Addressing the Challenge of Changing the Specificity of RNase T1 with Rational and Evolutionary Approaches

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Although ribonuclease T1 (RNase T1) is one of the best-characterized proteins with respect to structure and enzymatic action, numerous attempts at altering the specificity of the enzyme to cleave single-stranded RNA at the 3′-side of adenylic instead of guanylic residues by rational approaches have failed so far. Recently we generated and characterized the RNase T1 variant RV with a 7200-fold increase in adenylyl-3′,5′-cytidine (ApC)/guanylyl-3′,5′-cytidine (GpC) preference, with the guanine-binding loop changed from 41-KYNNYE-46 (wt) to 41-EFRNWN-46. Now we have introduced the asparagine residue at position 46 of the wild-type enzyme as a single-point mutation in variant E46N and in combination with the Y45W exchange also occurring in RV. Both variants show an improved ApC/GpC preference with a 1450-fold increase for E46N and a 2100-fold increase for Y45W/E46N in comparison to wild-type activity. We also addressed the challenge of altering enzyme specificity with an evolutionary approach. We have randomly introduced point mutations into the RNase T1 wild-type gene and into the gene of the variant RV with different mutation rates. Altogether we have screened about 100 000 individual clones for activity on RNase indicator plates; 533 of these clones were active. A significant change in substrate specificity towards an ApC preference could not be observed for any of these active variants; this demonstrated the magnitude of the challenge to alter the specificity of this evolutionary perfected enzyme.

Introduction

Ribonuclease T1 (RNase T1; EC 3.1.27.3) is one of the best known representatives of a ~25-member superfamily of related microbial extracellular ribonucleases/toxins and is well characterized with respect to structure and enzymatic action.[1] RNase T1 from the mould fungus *Aspergillus oryzae*, is a small one-domain protein consisting of 104 amino acids with a relative molecular mass of 11 085 Da. It cleaves single-stranded RNA at the 3′-side of guanosine in a very specific manner. In minimal RNA substrates, guanine is preferred over adenine by almost a million fold (GpN and ApN substrates, N = A, C, G or U), though the biological significance of the base specificity is unknown. The catalytic mechanism is composed of two steps, cyclization and subsequent cleavage of the 2′,3′-cyclic phosphate intermediate to yield 3′-phosphorylated reaction products. As determined by structural and biochemical data, the phosphodiester bond is cleaved by general acid–base catalysis, in which His40, Glu58, Arg77 and His92 are involved as catalytic residues.[2, 3]

The guanine binding site is referred to as the primary recognition site (PRS) and exhibits the highest affinity by far among other subsites contributing to substrate binding.[4] The crystal structure of RNase T1 complexed with 3′-GMP shown in Figure 1 A reveals that the guanine moiety is bound mainly by hydrogen bonding to main-chain atoms of the nucleotide-recognition site formed by residues 42–46 together with N98. The glutamate in position 46, which is strictly conserved within the RNase T1 family, is the only amino acid residue involved in base recognition by side-chain hydrogen bonding (Figure 1 A).

Due to the specific nature of its side-chain interactions with the nucleotide base, E46 became a main candidate for changing the base specificity of RNase T1. Theoretical molecular dynamics and free-energy-perturbation calculations led to the prediction that the variant E46Q would have a greater relative adenine affinity than native RNase T1.[5] The amide group of the glutamine side chain was designated to provide N–H donors for hydrogen bonding with N(1) of the adenine base. Nevertheless, construction and biochemical characterization of this variant revealed no alteration of substrate specificity towards an adenine preference as shown by adenylyl-3′,5′-cytidine (ApC) and guanylyl-3′,5′-cytidine (GpC) hydrolysis.[6] Additionally, the

