/K_M)_{ApC}$ (*26*) and were found to be 1.0 and 33 $M^{-1}\ s^{-1}$, respectively. The values of $(k_{cat}/K_M)_{GpC}$ for wild type and this mutant were 1.9×10^6 and $2.3 \times 10^4\ M^{-1}\ s^{-1}$, respectively (*14*). These values of k_{cat}/K_M for both substrates were used to calculate the specificity ratios, $(k_{cat}/K_M)_{GpC}/(k_{cat}/K_M)_{ApC}$, which were 1.9×10^6 for wild type and 7.0×10^2 for 8/3-RNase T_1. Therefore, the specificity ratio for this mutant has decreased about 3000-fold as compared with wild type.

Conclusions. The present study of RNase T_1 is the first probe into the structural/functional space of its PRS using combinatorial random mutagenesis. It is remarkable that only five variants (out of 799 possibilities) in the present study had enzyme activity comparable to that for wild type particularly when possible combinatorial effects were included. A primary role of the PRS is to anchor the substrate and provide favorable contacts of the guanosyl 2′-OH group at the active site (*37*). It is possible that this limited role of the PRS in catalysis might be optimal for RNase T_1 and cannot be dramatically improved beyond the minor enhancements observed for some variants in the present study. A basis for this apparent resistance to change might reflect the fact that the guanine moiety bound to the wild-type enzyme has essentially all of its hydrogen bonding potential satisfied with a major portion of these bonds involving the economic use of donors hydrogens on a contiguous main chain segment. Nevertheless, further studies are warranted to secure this point (see below). Even though a definitive conclusion regarding the perfection of the PRS is not possible at this time, it seems likely since greater than 99% of the studied variants are incompatible with its optimal functioning.

The specificity of the enzyme for guanine (i.e., GpC versus ApC) among the mutants with wild-type activity was either enhanced because of a *decreased* activity wth ApC (e.g., 11-fold with Asn43His-RNase T_1) or diminished (e.g., 7-fold with Glu46Pro/(insert)47Glu-RNase T_1) by an *increased* activity with ApC. For other mutants, dramatic increases in the relative activity with ApC is mainly due to decreases in activity with GpC rather than increases in activity with ApC.

Highly conserved Glu46 can be substituted by proline without loss of wild-type activity if a Glu47 is inserted; it is likely that the γ-carboxylate group of Glu47 assumes the role of wild type Glu46 in guanine binding. Combinatorial effects may explain the moderately high activity of Asn43Gln/Asn44Gly-RNase T_1 mutation, but none of the double substitution mutants matched or exceeded wild type activity which suggests the lack of significant functional complementation at these positions. Two unanticipated results of interest were the finding of a Tyr45Ser mutant with an unusually high value for k_{cat} with GpC and the Asn43Tyr mutant which evidences a dramatic structural variation.

Future Studies. Additional combinatorial random mutagenesis studies on the PRS of RNase T_1 are planned for Asn43/Tyr45 with wild type and Asn43/Asn44 starting with Glu46Pro/(insert)47Glu. Even though mutations in the present study were limited to a surface loop, it cannot be presumed that the catalytically active mutants have the same thermodynamic stability as the parent enzyme. Indeed, substitutions at Asn44 have been already shown to destabilize the protein by about 2 kcal/mol (*38*). [In the case of Asn43Tyr, evidence presented here suggests that even covalent differences (disulfide and/or peptide bonds) might have occurred.] Therefore, thermodynamic studies using differential scanning calorimetry are planned for these variants to assess this aspect of their "perfection."

ACKNOWLEDGMENT

The authors thank Dr. Dipak Dube for communicating his original work on random mutagenesis which motivated the present study. We also are grateful to Dr. Nick Pace for the plasmid pMcRT1 and to Dr. Frank Smith for insights into sampling statistics.

