Modification of Ribonuclease T1 Specificity by Random Mutagenesis of the Substrate Binding Segment[†,‡]

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ABSTRACT: Attempts to modify the guanine specificity of ribonuclease T1 (RNase T1) by rationally designed amino acid substitutions failed so far. Therefore, we applied a semirational approach by randomizing the guanine binding site. A combinatorial library of approximately 1.6 million RNase T1 variants containing permutations of 6 amino acid positions within the recognition loop was screened on RNase indicator plates. The specificity profiles of 180 individual clones showing RNase activity revealed that variant K41S/N43W/N44H/Y45A/E46D (RNaseT1-8/3) exhibits an altered preference toward purine nucleotides. The ApC/GpC preference in the cleavage reaction of this variant was increased 4000-fold compared to wild-type. Synthesis experiments of dinucleoside monophosphates from cytidine and the corresponding 2′3′-cyclic diesters using the reverse reaction of the transesterification step showed a 7-fold higher ApC synthesis rate of RNase 8/3 than wild-type, whereas the GpC synthesis rates for both enzymes were comparable. This study shows that site-directed random mutagenesis is a powerful additional tool in protein design in order to achieve new enzymatic specificities.

In the past, peptides and proteins with new functional properties have been generated by combining rational protein design methods with nonrational ones. By this approach, functionally important domains of peptides or proteins are altered by random substitutions, and the resulting variant proteins are screened for novel functions. This attempt is very useful since de novo and rational designs are often difficult to achieve or only partly successful due to limitations in the prediction of protein folding, availability of detailed structural data, or limited information of protein−ligand interactions. Recently this approach was chosen to design DNA binding proteins with novel sequence specificities (*1*, *2*) and in the artificial increase of diversity of recombinant antibody libraries (*3*, *4*). In addition to the creation of new binding affinities, approaches were taken to modify substrate specificity and enzymatic properties (*5*−*7*).

A well-established model protein to study protein/nucleic acid interactions is ribonuclease (RNase) T1. RNase T1 (EC 3.1.27.3) is one of the best characterized proteins with respect to structure and enzymatic action (*8*, *9*). Moreover, it is used as a model system for investigations of protein stability (*10*−*12*) and folding (*13*). RNase T1, originally isolated from the fungus *Aspergillus oryzae*, is a small globular endonuclease consisting of a single polypeptide chain of 104 amino acid residues with a relative molecular weight of 11 085. It hydrolyzes single-stranded RNA with high specificity on the 3′ side of guanylic residues in a two-step mechanism. In the first transesterification step, the P−O5′ bond is cleaved and a cyclic guanosine 2′,3′-phosphate intermediate is formed, which is hydrolyzed in a second step to yield terminal guanosine 3′-phosphate groups. As determined from biochemical and structural data, the phosphodiester bond is cleaved by general acid−base catalysis, where His40, Glu58, Arg77, and His92 are involved as catalytic residues (*14*−*18*).

The crystal structures of the enzyme complexed with the inhibitor 2′GMP[1] (*15*, *19*) or 3′GMP (*20*) and with the dinucleoside monophosphate inhibitor 2′5′GpG (*21*) have been determined at high resolution. The guanine base interacts with six amino acid residues of RNase T1 by hydrogen bonding of the binding loop region mainly to main chain atoms (Asn43NH···N7Gua, Asn44NH···O6Gua, Asn45NH···O6Gua), one side chain group (GuaN1H···Oϵ1Glu46 and GuaN2H1···Oϵ2Glu46), and Asn 98 (GuaN2H2···OAsn98) being located outside the recognition loop. Additionally, the side chains of Tyr42 and Tyr45 form a hydrophobic pocket which binds the guanine base by stacking interactions enhancing hydrogen bonding between protein and base.

Although the mode of substrate binding and base recognition has been investigated in great detail by crystal structure and NMR analysis (*22*), these data did not allow the prediction of variants with altered specificities by rational

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[1] Abbreviations: ApC, adenylyl-3′,5′-cytidine; GpC, guanylyl-3′,5′-cytidine; NpC, nucleoside-3′,5′-cytidine; wt, wild-type; 2′AMP, 2′-adenylic acid; 3′AMP, 3′-adenylic acid; 2′GMP, 2′-guanylic acid; 3′GMP, 3′-guanylic acid; 2′3′cAMP, 2′,3′-cyclic adenosine monophosphate; 2′3′cGMP, 2′,3′-cyclic guanosine monophosphate.