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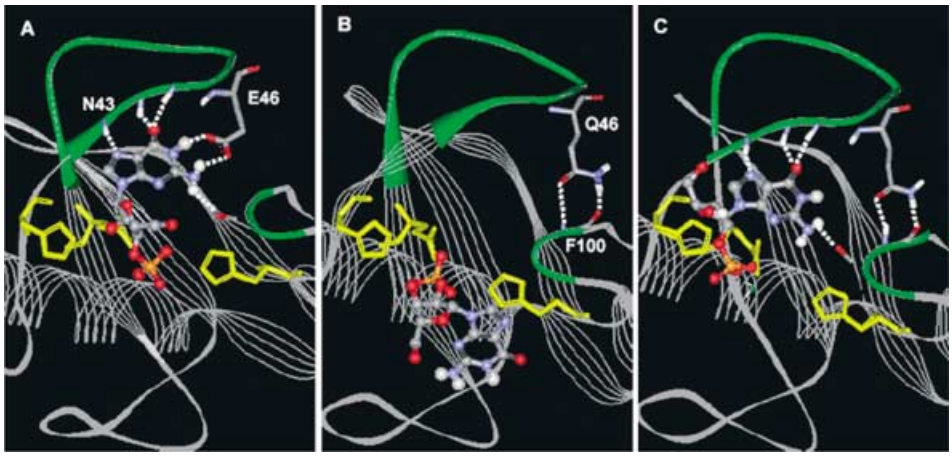

Figure 1. *Comparison of 2′-GMP binding in A) RNase T1 wt (ref. [23]), B) RNase T1 E46Q (ref. [6]) and C) RNase T1 9/5 (ref. [7]). Amino acid side chains are shown as sticks, inhibitors as balls and sticks and the protein backbone as a ribbon. Side-chains of amino acids His40, Glu58 and His92, involved in catalytic action, are depicted in yellow.*

crystallographic investigation of variant E46Q in complex with 2′-GMP (Figure 1 B) and 2′-AMP showed that the two nucleotides are not bound in the specific recognition site but at the 3′-subsite H92; this results in greatly reduced activity. The amide group of the new Q46 side chain thereby is tightly hydrogen bonded in a bidentate mode to main-chain atoms of F100 (Figure 1 B).

Within a semirational approach including the randomization of the guanine binding segment, an RNase T1 variant was isolated with the recognition segment changed from 41-KYNNYE-46 in the wild-type enzyme to 41-EFRNWQ-46 (variant 9/5).[7] The crystal structure of this variant shows that, although the glutamine residue in position 46 forms bidentate hydrogen bonds to the peptide backbone as in the single-point variant E46Q, the 2′-GMP inhibitor is bound to the PRS as observed for wild-type RNase T1, excluding H-bond interactions with residue 46 (Figure 1 C).

Recently we have introduced an asparagine residue at position 46 of RNase T1 9/5 to yield the variant RV. Crystallographic and biochemical characterization of this variant revealed a remarkable shift from guanine to purine specificity as shown by dinucleoside phosphate cleavage experiments.[8] Shortening of the side chain by a methylene group results in a 75-fold increased ApC cleavage accompanied by a 96-fold decreased GpC cleavage leading to a 7200-fold increased ApC/GpC preference in comparison to wild-type RNase T1. The structural basis for this alteration, deduced from the crystal structure of the variant, was that the amide bond of the asparagine participates in nucleobase recognition and is not fixed to the peptide backbone as observed for the glutamine residues in variants E46Q and 9/5.

Here we report the generation and characterization of the two RNase T1 variants E46N and Y45W/E46N containing amino acid substitutions in variant RV in order to investigate the influence of these particular substitutions on substrate specificity and enzyme activity.

Another way to alter a protein's phenotype in a desired direction is by directed molecular evolution. Within this new strategy for protein engineering, the natural evolution mechanism—random mutagenesis, recombination and selection (or screening)—is mimicked and has been successfully used for solving a diverse variety of biological design problems.[9] We decided to apply such a combinatorial approach to address the challenge of changing the substrate specificity of RNase T1 starting from wild-type protein and from variant RV.

