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Kinetic studies of guanine recognition and a phosphate group subsite on ribonuclease T_1 using substitution mutants at Glu46 and Lys41☆

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Abstract

pH-Dependent kinetic studies were performed with ribonuclease T_1 (RNase T_1) and its Glu46Ser, Lys41Met, and Lys41Thr mutants with GpC and polyinosinic acid (PolyI) as substrates. Plots of pH versus $\log(k_{cat}/K_M)$ for both substrates had ascending slopes that were significantly greater for RNase T_1 compared with Glu46Ser–RNase T_1, which indicated that the γ-carboxyl group of conserved Glu46 must be deprotonated (anionic) for maximal interaction with N(1)H and N(2)H of the guanine moiety of GpC or the N(1)H of the hypoxanthine moiety of PolyI. The involvement of the ε-ammonium group of nonconserved Lys41 at the **2p** subsite (i.e., for an RNA phosphate group two nucleotide positions 5′-upstream from the active site) was supported by comparisons of Lys41Met–RNase T_1 and Lys41Thr–RNase T_1 with wild-type. These mutants shared identical catalytic properties (i.e., k_{cat} and K_M) with wild-type using GpC as a substrate. However, k_{cat}/K_M for both were identical with each other but *lower* than those for wild-type when PolyI was the substrate (PolyI has a phosphate group that could interact at a putative **2p** site). The pH dependence of this latter difference can be interpreted as reflecting the loss of the **2p** subsite interaction with the wild-type enzyme upon deprotonation of the ε-ammonium group of Lys41. Subsite interactions for ribonucleases are shown to mainly increase k_{cat} and result in an attenuated pH dependence of k_{cat}/K_M.

Keywords: Ribonuclease T_1; Site-directed mutagenesis; Enzyme subsites; pH-Dependent kinetics

Ribonuclease T_1(RNase T_1)[2] catalyzes the depolymerization of RNA at guanylyl residues by a transesterification mechanism that yields a 3′-terminal, cyclic 2′,3′-guanosine phosphate residue [1,2]. The nature of guanine recognition at the primary recognition site has been elucidated in a variety of crystal structures and involves six hydrogen bonds including those for the guanine N(1)H and N(2)H with $O^{\varepsilon 1}$ and $O^{\varepsilon 2}$ of conserved Glu46 [2]. However, the protonated state of the γ-carboxyl group of this residue could not be determined from the crystal structures at the atomic resolutions obtained. Steady-state kinetic studies of the enzyme with minimal RNA substrates (GpNs; N = A, C, G, U) suggested that *two* carboxylates on the free enzyme need to be deprotonated for optimal substrate binding and/or catalysis on the basis of the pH dependence of k_{cat}/K_M [3]. One of these carboxylates is most likely that of Glu58 which interacts with the guanylyl 2′-hydroxyl group and acts as a general base in catalysis [2–4] but the identity of the other (among the remaining 12 carboxyls) is unknown. It has been proposed that Glu46 is ionized for its interaction with a guanine moiety based on the idea that ionic hydrogen bonds are stronger than non-ionic hydrogen bonds [5]. Nevertheless, there is no rigorous basis for assuming that a charged carboxylate of Glu46 can bind with a guanine group more tightly at the active site of RNase T_1 compared with its uncharged conjugate acid since countervailing hydration and/or steric/electrostatic effects might

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[2] *Abbreviations used:* RNase, ribonuclease; PolyI, polyinosinic acid; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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characterize this binding. In this regard, it has been shown that the binding of protonated (cationic) and neutral cytosine moieties (i.e., of 3′-CMP and 2′-CMP) surprisingly had the same affinity with pyrimidine-specific bovine pancreatic RNase A, even though the binding of the cationic cytosine would likely involve an ionic hydrogen bond between the cytosine $N(3)H^+$ and O^γ of threonine 45 [6,7]. In any event, one obvious approach toward elucidating the functional importance of Glu46 ionization is site-directed mutagenesis involving its substitution by an uncharged residue, followed by pH-dependent kinetic studies to test whether only *one* carboxylate is needed for optimal binding/catalysis of the mutant enzyme versus the wild-type. A number of such mutants (e.g., substitutions with either Ala or Gln) have been prepared but the necessary kinetic studies to resolve this issue were not reported [5]. In the present study we prepared Glu46Ser–RNase T_1 to perform such experiments and the results strongly support the view that the second carboxylate involved in substrate binding and/or catalysis is that of Glu46.