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design approaches. Attempts to change the substrate specificity of this enzyme by rational substitutions have failed so far. Thus, a number of single amino acid substitutions within positions 42−46, which belong to the specificity center, indeed showed effects on enzyme activity, whereas the specificity was not affected (*23*, *24*). Molecular dynamics and free energy perturbation calculations resulted in the prediction that the Glu46Gln substitution renders the enzyme specific for adenine (*25*). The biochemical characterization of this variant revealed no change in enzymatic specificity toward the substrates ApC and GpC. Crystal structure analysis showed that 2′GMP did not bind to the specific guanine recognition site, but was localized, like 2′AMP, at the 3′ subsite (*26*). This result indicated that a single amino acid substitution can affect the tertiary structure in a nonpredictable way, changing the guanine recognition site no more able to interact with the substrate. In this work, we describe a semirational approach to change the base specificity of RNase T1 by the randomization of the substrate binding loop.

EXPERIMENTAL PROCEDURES

Materials. Restriction endonucleases, $Vent_R$ DNA polymerase, and T4 DNA ligase were purchased from MBI Fermentas or New England Biolabs. Nucleotides and enzyme substrates were from Sigma or Pharma Waldhof. High molecular weight RNA was from Merck. Media for bacterial growth were from Gibco BRL; yeast-RNA and toluidine blue O used for RNase indicator plates were from Boehringer Mannheim and Fluka, respectively. Oligonucleotides used for mutagenesis experiments and DNA sequencing were purchased from TIB Molbiol. T4 DNA sequencing kit and materials for protein purification were from Pharmacia. Chemicals were of pure and ultrapure quality and were obtained from commercial sources. *Escherichia coli* strain DH5αF′ [F′*ϕ*80d*lac*ZΔM15_Δ(*lac*ZYA-*arg*F)U169 *end*A1 *rec*A1 *hsd*R17(rK^-, mK^+) *deo*R *thi*-1 supE44 *λ-gyr*A96 *rel*A1] was purchased from Gibco BRL.

Mutagenesis. Site-directed random mutagenesis was carried out by a two-step PCR technique (27). Primers used were A2vo (5′-TAC GGA TTC ACT GGA ACT C−3′), A2hi (5′-CAT CTT AGC AGC CTG AAC-3′), and the mutagenesis primer 41−46* (5′-CAC AGA GAA ATC AAA ACC SNN SNN SNN SNN SNN SNN GTG TGG GTA AGA ATT GG-3′), where N means an equimolar mixture of all four nucleotides and S a mixture of 50% G and C each. In the first PCR step, 100 ng of DNA from plasmid pA2T1 (*28*, *29*) or its derivative coding for the inactive variant, the universal primer A2vo, and the random mutagenesis primer 41−46* were applied for amplification. The reaction was performed in a Minicycler (MJ Research) with initial denaturation (5 min at 98 °C), followed by 25 cycles with 1 min at 98 °C, 1 min at 45 °C, and 1 min at 72 °C. To complete polymerization, a final extension step (5 min at 72 °C) followed. The second PCR was performed with half of the purified product of the first step and the universal primer A2hi under identical conditions except that 50 °C was chosen as annealing temperature. Both reactions were carried out with $Vent_R$ DNA polymerase in a total volume of 50 μL under conditions recommended by the manufacturer.

Molecular Cloning of Randomized RNase T1 Genes. The DNA obtained from 10 amplification aliquots of the second PCR step was purified twice by gel electrophoresis on 2% agarose. Gel slices containing the resulting DNA fragments were cut out and electroeluted in an elution chamber (Biometra). The DNA was twice extracted with phenol/chloroform, subjected to *Xba*I/*Hin*dIII digestion, and subsequently purified as described above. The pIN-III-*ompA*2 vector DNA (*30*) was prepared by digestion with the same enzymes. Vector DNA was purified by gel electrophoresis, electroeluted onto DEAE-cellulose membranes, and subjected to phenol/chloroform extraction. Ligation was performed in a final volume of 15 μL with a total of 9 μg of DNA in a molar ratio of 1:10 (vector:insert) at 12 °C overnight.