Results

Purification and kinetic characterization of RNase T1 variants E46N and Y45W/E46N

Recombinant enzymes were isolated from the periplasm of the overproducers and were purified in two main steps. As shown in Figure 2, an acceptable purity for both variants was indicated by single bands after SDS-PAGE showing the same mobility as the wild-type protein. The yields of the enzyme variants were significantly lower ($\sim 7\ mg\,L^{-1}$) than those of the wild-type ribonuclease ($15\ mg\,L^{-1}$). The measured molecular masses of the variants were determined by MALDI mass spectrometry and agreed with those calculated from the amino acid composition.

In order to determine the substrate specificities of both RNase T1 variants, we investigated dinucleoside phosphate cleavage reactions. After 30 min incubation at room temperature, we could detect significant hydrolysis of GpC and ApC for both variants, whereas the wild-type enzyme only showed cleavage

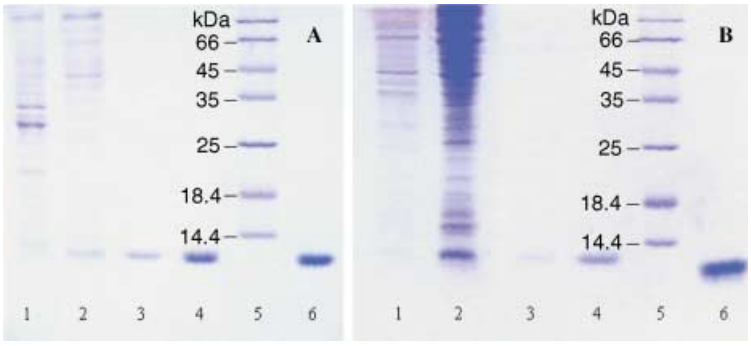

Figure 2. *15% denaturing SDS-PAGE of RNase T1 variants A) E46N and B) Y45W/E46N for different steps of purification. Lane 1, periplasmic fractions; lane 2, pools after diethylaminoethyl (DEAE) anion-exchange chromatography; lane 3, pools after gel filtration; lane 4, 10 μg of lyophilized protein; lane 5, standard; lane 6, 10 μg RNase T1 wt protein.*

products in the case of GpC (data not shown). For a more detailed characterization of the kinetic properties, we investigated both dinucleoside phosphate cleavage reactions spectrophotometrically. The resulting Michaelis – Menten diagrams are shown in Figure 3. No substrate saturation could be observed for either substrate or either variant within the investigated range of concentration. It is not possible to apply higher substrate concentrations within this photometric assay due to the high intrinsic absorption of the substrates. Therefore we were limited to determining only k_{cat}/K_m values from the initial slope of Michaelis – Menten diagrams. Those data together with the determined RNA-hydrolysis activities are summarized in Table 1 and compared to the kinetic parameters of wild-type RNase T1, and variants E46Q, 9/5 and RV. The specific activities for both former variants on high-molecular-weight RNA are much lower in comparison to the wild-type enzyme (3.3% for RNase T1 E46N and 3.7% for RNase T1 Y45W/E46N), but significantly higher than for variant RV with an activity of 1.5% compared to wild-type RNase T1. With GpC as a substrate, the situation is different. Here k_{cat}/K_M values are fourfold (E46N) and 2.5-fold (Y45W/E46N) lower than the value of variant RV which comprises 1% of the wild-type activity. Both variants show increased activities on ApC as a substrate. Compared to the wild-type enzyme, k_{cat}/K_M for ApC is approximately fourfold higher for E46N and ninefold higher in the case of Y45W/E46N, but does not reach the remarkable 75-fold higher ApC-cleavage activity of variant RV. The resulting specificity ratios are shifted 1450-fold (E46N) and 2100-fold (Y45W/E46N) towards an ApC preference compared to wild-type activity, but are not comparable to the specificity change in variant RV with a 7200-fold improved specificity ratio.