REFERENCES

1. Takahashi, K., and Moore, S. (1982) *The Enzymes*, Vol. XV, pp 435−468, Academic Press Inc., New York.
2. Irie, M. (1997) in *Ribonucleases* (D'Alessio, G., and Riordan, J. F., Eds.) pp 101−130, Academic Press, San Diego.
3. Walz, F. G., Jr., Osterman, H. L., and Libertin, C. (1979) *Arch. Biochem. Biophys. 195*, 95−102.
4. Walz, F. G., Jr. (1997) *Biochim. Biophys. Acta 1350*, 183−188.
5. Arni, R., Heinemann, U., Tokuoka, R., and Saenger, W. (1988) *J. Biol. Chem. 263*, 15358−15368.
6. Kostrewa, D., Choe, H. W., Heinemann, U., and Saenger, W. (1989) *Biochemistry 28*, 7592−7600.
7. Walz, F. G., Jr. (1992) *Biochim. Biophys. Acta 1159*, 327−334.
8. Ikehara, M., Ohtsuka, E., Tokunaga, T., Nishikawa, S., Uesugi, S., Tanaka, T., Aoyama, Y., Kikyodani, S., Fujimoto, K., Yanase, K., Fuchimura, K., and Morioka, H. (1986) *Proc. Natl. Acad. Sci. 83*, 4695−4699.
9. Hakoshima, T., Toda, S., Sugio, S., Tomita, K., Nishikawa, S., Morioka, H., Fuchimura, K., Kimura, T., Uesugi, S., Ohtsuka, E., and Ikehara, M. (1988) *Protein Eng. 2*, 55−61.
10. Steyaert, J., Opsomer, C., Wyns, L., and Stanssens, P. (1991) *Biochemistry 30*, 494−499.
11. Hakoshima, T., Tanaka, M., Itoh, T., Tomita, K., Amisaki, T., Nishikawa, S., Morioka, H., Uesugi, S., Ohtsuka, E., and Ikehara, M. (1991) *Protein Eng. 4*, 793−799.
12. Grunert, H.-P., Landt, O., Zirpel-Geiesebrecht, M., Backmann, J., Heinemann, U., Saenger, W., and Hahn, U. (1993) *Protein Eng. 6*, 739−744.
13. Loverix, S., Doumen, J., and Steyaert, J. (1997) *J. Biol. Chem. 272*, 9635−9639.
14. Hubner, B., Haensler, M., and Hahn, U. (1999) *Biochemistry 38*, 1371−1376.
15. Osterman, H. L., and Walz, Jr., F. G. (1979) *Biochemistry 18*, 1984−1988.
16. Day, A. G., Parsonage, D., Ebel, S., Brown, T., and Fersht, A. R. (1992) *Biochemistry 31*, 6390−6395.
17. Noguchi, S., Satow, Y., Uchida, T., Sasaki, C., and Matsuzaki, T (1995) *Biochemistry 34*, 15583−15591.
18. Kumar, K., and Walz, Jr., F. G. (1998) *FASEB J. 12*, A1366.
19. Steyaert, J., Hallenga, K., Wyns, L., and Stanssens, P. (1990) *Biochemistry 29*, 9064−9072.
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
21. Black, M. E., and Loeb, L. A. (1993) *Biochemistry 32*, 11618−11626.
22. Shirley, B. A., and Laurent, D. V. (1990) *J. Biochem. Biophys. Methods 20*, 181−188.
23. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) *Protein Sci. 4*, 2411−2423.
24. Quaas, R., Landte, O., Grunert, H. P., Beinicke, M., and Hahn, U. (1989) *Nucl. Acids Res. 17*, 3318.
25. Oshima, T., Uenishi, N., and Imahori, K. (1976) *Anal. Biochem. 71*, 632−634.
26. Walz, Jr., F. G., and Kitareewan, S. (1990) *J. Biol. Chem. 265*, 7127−7137.
27. Irie, M., and Ohgi, K. (1976) *J. Biochem. 80*, 39−43.
28. Okorov, A. L., Panov, K. I., Kolbanovskaya, E. Y., Karpeisky, M. Y., Polyakov, K. M., Wilkinson, A. J., and Dodson, G. (1996) *FEBS Lett. 384*, 143−146.
29. Bornscheuer, U. T. (1998) *Angew. Chem., Int. Ed. 37*, 3105−3108.
30. Shlyapnikov, S. V., and Dementiev, A. A. (1993) *Chem. Pept. Proteins 516* (Pt. B), 663−673.
31. Dementiev, A. A., Moiseyev, G. P., and Shlyapnikov, S. V. (1993) *FEBS Lett. 334*, 247−249.

32. Lebedev, A. A., Shlyapnikov, S. V., Pustobaev, V. N., Kirpichnikov, M. P., and Dementiev, A. A. (1996) *FEBS Lett. 392*, 105−109.
33. Hebert, E. J., Grimsley, G. R., Hartley, R. W., Horn, G., Schell, D., Garcia, S., Both, V., Sevcik, J., and Pace, C. N. (1997) *Protein Expression Purif. 11*, 162−168.
34. Walz, Jr., F. G., and Hooverman, L. L. (1973) *Biochemistry 12*, 4845−4851.
35. Höschler, K., Hoier, H., Hubner, B., Saenger, W., Orth, P., and Hahn, U. (1999) *J. Mol. Biol. 294*, 1231−1238.
36. Yakovlev, G. I., Moiseyev, G. P., Bezborodova, S. I., Both, V., and Sevcik, J. (1992) *Eur. J. Biochem. 204*, 187−190.
37. Arni, R. K., Watanabe, L., Ward, R. J., Kreitman, R. J., Kumar, K., and Walz, F. G., Jr. (1999) *Biochemistry 38*, 2452−2461.
38. Hebert, E. J., Giletto, A., Sevcik, J., Urbanikova, L., Wilson, K. S., Dauter, Z., and Pace, C. N. (1998) *Biochemistry 37*, 16192−16200.

BI002837C