Screening for Clones Secreting Active RNase T1 Variants. Competent *E. coli* DH5αF′cells were prepared by the method of Inoue et al. (*31*), yielding a transformation efficiency of 5×10^7 colony forming units/μg of pB*R*322. Cells were transformed with 40 ng of the ligation mixture per 100 μL cell aliquot. Transformants were screened for RNA hydrolysis activity on RNase indicator plates (*32*) containing Luria−Bertani medium supplemented with 100 mg of ampicillin, 75 mg of toluidine blue O, and 2 g of yeast RNA per liter. Plates were incubated 16−17 h at 37 °C. Colonies secreting active RNase variants could be identified by red haloes.

Determination of Substrate Specificity. Active clones obtained from indicator plates were grown in 5 mL of 2 × YT/ampicillin (100 μg/mL) medium at 37 °C overnight. Cells were harvested, and their periplasm was isolated by osmotic shock (*28*). Periplasmic fractions were incubated with each of the synthetic dinucleoside phosphates ApC, CpC, GpC, and UpC (1 μg of NpC and 9 μL of each periplasmic fraction, respectively) for 15 min at room temperature. The hydrolysis products were separated by thin-layer chromatography as described (*17*).

Enzyme Purification. Recombinant RNase T1 and variants were isolated from *E. coli* periplasm by DEAE anion exchange and size-exclusion chromatography (*33*). Proteins were purified to homogeneity as judged by SDS−polyacrylamide gel electrophoresis and silver staining. The concentrations of wt RNase T1 were determined spectrophotometrically using an extinction coefficient of $\epsilon_{278} = 17\,300\ M^{-1}\ cm^{-1}$ (*16*). To consider the Tyr/Trp exchange within the recognition loop of variant T1-8/3, the extinction coefficient of $\epsilon_{280} = 22\,400\ M^{-1}\ cm^{-1}$ of RNase T1 variant Tyr45Trp (*23*) was used.

RNA Hydrolysis Activity. RNase activity toward high molecular weight RNA was determined by the method of Anfinsen et al. (*34*) with the following modifications: a total volume of 250 μL, containing 100 μg of yeast RNA in 50 mM Tris/HCl (pH 7.5), 2 mM EDTA, was incubated with variant 8/3 (20−100 μM) for 15 min at 37 °C. By addition of 250 μL of precipitation solution (1% lanthane nitrate in 12.5% perchloric acid), the nonhydrolyzed RNA was precipitated on ice for 20 min. After centrifugation, the extinction coefficient of the supernatant was determined at 260 nm. These values were compared with a calibration curve obtained from wt enzyme (0.5−2 μM).

HPLC Analysis. HPLC analysis was performed as described earlier (*35*). In the case of ApC synthesis and cleavage, the components were separated by gradient elution with 0.01 M potassium dihydrogen phosphate (pH 4.15) and

0−30% methanol within 10 min followed by an increase in methanol concentration up to 50% within a further 5 min. 3′AMP, 2′3′cAMP, and ApC were detected at 268 nm. 3′GMP, 2′3′cGMP, and GpC were detected at 252 nm. ApC and GpC concentrations were determined by calibration curves obtained from pure standards. The 2′3′cGMP concentrations were calculated using the extinction coefficient $\epsilon_{352} = 13\,400\ M^{-1}\ cm^{-1}$ for 3′GMP (*17*), and $\epsilon_{258} = 14\,700\ M^{-1}\ cm^{-1}$ was used for 2′3′cAMP and 3′AMP.[2] To determine the concentration of ApC and GpC, the molar extinction coefficients of $\epsilon_{260} = 10\,600\ M^{-1}\ cm^{-1}$ (*36*) and $\epsilon_{280} = 12\,600\ M^{-1}\ cm^{-1}$ (*37*) were used.

Dinucleoside Phosphate Cleavage. ApC and GpC cleavage was carried out in 100 mM MES, 100 mM NaCl, 2 mM EDTA (pH 6.0) with a final concentration of 600 μM of each substrate. Enzyme concentrations varied between 5 nM and 5 mM. At different times, aliquots were taken from the reaction mixture and analyzed by HPLC immediately.