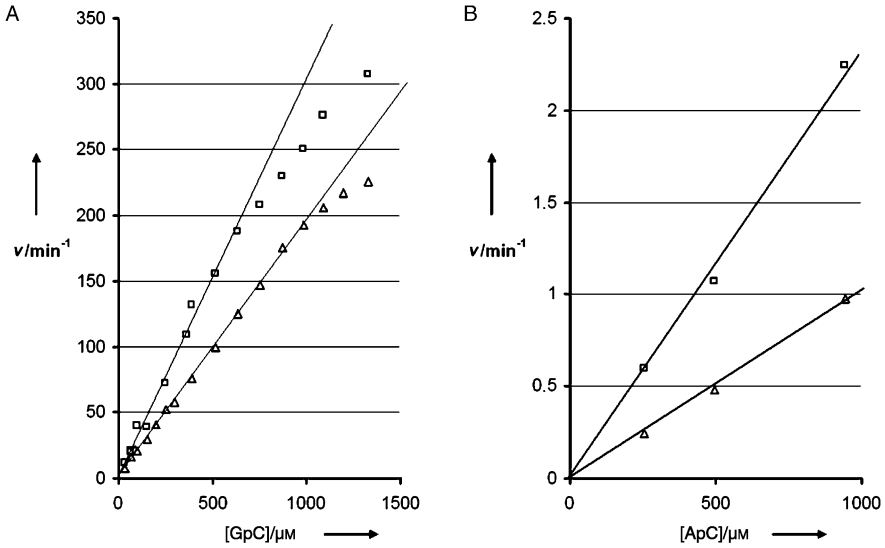

Figure 3. Michaelis – Menten diagrams of ApC and GpC hydrolyses by RNase T1 variants A) E46N and B) Y45W/E46N. (Δ) RNase T1 E46N; (□) RNase T1 Y45W/E46N.

Addressing a specificity change of RNase T1 with an evolutionary approach

Construction of the permutational libraries

As the starting point for an evolutionary approach to change the substrate specificity of RNase T1 towards an adenosine preference, we have used the genes of wild-type RNase T1 and the variant RV. Due to the shortness of the enzyme, we decided to use a high mutation rate by combining error-prone PCR with DNA shuffling. All manipulation steps leading to randomized full-length product are shown in Figure 4. As a template for the initial PCR we used the expression secretion vector pA2T1,[10, 11] which is a derivative of the vector pIN-III-ompA2[12] containing the chemically synthesized RNase T1 gene fused to the region encoding the signal peptide of the major outer-membrane protein of *Escherichia coli*. After mutagenesis, purified PCR product was cloned via *Xba*I/*Hin*dIII into vector pIN-III-ompA2 and transformation of TOP10F′ with a vector background of less than 1% was carried out by electroporation. Sequencing of 12 randomly chosen clones revealed a mutation rate of 1.5%.

Table 1. *Kinetic parameters for variants E46N and Y45W/E46N in comparison to wild-type and other RNase T1 variants.*

Enzyme	RNA hydrolysis[c] Specific activity [units mg^{-1}]	GpC K_M [μM]	GpC k_{cat} [min^{-1}]	GpC k_{cat}/K_M [min^{-1} M^{-1}]	ApC K_M [μM]	ApC k_{cat} [min^{-1}]	ApC k_{cat}/K_M [min^{-1} M^{-1}]	Specificity ratio k_{cat}/K_M (GpC)/ k_{cat}/K_M [ApC]
RNase T1 (wt)[d]	384000 (100)	135	10200	75.0×10^6	–[b]	–[b]	264	284000
variant E46Q[e]	n.d.[a]	116	20	170.7×10^3	n.d.[a]	n.d.[a]	n.d.[a]	
variant 9/5[f]	7091 (1.8)	979	444	453.5×10^3	n.d.[a]	n.d.[a]	n.d.[a]	
variant RV[g]	5644 (1.5)	418	327	782.5×10^3	803	16	19925	40
variant E46N	12640 (3.3)	–[b]	–[b]	195.0×10^3	–[b]	–[b]	1000	195
E46NY45W	14180 (3.7)	–[b]	–[b]	311.7×10^3	–[b]	–[b]	2280	137

[a] Not determined. [b] Only k_{cat}/K_M could be determined. [c] Percent of wild-type values are in parentheses. [d] Ref. [24]. [e] Ref. [6]. [f] Ref. [7]. [g] Ref. [8].