The existence and catalytic importance of subsite interactions beyond the active site of RNase T_1 have been reported [8]. These can be illustrated in terms of the RNA sequence (5′)...**2N-2p-1N-1p-G-p1-N1-p2-N2**...(3′) where **G-p1** represents the primary recognition site for the guanine base as well as sites for ribose and phosphate interactions at the active site, whereas, numerated **N** and **p** indicates putative subsites for nucleoside and phosphate groups, respectively. A **1p** site does not appear to exist but binding studies of the enzyme with d(pApG)/d(ApG) and d(pTpG)/d(TpG) pairs suggested the existence of a **2p** site which contributes about −0.8 kcal/mol for binding [8]. We examined three-dimensional models of RNase T_1 complexed with pApG having its guanine moiety bound at the **G** site and found that the terminal 5′-phosphomonoester group could interact with the ε-ammonium group of nonconserved Lys41. To test this hypothesis we prepared substitution mutants at position 41 with either methionine (having a similar methylene chain as lysine) or threonine (found at this position in three orthologous forms of the enzyme [2]) and studied their kinetic properties using PolyI and GpC that are substrates with and without a phosphate at the **2p** position, respectively; the results support this hypothesis.

Materials and methods

The glutamine-25 variant of the plasmid pMcRT1 ([9,10]; a gift of N. Pace) was used for mutation and expression of wild-type and mutant versions of RNase T_1. All site-directed mutagenesis enzymes and reagents were from Stratagene. Restriction enzymes used for digests of transformants were from New England Biolabs. Oligodeoxynucleotides were from NBI. General methods for recombinant DNA manipulations were performed as previously reported [11].

Site-directed mutagenesis was carried out using the QuikChange site-directed mutagenesis protocol (Stratagene). Primers for this procedure were designed to incorporate both the desired mutation and a new restriction enzyme recognition site to facilitate identification of positive clones. The primer sets used for each reaction were complementary pairs with the forward primers having the following sequences: for K41M, 5′-CCTTCGTAGTTGTTGTA**C**ATGTGTGGGTAAGAATTGGATCC-3′; for K41T, 5′-CCTTCGTAGTTGTA**C**GTGTGTGGGTAAGAATTGGATCC-3′; for E46S, 5′-GCTCACAGAGAAATCAAAACCT**GA**GTAGTTGTT**G**TACTTGTGT-3′. Base changes are shown in boldface type and the new *Rsa*I restriction sites are underlined. The PCR mixture included the following: 10–50 ng of miniprep pMcT1-Q plasmid DNA, 5 μL of 10X reaction buffer, 125 ng of each primer (forward and reverse), 50 μM each dNTP and H_2O for a final volume of 50 μL. PCR was done using the following program: denaturation at 95 °C for 30 s; annealing at 50 °C for 1 min; extension at 68 °C for 10 min; for 18 cycles. After a 1 h digestion with *Dpn*I, 1 μL of each reaction mixture was transformed into 50 μL of Epicurian Coli XL1-Blue supercompetent cells and the entire reaction mixtures plated onto LB agar plates containing chloramphenicol (250 μg/mL). Transformants containing the desired mutations were tentatively identified by restriction enzyme digests using *Rsa*I. Plasmid DNA was isolated and purified using a kit (Qiagen) and DNA sequencing was as previously described [10].