Synthesis Experiments. ApC and GpC syntheses were performed in 1.5 mL polypropylene tubes in a total volume of 100 μL in solution if performed at 0 °C, and in 50 μL for the frozen state at −10 °C. 2′,3′cAMP and 2′,3′cGMP (2.75 mM) each and cytidine (0.275 M) were dissolved in 0.1 M Tris/HCl (pH 7.0). Frozen state samples were cooled to 0 °C before enzyme solution was added. The reaction mixtures were rapidly shaken, placed in liquid nitrogen for 30 s, and incubated in a cryostat at −10 °C. Reactions were stopped by the addition of 50 μL of 0.4 M $ZnSO_4$ and analyzed by HPLC immediately. Synthesis reactions performed at 0 °C in an ice bath were carried out without freezing.

RESULTS

Construction of the Permutational Library. For library construction, we randomized the polypeptide segment between positions 42 and 46, which is mainly involved in guanine binding. Additionally we included in this permutation the amino acid residue Lys41 to ensure maximal structural flexibility of the recognition site with respect to its proximity to the catalytic residue His40. We chose a dual mutagenesis approach for library construction in order to circumvent possible background activity caused by wt enzyme contaminations. Thus, in preliminary experiments, the expression secretion vector pA2T1 (*28*, *29*) was used as template for PCR mutagenesis. pA2T1 is a modified derivative of the vector pIN-III-*ompA*2 (*30*) containing the chemically synthesized RNase T1 gene fused to the region encoding the signal peptide of the major outer membrane protein of *E. coli*. The first mutagenesis was performed as described using the mutagenesis primer 41−46*. By the application of RNase indicator plates, a colony expressing an inactive variant was randomly chosen. The corresponding gene was analyzed by DNA sequencing to prove the correctness of mutagenesis in the target region without affecting other parts. This inactive variant (K41stop/Y42S/N43A/N44R/Y45M/E46Y) was chosen as template for the permutational library. Mutagenesis was performed as described, and DNA fragments encoding the permutated RNase T1 variants fused to the ompA signal peptide coding region were cloned via *Xba*I/*Hin*dIII sites into the pIN-III-*ompA*2 vector. Transformation of competent *E. coli* DH5αF′ with an aliquot of the ligation mixture resulted in a total of 3.2×10^6 independent transformants with a vector background from self-ligation of less than 1%. The randomness of the mutated codons was confirmed by DNA sequencing of 10 clones from the library.

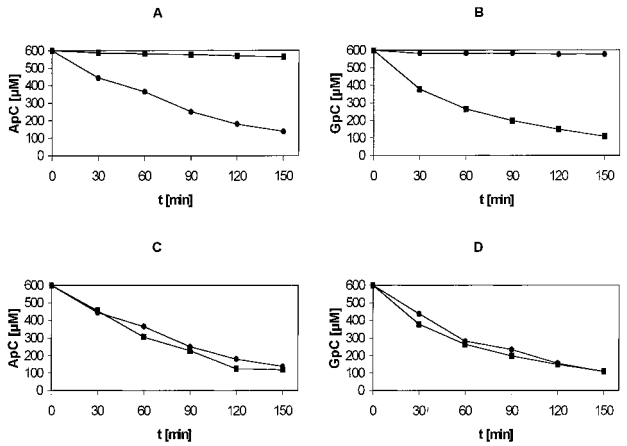

FIGURE 1: Time courses for ApC and GpC degradation catalyzed by RNase T1-8/3 (●) and wt enzyme (■). ApC degradation was performed with enzyme concentrations of 5 μM each (A) and GpC degradation with enzyme concentrations of 5 nM (B). To evaluate the relative activity of RNase T1-8/3, both enzyme concentrations were varied to obtain similar time courses for ApC (C) and GpC (D) degradation. Enzyme concentrations used were 5 μM (RNase T1-8/3) and 200 μM (wt) in ApC and 500 nM (RNase T1-8/3) and 5 nM (wt) in GpC cleavage. Hydrolysis experiments were performed in 100 mM MES (pH 6.0), 100 mM NaCl, 2 mM EDTA.