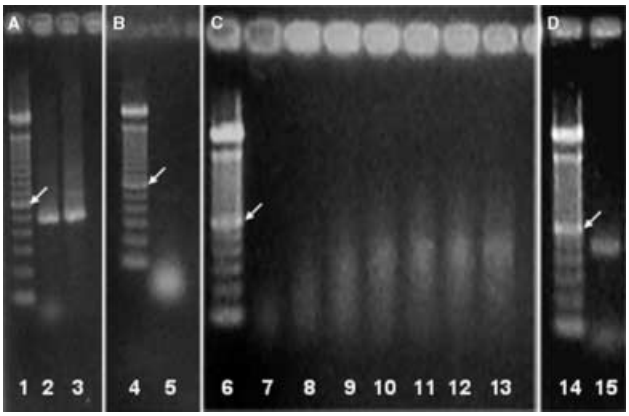

Figure 4. *DNA shuffling of RNase T1 genes. A) PCR amplification of the RNase T1 gene. PCR can be run under mutagenic conditions in order to increase the mutation rate. Lane 1, 100 bp ladder; PCR-amplified RNase T1 gene before (lane 2) and after (lane 3) purification. B) Gene fragmentation by DNase I digestion. Lane 4, 100 bp ladder; lane 5, RNase T1 gene after DNase I digestion. C) Reassembly PCR without primers. Lane 6, 100 bp ladder; lanes 7 – 13, aliquots of reassembly PCR after 20 (7), 25 (8), 30 (9), 35 (10), 40 (11), 45 (12) and 50 (13) cycles. D) PCR with primers: 1:40 dilution of the reassembly PCR after 50 cycles. Lane 14, 100 bp ladder; lane 15, PCR product after 15 additional cycles with primers. The arrows mark the 500 bp band within the standard lanes.*

Selection of active enzymes and determination of their base specificity

Randomized RNase T1 libraries were expressed and secreted by *E. coli* cells by using the vector pA2T1 so that active mutants could be easily identified through the formation of red haloes on RNase indicator plates.[13] Starting from the wild-type gene, we screened about 19000 clones of which 194 (1%) were active; starting from variant RV, we tested 28000 clones of which 284 (1%) showed activity. As judged by the diameter of red haloes surrounding the colonies and by the time course of halo formation, activity within the libraries ranged from about 100% to less than 1% of wild-type activity. We tested all 478 active clones found within the libraries for their ability to cleave substrates on the 3′-site of adenylic residues by incubating periplasmic fractions of all clones with the dinucleoside monophosphate ApC. Thin-layer chromatography analysis revealed that in none of the reactions derived from the wild-type library cleavage products could be identified after incubation times of upto 90 min. We also constructed a wild-type library with an even further increased mutation rate by using two consecutive error-prone PCRs combined with DNA shuffling. The percentage of active clones decreased to 0.1%, but apart from the 55 active clones out of a total of 52000, no ApC-cleaving activity could be identified.

Starting from variant RV, about 40% of active clones already showed ApC cleavage after 20 min, but in none of the reactions was a significantly increased amount of cleavage product observed in comparison to variant RV. Furthermore all periplasmic fractions of ApC-cleaving clones still showed high activity with GpC as a substrate after 15 min incubation. Therefore we were also unable to identify variants with a significantly increased GpC/ApC cleavage ratio within the library sizes we screened starting from RV.