Expression and purification of proteins were carried out essentially as described [12]. Proteins were purified at room temperature using anion-exchange chromatography followed by gel filtration. The anion-exchange column consisted of four 5-mL HiTrap Q columns (Pharmacia Biotech) connected in series. The column was prepared according to the manufacturers directions with the start buffer being 0.10 M NaCl, 25 mM Tris, pH 7.5. Each supernatant was made 0.10 M in NaCl before being applied to the column. Column effluent was monitored using an RNase plate assay [10,13] to assure that the proteins were binding to the column and that the column was not being overloaded. Protein was loaded onto the column at a rate of 5 mL per minute. The column was then washed with five column volumes of start buffer and the protein was eluted using a linear salt gradient (0.1–0.6 M NaCl). The eluent was collected in 0.5-mL volumes and fractions containing RNase activity (as indicated by the RNase plate assay) were pooled and the volume reduced to 0.5–1.0 mL using a Gelman centrifugal concentrator (3 K cutoff). This volume was then applied directly to a preequilibrated HiPrepSephacryl S-100 gel filtration column (Pharmacia) using 50 mM ammonium carbonate as the equilibration

buffer. Chromatography was monitored using UV absorbance at 280 nm and fractions (0.5 mL volumes) exhibiting RNase T_1 activity were pooled, extensively dialyzed and lyophylized.

Protein characterization involved UV spectroscopy (240–320 nm) and the ratio of maximum (≅278 nm) and minimum (≅ 251 nm) absorbancies were all $\geqslant 3.2$. Enzyme concentrations were determined at 278 nm using $\varepsilon_{278\,nm} = 19{,}290\,M^{-1}\,cm^{-1}$ for all proteins [10]. Protein purity was verified using SDS–PAGE with a Bio-Rad Mini-Protean II system and Tris–glycine 8–16% polyacrylamide precast gels (Bio-Rad). Molecular masses of the recombinant proteins were determined by electro spray–mass spectroscopy using a Brucker Esquire LC-MS instrument as previously described [10] and were all found to be within 1 Da of the expected value.

Kinetic studies on the transesterification of GpC by wild-type and mutant proteins were performed by recording the absorbance increase at 280 nm for (first-order) progress curves using an Olis (Cary 118) UV/VIS spectrophotometer to determine k_{cat}/K_M from the apparent first-order rate constant as previously reported [10,14]. The buffers used in the pH range 4–9 were 0.002 M EDTA, 0.05 M Tris, 0.1 M potassium chloride, and 0.05 M sodium acetate titrated to the desired pH using glacial acetic acid and for pH values <4 sodium lactate–lactic acid replaced sodium acetate–acetic acid [3]. GpC concentrations were at least 20-fold lower than the reported K_M for wild-type RNase T_1 [10]. Enzyme concentrations ranged from 2.75×10^{-9} to 1.98×10^{-7} M depending on the activity of the mutants. Reactions were carried out in parafilm-sealed, 1-cm pathlength cuvettes at 25 °C and allowed to proceed through at least 3.5 half-lives. All reactions were performed at least in duplicate and were reproducible within 7%. Initial velocity steady-state kinetic experiments were performed at pH 6 and EnzFitter (Biosoft) was used to evaluate k_{cat} and K_M [10].

Kinetic studies of the depolymerization of polyinosinic acid (polyI) by wild-type and mutant proteins were determined using (first-order) progress curves as described above except that the increase in absorbance at 248 nm was measured. PolyI (nucleotide) concentrations were determined using $\varepsilon_{248} = 10{,}000\,M^{-1}\,cm^{-1}$ [15] and these were at least 20-fold less than K_M for the wild-type enzyme. Enzyme concentrations ranged from 7.09×10^{-9} to 5.42×10^{-7} M depending on the activity of the mutants. Initial velocity steady state kinetic experiments were performed at pH 6.0 to determine k_{cat} and K_M; the absorbance increase at 248 nm was measured and $\Delta\varepsilon = 1330\,M^{-1}\,cm^{-1}$ was used to calculate the initial molar velocities [15].