Selection of Active Enzymes and Determination of Their Base Specificity. RNase variants were expressed and secreted by *E. coli* cells using the expression/secretion vector pA2T1, and active RNases could be detected on RNase indicator plates (*32*). Thus, large numbers of variants could be tested for RNA hydrolysis activity by colony screening. In several rounds of transformation and plating on RNase indicator plates, 180 active variants were detected from a total number of 1.6×10^6 independent transformants. As judged by the diameter of red haloes surrounding the colonies, all active clones showed equal or lower RNA hydrolysis activity than wt. The substrate specificities of these 180 active variants obtained from this library were determined by their ability to cleave different dinucleoside monophosphates (ApC, CpC, GpC, and UpC) using periplasmic fractions of all clones. The hydrolysis profiles analyzed indicated that the RNase T1 variant RNase T1-8/3 (K41S/N43W/N44H/Y45A/E46D) was cleaving substrates with guanine and adenine in the 5′ position, whereas all other variants still exhibited exclusively guanine specificity.

Cleavage Experiments. RNase T1-8/3 was purified to homogeneity as judged by SDS−PAGE and silver staining. Mass spectrometric measurements confirmed the calculated molecular weight of 11 039. RNA hydrolysis of this variant under standard conditions at pH 7.5 (*37*) showed 1.2% of wt activity. To evaluate the activity toward GpC and ApC, their decrease was determined at various times by RP-HPLC. Relative to wt enzyme, RNase T1-8/3 had a significantly higher activity in ApC cleavage (Figure 1A) while the GpC cleavage activity was reduced (Figure 1B). To evaluate the ApC hydrolysis activity of each enzyme, concentrations were chosen in which wt showed a cleavage rate comparable to

[2] Sigma Chemical Co., personal communication.

Table 1: wt RNase T1 and RNase T1-8/3 Cleavage Rates[a]

enzyme	RNA (%)	NpC		ApC/GpC preference
		ApC (%)	GpC (%)	
wt-RNase T1	100	2.5×10^{-3}	100	2.5×10^{-5}
RNase T1-8/3	1.2	0.1	1.0	0.1

[a] To evaluate enzyme activity, different substrates were used. NpC cleavage rates are indicated with reference to the GpC cleavage rate of wt (=100%).

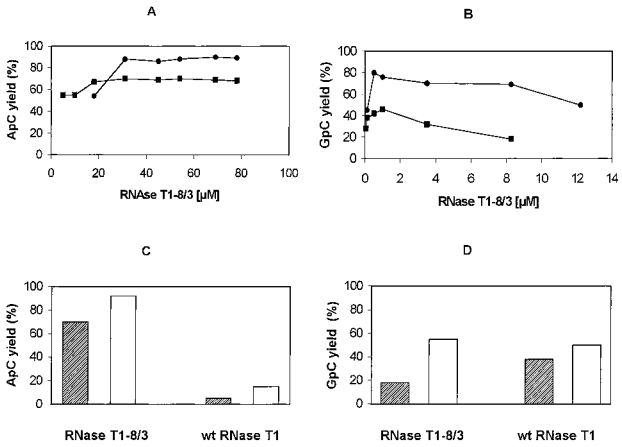

FIGURE 2: Synthesis yields of ApC and GpC catalyzed by wt RNase T1 and RNase T1-8/3. Depicted is the dependence of ApC (A) and GpC (B) synthesis on the enzyme concentration of RNase T1-8/3. Reactions were performed in liquid (0 °C, ■) and frozen (−10 °C, ●) mixtures. The synthesis rates for T1-8/3 and wt enzyme (C, D) were determined using enzyme concentrations which have been proven to be optimal in ApC synthesis catalyzed by RNase T1-8/3, and in wt-catalyzed GpC synthesis (*42*), respectively. ApC (C) and GpC (D) syntheses were performed in liquid (0 °C, 24 h reaction time, hatched bars) and frozen mixtures (−10 °C, 48 h reaction time, open bars) with the following enzyme concentrations: (C) 18.2 μM (0 °C), 68.2 μM (−10 °C); (D) 0.009 μM (0 °C), 0.082 μM (−10 °C). All experiments were performed with final concentrations of 2.75 mM 2′3′cGMP and 0.275 M cytidine in 100 mM Tris/HCl (pH 7.0).

that of RNase T1-8/3 (Figure 1C). Corresponding experiments were carried out to determine GpC cleavage rates (Figure 1D). A summary of the determined cleavage rates for various substrates is given in Table 1.