Discussion

With RNase T1 variant RV we have recently characterized the first rationally designed RNase T1 variant with a significant alteration in substrate specificity towards a preference for adenine. To the best of our knowledge, this is the most effective variant regarding the Apc/GpC-specificity ratio characterized so far. Introduction of the asparagine residue into the wild-type enzyme in variant E46N also led to a remarkable change in substrate specificity. Therefore it seems to be evident that the amide group of the asparagine residue, in contrast to the amide group of the glutamine side chain within the almost inactive variant E46Q (Figure 1), participates in substrate binding leading to increased ApC- and strongly decreased GpC-cleavage activity. Addition of the Y45W exchange, which also exists in variant RV, strengthens this effect. Introduction of a tryptophan residue at position 45 within the wild-type enzyme leads to an increased k_{cat}/K_M value for GpC cleavage caused by an increased k_{cat}.[14] With reference to the Michaelis – Menten diagram, the same effect seems to occur when introducing this amino acid exchange in variant E46N. The k_{cat} value is distinctly increased, whereas K_M seems to be unchanged. Moreover, a slightly increased ApC cleavage could also be detected for RNase T1 variant Y45W.[15] Regarding the specificity ratio, variant RV is still about three times better than variant Y45W/E46N. Therefore the combination of all five amino acid exchanges within the primary recognition site of RNase T1 RV seems to be responsible for altering the enzyme specificity to the extent seen. Of course it needs to be pointed out that, in all specificity variants published so far, the alteration in substrate recognition was always made at the expense of activity.

In addition to addressing the challenge of substrate-specificity modification of RNase T1 with rational means, we also applied an evolutionary approach. We were not able to detect variants with specificities shifted towards an adenosine preference whether we started from the wild type or from the gene of variant RV. Library sizes of about 10000 per round of selection have previously been sufficient for many successful applications of DNA shuffling. Although we have clearly surpassed that number, our selection was not successful. Much larger libraries seem to be necessary to identify sets of RNase T1 variants with the desired specificity shift as the starting point for further rounds of selection. The main disadvantage of the screening introduced here is the necessity of first identifying RNase variants active on high-molecular-weight RNA and, secondly, of looking for specificity changes. We are currently establishing a selection system that allows direct identification of variants cleaving RNA on the 3′-site of adenosine combined with a higher throughput.

Experimental Section

Materials: Guanylyl-3′,5′-cytidine (GpC) and adenylyl-3′,5′-cytidine (ApC) were from Pharma-Waldhof (Düsseldorf, Germany). Muta-

genesis and standard primers were from MWG-Biotech (Ebersberg, Germany). The QIAEX II gel extraction kit was from Qiagen (Hilden, Germany). All enzymes and standards were from MBI Fermentas (Vilnius, Lithuania), *E. coli* strain TOP10F′ was from Invitrogen (Karlsruhe, Germany). All other chemicals were from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany).

Mutagenesis and enzyme purification: RNase T1 variants E46N and Y45W/E46N were obtained by site-directed mutagenesis by using two-step PCR.[16] The mutagenesis primers were 5′-CACAGAGAAAT-CAAAACCGTTGTAGTTGTTGTATTT-3′ for E46N and 5′-CACAGAGAAAT-CAAAACCGTTCCAGTTGTTGTATTT-3′ for Y45WE46N (base substitutions underlined). A2vo (5′-TACGGATTCACTGGAACTCTAGA-3′) and A2hi (5′-CATCTTAGCAGCCTGAAC-3′) were used as standard primers. Both variants as well as the wild-type enzyme were overproduced in *E. coli* TOP10F′ harbouring the corresponding plasmids.[11] The enzymes were isolated from the periplasm by osmotic shock and purified to homogeneity by DEAE-anion-exchange and size-exclusion chromatography as described previously.[10] In the case of variant Y45W/E46N, the size-exclusion chromatography step needed to be repeated once. Homogeneity and protein sizes were judged by SDS-PAGE and MALDI mass spectrometry.

Error-prone PCR: Random mutagenesis was introduced by error-prone PCR.[16] The primers A2vo and A2hi were used to amplify the full-length genes of RNase T1 wild-type and variant RV. The restriction sites for *Xba*I and *Hin*dIII allowed the PCR products to be ligated into vector pIN-III-ompA2[12] digested with the same enzymes. The resulting mutation rate per one error-prone PCR was determined to be 0.7%.

DNA shuffling: DNA shuffling was carried out as described previously with minor modifications.[18] DNA substrate was digested with DNase I (0.2 units) for 20 min at room temperature. Fragments (approximately 50 – 100 bp) were purified from a 2% agarose gel by using a QIAEX II gel extraction kit. Assembly PCR was extended to 50 cycles. The rate of point mutations per one DNA shuffling was 0.8%.