Results and discussion

k_{cat}/K_M from first-order progress curves. The pH dependence of k_{cat}/K_M can directly reflect the titration of acid–base groups on the free enzyme and free substrate that are important in enzyme–substrate binding and catalysis [3]. An efficient way to measure this parameter is to observe >90% of the progress curve (time-course) of the reaction where the rate law can be approximated as a first-order reaction (i.e., $d[S]/dt \approx (k_{cat}/K_M)[E]_0[S]$, where $[E]_0$ is the total enzyme concentration and the substrate concentration, [S], is initially at least 20-fold less than K_M). We verified the validity of this method for both GpC and PolyI substrates by independently determining k_{cat} and K_M from initial velocity experiments which were conducted at pH 6 where the lowest value of K_M (for GpC) was observed [3]. As shown in Table 1, the values of k_{cat}/K_M derived from initial velocity experiments agree with those from the first-order progress curves in all cases which validates the use of the latter method at other pH values. Also, the values k_{cat} and K_M for wild-type enzyme agree very well with those previously reported under comparable conditions for GpC [3,16] and PolyI [17]. The value of k_{cat}/K_M for Glu46-Ser–RNase T_1 with GpC is almost identical to that for Glu46Gln–RNase T_1, ~2-fold greater than that for Glu46Ala–RNase T_1 and ~10-fold lower than that for Glu46Asp–RNase T_1 [5].

The conjugate base of the γ-carboxyl group of Glu46 is required for tight binding of guanine and hypoxanthine moieties at the primary recognition site of RNase T_1. The pH dependencies of $\log(k_{cat}/K_M)$ for RNase T_1 and

Table 1
Steady-state kinetic parameters for wild-type RNase T_1, K41M–RNase T_1, K41T–RNase T_1, and E46S–RNase T_1 determined from initial velocity experiments with GpC and PolyI as substrates at pH 6.0 and 25 °C

Enzyme	GpC			PolyI		
	k_{cat} (s^{-1})	K_M ($\times 10^4$ M)	k_{cat}/K_M ($\times 10^{-6}\,M^{-1}\,s^{-1}$)	k_{cat} (s^{-1})	K_M ($\times 10^3$ M)	k_{cat}/K_M ($\times 10^{-5}\,M^{-1}\,s^{-1}$)
WT	392 ± 20	1.67 ± 0.02	2.34 ± 0.15 (2.51 ± 0.05)[a]	973 ± 79	1.53 ± 0.32	6.36 ± 2.33 (4.33 ± 0.06)[a]
K41M	416 ± 34	1.73 ± 0.01	2.40 ± 0.22 (2.40 ± 0.01)[a]	706 ± 101	3.04 ± 0.52	2.32 ± 0.88 (2.59 ± 0.04)[a]
K41T	365 ± 14	2.01 ± 0.05	1.82 ± 0.11 (2.75 ± 0.02)[a]	777 ± 162	3.06 ± 0.79	2.53 ± 1.61 (3.20 ± 0.06)[a]
E46S[b]	—	—	0.0139 ± 0.0002 (0.0165 ± 0.0014)[a]	—	—	0.243 ± 0.0085 (0.184 ± 0.0020)[a]

[a] Determined from first-order progress curves.

[b] Plots of initial velocity versus initial substrate concentration were linear for this mutant over all substrate concentrations tested. Values of k_{cat}/K_M were calculated from slopes of these curves using linear regression analysis. Values of K, were estimated by assuming no change in k_{cat} compared to the wild-type and these were 2.8×10^{-2} and 3.9×10^{-2} M for GpC and PolyI, respectively.

Glu46Ser–RNase T_1 with GpC and PolyI as substrates are shown in Figs. 1 and 2, respectively. The pH dependence of $\log(k_{cat}/K_M)$ for GpC with wild-type enzyme and corresponding slopes in Fig. 1 are in close agreement with those reported previously from initial velocity studies [3]. Differences in the pH dependence comparing this mutant with the wild-type enzyme for both substrates were substantiated by calculating ascending and descending slopes $(\Delta(\log(k_{cat}/K_M))/\Delta pH)$ for these data which are reported in these figures. In all cases, the absolute values for the ascending and descending slopes were significantly greater than unity with the exception of the ascending slopes for Glu46Ser–RNase T_1 which were either one or less than one for both substrates. On the other hand, the corresponding descending slopes are not significantly different. These results clearly support the view that two carboxyl groups are required in their conjugate base form for optimal substrate binding/catalysis for the wild-type enzyme with GpC and PolyI substrates, whereas, only one carboxylate is correspondingly required for the Glu46-Ser–RNase T_1 mutant.