Synthesis Experiments. Further studies on substrate specificity were carried out by using the reverse transesterification reaction of the enzymes for synthesis experiments. Recently, we established that RNase T1-catalyzed GpC synthesis can take advantage of the yield-increasing effect of freezing the reaction mixtures (*35*), as already reported for protease-catalyzed peptide synthesis (*39*). In this work, we observed a strong influence of the wt enzyme concentration on GpC yield (*35*). Therefore, we studied the synthesis of ApC and GpC starting from the 2′3′-cyclic phosphodiesters and cytidine at various concentrations of RNase T1-8/3 in solution at 0 °C as well as in frozen reaction systems at −10 °C. As shown (Figure 2A,B), freezing the reaction mixture resulted in a substantial increase of the yield for GpC and ApC synthesis. The influence of the enzyme concentrations on the dinucleoside monophosphate yield was more distinct for GpC than for ApC synthesis. Furthermore, we compared the synthesis potentials of RNase T1-8/3 and wt enzyme using for both enzymes concentrations which have been proven to be optimal in ApC synthesis catalyzed by RNase T1-8/3, and in wt-catalyzed GpC synthesis (*35*). No remarkable differences between wt and variant could be observed concerning GpC synthesis, whereas ApC synthesis catalyzed by RNase T1-8/3 showed a 7-fold increased yield compared to the wt enzyme (Figure 2C,D).

DISCUSSION

Changing enzyme specificity by rational protein design approaches is often limited by several factors. For example, it is often very time-consuming to predict amino acid substitutions leading to altered functions by computational approaches. Theoretical predictions are difficult especially if a complex recognition mode between enzyme and substrate occurs. Moreover, many approaches fail because of nonpredictable influences of amino acid substitutions on the protein structure which may result in a significant loss in enzymatic activity.

With this in mind, we attempted to change the substrate specificity of RNase T1. The guanine specificity of RNase T1 is mainly caused by a loop segment between residues Tyr42 and Glu46. The complex recognition mode between the loop segment and guanine involves six hydrogen bonds. Two bonds are formed between substrate and the carboxylate side chain of Glu46, whereas the remaining four are formed with peptide backbone atoms. In addition to hydrogen bonding, the substrate is fixed by hydrophobic interactions with Tyr42 and Tyr45. This substrate binding mode, mainly based on peptide backbone atoms, was expected to render attempts to change the mode of substrate recognition very difficult. Kinetic studies showed that replacements of amino acids of the substrate binding loop not only affect the Michaelis constant, K_m, suggesting differences in the substrate binding mode, but also affected k_{cat}, possibly due to the induction of conformational changes in the enzyme's framework (*40*). Most investigated substitutions within the substrate binding site result in a significant loss of enzymatic activity. For example, the substitution of Tyr42 or Glu46 by Ala led to 5110-fold and 530-fold decreased specificity constants (k_{cat}/K_m) compared to wt enzyme (*24*).

First we created putatively new substrate binding segments in RNase T1 by simultaneous randomization of six positions of the guanine binding segment. It is remarkable that a relatively low number of the screened variants (0.01%) exhibit RNase activity. Therefore, one might conclude that the structural diversity is strongly limited in active enzyme variants.