Screening for RNase T1 variants with an altered specificity: The randomized RNase T1 genes were cloned into plasmid pIN-III-ompA2, and TOP10F′ was transformed with the ligated DNA by electroporation. Libraries of transformants were spread on RNase indicator plates[13] with approximately 1000 colonies per 8 cm plate and incubated at 37 °C for 14 – 20 h. Colonies secreting active RNase T1 variants, identified by the formation of red haloes on the indicator plates, were cultured to an OD_{600} of 0.5 at 37 °C, induced with isopropyl-β-D-1-thiogalactopyranoside (0.1 mM) and allowed to grow for 16 h at 30 °C overnight. Cells were harvested, and periplasm was isolated by osmotic shock as described previously.[10] Periplasmic fractions (40 μL) were incubated with ApC or GpC (10 μL, 5 mg mL^{-1}) for 15 – 90 min at room temperature. Cleavage products were analysed by thin-layer chromatography spotting 10 μL from each reaction mixture.[3]

RNA hydrolysis activity: RNase activity towards high-molecular-weight RNA was determined by the method of Anfinsen et al.[19] with the following modifications. A sample (1 mL) of Tris buffer (50 mM, pH 7.5) containing yeast RNA (3 mg) and ethylenediamine tetraacetate (EDTA, 2 mM) was incubated with the enzyme for 15 min at 37 °C. The nonhydrolyzed RNA was precipitated on ice for 20 min by addition of ice-cold lanthane nitrate (2.5%, 250 μL) in 30% perchloric acid. After centrifugation, the extinction of the 1:20 diluted supernatant was determined at 260 nm. The specific activity (U [mg^{-1}]) of the enzyme was calculated by using the following equation:

$$U = \frac{E_{260\,\mathrm{nm\ (measured\ value)}} - E_{260\,\mathrm{nm\ (blank)}} \times 25}{\mathrm{mg\ protein}}$$

Dinucleoside phosphate cleavage: Substrate specificities were tested by performing cleavage experiments on both 3′,5′-dinucleoside phosphates GpC and ApC. The pure enzymes (9 μM) were incubated with the dinucleoside phosphates (1 mg mL^{-1}) for 30 min at room temperature. Cleavage products were analysed by thin-layer chromatography.[3]

To determine the kinetic parameters for dinucleoside phosphate cleavage, the hyperchromic effect during transesterification was measured spectrophotometrically. The reaction was carried out in a Beckman Coulter spectrophotometer DU640 B in 2-(4-morpholino)-ethane sulfonic acid (MES) buffer (100 mM, pH 6.0) containing NaCl (100 mM) and EDTA (2 mM) at 25 °C. The initial velocities were measured at least as duplicates at 280 nm for GpC transesterification by using the difference extinction coefficient $\varepsilon_{280\mathrm{nm}} = 2200\,\mathrm{M^{-1}\,cm^{-1}}$[19] and at 268 nm for ApC transesterification using the difference extinction coefficient $\varepsilon_{268\mathrm{nm}} = 2120\,\mathrm{M^{-1}\,cm^{-1}}$[21] at substrate concentrations up to 1300 μM for GpC hydrolysis and up to 950 μM for ApC hydrolysis. Depending on activity, the enzyme concentrations were 80 nM for GpC hydrolysis and 900 nM for ApC hydrolysis. The enzyme concentrations were determined spectrophotometrically by using a calculated absorption coefficient of $\varepsilon_{280\mathrm{nm}} = 17\,300\,\mathrm{M^{-1}\,cm^{-1}}$ for variant E46N and $\varepsilon_{280\mathrm{nm}} = 21\,680\,\mathrm{M^{-1}\,cm^{-1}}$ for variant Y45W/E46N.[22] The mixture without enzyme was pre-incubated for about 3 min to ensure that autohydrolysis did not occur, and the reaction was started by adding the enzyme.

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Keywords: enzymes · protein design · ribonuclease T1 · structure – activity relationships · substrate recognition

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