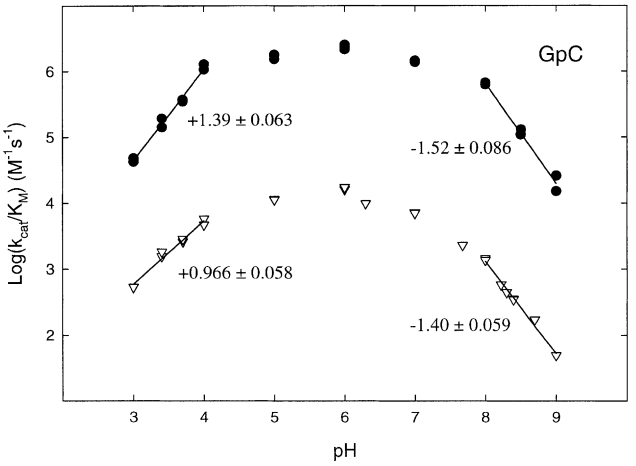

Fig. 1. Plot of $\log(k_{cat}/K_M)$ versus pH for enzyme catalyzed transesterification of GpC. k_{cat}/K_M values were determined from first-order progress curves. All individual (duplicate) data points are presented: (●) RNase T_1; (▽) Glu46Ser–RNase T_1. Slopes and standard errors were calculated using all data points for the regions indicated. Other conditions are found under Materials and methods.

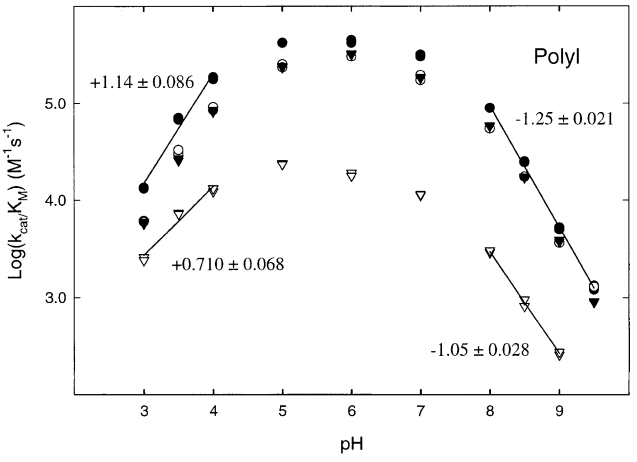

Fig. 2. Plot of $\log(k_{cat}/K_M)$ versus pH for enzyme-catalyzed transesterification of PolyI. k_{cat}/K_M values were determined from first-order progress curves. All individual (duplicate) data points are presented: (●) RNase T_1; (○) Lys41Met–RNase T_1; filled inverted triangles (▼) Lys41Thr–RNase T_1; inverted triangles (▽) Glu46Ser–RNase T_1. Slopes and standard errors (for RNase T_1 and Glu46Ser–RNase T_1 only) were calculated using all data points for the regions indicated. Other conditions are found under Materials and methods.

The pK_A values for 11 carboxyl groups of RNase T_1 have been determined from NMR titration studies, whereas that for the α-carboxyl group of Thr104 was not reported and the β-carboxyl of Asp76 did not appear to titrate between pH 2 and pH 10 [23]. Interestingly, the pK_A for Glu58 (3.96) was found to be greater than that for Glu46 (3.62) in this NMR study which was also the case for putative Glu58 (4.3 ± 0.2) and the "unknown" carboxylate group (3.4 ± 0.5) deduced from earlier kinetic studies [3]. Therefore, all evidence to date is consistent with the conclusion that the unknown carboxylate is that of Glu46 and it might be concluded that hydrogen-bonding interactions of guanine or hypoxanthine bases with the enzyme are greatly enhanced when the γ-carboxyl group of Glu46 is ionized. It is not possible to deduce the difference in ΔG for binding of the guanine moiety comparing enzyme species with protonated and deprotonated Glu46 in the present study but the total contribution of Glu46 to this interaction is about 4 kcal/mol [5].