The sequence alignment in Table 2 illustrates possible common features of substrate binding segments of the RNase T1 variants described here and homologous RNases belonging to the RNase T1 family (*8*, *41*, *42*). Thus, in position 41, the variants isolated from the library (Table 2A) as well as the eukaryotic (Table 2B) and prokaryotic members of the RNase T1 family (Table 2C) showed no amino acid preference. In position 42, a large fraction of hydrophobic amino acids can be detected. While most of the variants obtained in our experiments exhibit Tyr residues in that position, this residue is conserved in all eukaryotic members, whereas Phe residues are present in all prokaryotic RNases. In position 43, the diversity is limited for polar or charged amino acids. Variants obtained in this work show a lower degree of conservation. In contrast to the highly conserved

Table 2: Sequence Alignment and Specificities of Active RNase T1 Variants Randomly Selected from the Library Described in This Work (A) and Eukaryotic (B) and Prokaryotic (C) RNases from the T1 Family (8)[a]

			position						
	variant	specificity	–	41	42	43	44	45	46 –
A									
	8/3	(R)	–	S	Y	W	H	A	D –
	9/5	(G)	–	E	F	R	N	W	Q –
	9/4	(G)	–	T	Y	H	H	L	E –
	9/3	(G)	–	V	Y	G	G	Y	E –
	9/1	(G)	–	P	Y	S	D	K	E –
	9/7	(G)	–	R	Q	L	N	R	E –
	9/8	(G)	–	V	N	H	N	R	E –
	8/6	(G)	–	R	L	Y	N	M	E –
	8/7	(G)	–	W	Y	L	N	N	E –
	8/9	(G)	–	T	L	S	N	K	E –
	9/9	(G)	–	I	Y	R	A	N	D –
	9/10	(G)	–	E	Y	L	S	M	E –
	9/12	(G)	–	E	Y	L	A	L	E –
B									
	T1	(G)	–	K	Y	N	N	Y	E –
	Ap1	(G)	–	Q	Y	R	N	Y	E –
	C2	(G)	–	Q	Y	R	N	Y	E –
	Pb1	(G)	–	E	Y	H	N	Y	E –
	Pch1	(G)	–	E	Y	R	N	Y	E –
	Ms	(N)	–	E	Y	H	D	Y	E –
	Th1	(G)	–	V	Y	N	N	Y	E –
	F1	(G)	–	T	Y	N	N	Y	E –
	Fl1	(G)	–	T	Y	N	N	Y	E –
	Fl2	(G)	–	T	Y	H	N	Y	E –
	U1	(G)	–	T	Y	N	N	Y	E –
	U2	(R)	–	Q	Y	Y	D	–	E –
C									
	Ba	(N)	–	I	F	S	N	R	E –
	Bi	(N)	–	V	F	S	N	R	E –
	Sa	(G)	–	V	F	Q	N	R	E –
	St	(G)	–	V	F	E	N	R	E –

[a] Shown are the segments mainly responsible for substrate recognition (RNase T1 numbering). Abbreviations: (G), guanine specificity; (R), purine specificity; (N), no specificity; T1, RNase T1 from *Aspergillus oryzae*; Ap1, RNase AP1 from *Aspergillus pallidus*; C2, RNase C2 from *Aspergillus clavatus*; Pb1, RNase Pb1 from *Penicillium brevicompactum*; Pch1, RNase Pch1 from *Penicillium chrysogenum*; Ms, RNase Ms from *Aspergillus saitoi*; Th1, RNase Th1 from *Trichoderma harzianum*; F1, RNase F1 from *Fusarium moniliforme*; Fl1, RNase Fl1 from *Fusarium lateriticum*; Fl2, RNase Fl2 from *Fusarium lateriticum*; U1, RNase U1 from *Ustilago sphaerogena*; U2, RNase U2 from *Ustilago sphaerogena*; Ba, RNase Ba (Barnase) from *Bacillus amyloliquefaciens*; Bi, RNase Bi from *Bacillus intermedius* 7p; Sa, RNase Sa from *Streptomyces aureofaciens*; St, RNase St from *Streptomyces erythreus*.

aromatic amino acid in position 42 of the RNase T1 variants, there is no preference in position 45, although this residue is conserved in nearly all members of the eukaryotic RNases. The residue Glu46, which is conserved in all members of the RNase T1 family, was also strongly preferred in the variants obtained in our study. A negatively charged amino acid at position 46 is probably important for activity. Thus, 10 out of 12 active variants exhibit a Glu residue in this position. This is not surprising as Glu46 in wt RNase T1 is the only amino acid residue involved via its side chain interactions in guanine recognition. RNases 8/3 and 9/9 both show an Asp in this position. Since RNase 9/9 is nevertheless guanine-specific, this suggests that the Glu46Asp exchange is not sufficient for a change in RNase T1 specificity.

The RNase T1 variant K40S/N43W/N44H/Y45A/E46D (RNaseT1-8/3) shows a remarkable shift from guanine to purine specificity as demonstrated by cleavage of the dinucleoside monophosphates ApC and GpC and the synthesis of these compounds in the reverse reaction of the enzymes.

Cleavage experiments using the variant 8/3 showed that the activity toward RNA and GpC substrates is about 1% with respect to the wt enzyme. This decrease in enzymatic activity has already been described for other variants containing substitutions in the guanine binding segment (*24*, *26*, *44*). RNase T1-8/3 exhibits a 10-fold lower ApC cleavage activity than determined for GpC. Thus, the ApC/GpC cleavage ratio showed a remarkable shift from 2.5×10^{-5} (wt enzyme) to 1×10^{-1} (RNase T1-8/3). The estimated value for wt was in good agreement with the value of Walz et al. (*43*), who determined a ratio of 2×10^{-6} under different conditions. In addition to the estimation of enzyme activity by HPLC described here, we determined the kinetic constants of GpC cleavage by the increase of the absorbance due to the hyperchromic effect during transesterification (*37*). In GpC cleavage, we found a K_m value of 1085 μM and a k_{cat} of 25 s^{-1} for variant 8/3 (wt enzyme 129 μM and 249 s^{-1}, respectively). The resulting specificity constant of RNase T1-8/3 was about 1% in comparison to wt enzyme, which was in good agreement with our data determined by HPLC analysis. The kinetic constants for ApC cleavage could not be determined accurately using this assay, presumably due to the high absorbance of enzyme at the concentration necessary for these experiments.

To further investigate the substrate specificity of RNase T1-8/3, we additionally analyzed the reverse reaction for the synthesis of dinucleoside monophosphates from cytidine and the corresponding 2′,3′-cyclic diesters. In these experiments, the variant showed a significantly higher ApC synthesis rate than the wt enzyme, whereas the yield in GpC synthesis of both enzymes was almost equal. At 0 °C, the yield in ApC synthesis of variant 8/3 was even higher than the GpC yield using wt enzyme. All synthesis reactions were not disturbed by the second step of RNase T1 action, namely, the irreversible hydrolysis of the cyclic diesters, since no 3′-monophosphates could be detected (data not shown). This has already been reported for wt enzyme where the specificity constant for the transesterification reaction was determined to be 3 orders of magnitude higher in comparison to the hydrolysis reaction (*17*). Thus, the yield-limiting factor of both enzymes was the transesterification of the newly formed phosphodiester bonds. Obviously the changed protonation state of catalytic residues in variant 8/3 is the reason why synthesis rates show relatively high yields, whereas the cleavage reaction is drastically reduced. The relatively high yield in ApC synthesis compared to GpC synthesis is possibly caused by a tighter binding of 2′3′cAMP in comparison to 2′3′cGMP; otherwise, ApC would be much better cleaved.

Most eukaryotic members of the RNase T1 family cleave single-stranded RNA specifically after guanine residues, except RNases Ms and U2. RNase Ms, originally isolated from *Aspergillus saitoi*, cleaves single-stranded RNA without absolute base specificity. The ApC:GpC ratio was determined to be 1:460 (*44*). RNase T1-8/3 shows a value of 1:10 and is therefore less specific. RNase U2 from *Ustilago sphaerogena* cleaves single-stranded RNA specifically after purine nucleotides with a slight preference for adenosine (*45*). Our data show that the discrimination of RNase T1-8/3 is comparable with that of RNase U2 with the exception that guanine is the preferred base. Thus, RNase T1-8/3, obtained in this work, seems to be the only member in the T1 family

with a specificity toward purine nucleotides with a slight preference for guanine.

Using a random mutagenesis approach, we have been able to bypass the problems inherent in rational protein design. Thus, we randomly created a new substrate binding site in RNase T1 resulting in an alteration in substrate recognition and enzymatic activity. The reasons for modified specificity of RNase T1-8/3 and its discrepancy between cleavage and synthesis rates cannot be explained in detail without understanding the effects of these substitutions on the three-dimensional structure. Unfortunately, crystallization experiments have not been successful so far.

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