Kinetic evidence for a specific subsite interactions of RNase T_1 with PolyI. The data in Table 1 also provide information on subsite interactions of RNase T_1 with PolyI; i.e., the value of k_{cat}/K_M for PolyI is 10- to 100-fold greater than those for IpY (Y = C or U) substrates obtained under the same conditions [16]. This difference is almost entirely due to an increase in k_{cat} for the polymeric substrate; in fact, k_{cat} for PolyI is even 2.5-fold greater than that for GpC which is the most efficiently catalyzed minimal RNA substrate [16]. Subsite interactions of ribonucleases that substantially increase k_{cat} are common and these might enhance catalysis by maintaining the substrate in stereo-electronic conformations that favor reactivity at the active site [18–21].

It was noted that the pH dependencies of $\log(k_{cat}/K_M)$ for PolyI were attenuated vis á vis GpC such that the ascending and descending slopes in Fig. 2 were significantly less than corresponding slopes for GpC as shown in Fig. 1. The absolute decreases in both slopes were 18% for wild-type and 26% (ascending) and 25% (descending) for Glu46Ser–RNase T_1; the greater attenuation for the mutant could reflect the fact that subsite effects are more pronounced when binding at the primary recognition site is decreased. This latter phenomenon and enhanced values of k_{cat} were previously

observed for RNase T_1 with ApGpC [19] or GpCpC (H.L. Osterman and F.G. Walz, Jr., unpublished experiments) vis á vis GpC; and for RNase A with uridine-2′,3′-(cyclic)-5′-diphosphate (vis á vis uridine-2′, 3′-(cyclic)-phosphate [18]). The attenuated pH dependence of k_{cat}/K_M can be interpreted to reflect subsite interactions that permit parallel reaction paths in the pH-dependent mechanism reflecting that additional protonated states at the active site can bind with the substrate [19]. A less stringent pH dependence of k_{cat}/K_M would be advantageous for catalysts like extracelluar RNase T_1 that might operate under variable pH conditions.

Lys41 is involved in a subsite interaction with a phosphodiester group of PolyI. The results in Table 1 clearly indicate that Lys41Met–RNase T_1 and Lys41Thr–RNase T_1 are indistinguishable from wild-type when GpC is a substrate but have lower values of k_{cat}/K_M compared with wild-type when PolyI is the substrate. The difference in ΔG for binding a *phosphomonoester* group at the **2p** subsite is ~ 0.6 kcal/mol [8] which compares well with that deduced from the difference in k_{cat}/K_M for RNase T_1 and the Lys41 mutants (~ 0.4 kcal/mol) for the putative interaction of the enzyme with a *phosphodiester* group of PolyI in the present study, between pH 3 and pH 8 (see Fig. 2). It was noted that this difference progressively decreases at pH values >8 (Fig. 2; e.g., compare pH ranges 3–4 and 8.5–9.5) which could reflect the titration of the ε-ammonium group of Lys41 which had previously been assigned a pK_A value of 8.6 [22].

It is obvious from the crystal structure of RNase T_1 that cationic Lys41 is available for interaction at the **2p** position in view of the relative positions of the **G-p1-N1** sequence at the active site. It is interesting that position 41 in 13 fungal members of the RNase T_1 superfamily is among the least conserved being occupied by one Arg, one Lys, two Val, three Glu, three Gln, and three Thr residues [2]. Minor subsite interactions of the enzyme could also occur with PolyI at the **p2** and **N2** positions which were previously shown to exert marginal effects with GpCpC as a substrate (H.L. Osterman and F.G. Walz, Jr., unpublished experiments). However, it is likely that the number of nucleotide residues in PolyI that significantly interact with the enzyme to form a productive complex does not exceed 3 [20].

In summary, the present work demonstrates that the anionic γ-carboxylate of Glu46 is the species that interacts strongly with guanine or hypoxanthine moieties at the active site of RNase T_1, presumably as a hydrogen bond acceptor for base group N1H and N2H donors. The role of Lys41 as a subsite for a phosphodiester group has been established and this most likely involves the **2p** position of an RNA substrate. This is the first ribonuclease subsite discovered that appears to involve a non-conserved residue. The general role of ribonuclease subsites in enhancing catalytic turnover and resulting in an attenuated pH dependence of k_{cat}/K_M has been further substantiated.

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