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Subsite Interactions of Ribonuclease T_1: Viscosity Effects Indicate That the Rate-Limiting Step of GpN Transesterification Depends on the Nature of N†

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ABSTRACT: We report on the effect of the viscogenic agents glycerol and ficoll on the RNase T_1 catalyzed turnover of GpA, GpC, GpU, and *Torula* yeast RNA. For wild-type enzyme, we find that the k_{cat}/K_m values for the transesterification of GpC and GpA as well as for the cleavage of RNA are inversely proportional to the relative viscosity of glycerol-containing buffers; no such effect is observed for the conversion of GpU to cGMP and U. The second-order rate constants for His40Ala and Glu46Ala RNase T_1, two mutants with a drastically reduced k_{cat}/k_m ratio, are independent of the microviscosity, indicating that glycerol does not affect the intrinsic kinetic parameters. Consistent with the notion that molecular diffusion rates are unaffected by polymeric viscogens, addition of ficoll has no effect on the k_{cat}/K_m for GpC transesterification by wild-type enzyme. The data indicate that the second-order rate constants for GpC, GpA, and *Torula* yeast RNA are at least partly limited by the diffusion-controlled association rate of substrate and active site; RNase T_1 obeys Briggs–Haldane kinetics for these substrates ($K_m > K_s$). Calculations suggest that the equilibrium dissociation constants (K_s) for the various GpN–wild-type enzyme complexes are virtually independent of N whereas the measured k_{cat} values follow the order GpC > GpA > GpU. This is also revealed by the steady-state kinetic parameters of Tyr38Phe and His40Ala RNase T_1, two mutants that follow simple Michaelis–Menten kinetics because of a dramatically reduced k_{cat} value (i.e., $K_m = K_s$). The data confirm the hypothesis that RNase T_1 contains a subsite with a preference for the leaving nucleoside cytidine; interactions at this subsite are reflected in k_{cat} rather than K_m. The contribution of the phenolic hydroxyl of Tyr38 to catalysis was hitherto not recognized.

Ribonuclease T_1 (RNase T_1) from *Aspergillus oryzae* (EC 3.1.27.3) is the best known representative of a family of homologous ribonucleases from various microbial sources (Hartley, 1980; Hill et al., 1983). The enzyme cleaves the 3′-O5′ ester bond of GpN[1] sequences (Sato & Egami, 1957). The first step in the reaction consists of a transesterification and results in a terminal 2′,3′-cyclic guanosine phosphate. This cyclic phosphodiester may, in a second step, be hydrolyzed to yield 3′-guanylic acid. In the transesterification reaction, Glu58 and His92 provide general-base and general-acid assistance, respectively. The protonated His40 imidazole probably acts as an electrostatic catalyst (Steyaert et al., 1990).

The mode of 2′-GMP binding observed in the RNase T_1–2′-GMP crystal complex (Heinemann & Saenger, 1982; Arni et al., 1988) provides a structural basis for understanding the guanosine specificity of the primary nucleotide binding site. The hydrogen-bonding potential of the guanine base is saturated by complementary donor/acceptor sites on the enzyme. The Glu46 carboxylate and the Asn98 main-chain carbonyl contribute 5.0 kcal/mol to guanine binding (Steyaert et al., 1991a).

It is quite well established that enzymes degrading polymeric substrates (including proteases, lysozyme, and staphylococcal nuclease) contain subsites for secondary substrate units, the interaction being mostly used to increase k_{cat} rather than to increase the binding of the substrate (Fersht, 1985). A series of binding and kinetic studies suggested that RNase T_1 contains a subsite for the leaving nucleoside N of GpN substrates with a preference for cytidine (Walz et al., 1979, and references cited therein). This conclusion has always been compromised by the observation that the k_{cat}/K_m values characterizing the four GpN substrates are virtualy identical, due to compensatory changes in k_{cat} and K_m ($k_{cat}/K_m \sim 1.5 \times 10^6$ M^{-1} s^{-1}; Osterman & Walz, 1978). Both the k_{cat} and K_m values vary about an order of magnitude and follow the sequence GpC > GpA > GpG > GpU. Walz and co-workers (1979) put forward the possibility that $K_m > K_s$ for some dinucleoside phosphates, an inequality resulting from a rate constant of product formation being comparable to or exceeding the rate constant of dissociation of the enzyme–substrate complex. In the latter case, the parameter k_{cat}/K_m is essentially determined by the bimolecular enzyme–substrate association rate constant.

To investigate this hypothesis, we studied the effect of solvent viscosity on the kinetic properties of RNase T_1. The results indicate that the magnitude of k_{cat}/K_m for some substrates is limited by the bimolecular rate for the diffusion-controlled encounter between substrate and active site. At low concentrations of substrate this is the criterion for an optimally efficient catalyst (Albery & Knowles, 1976).

Experimental Procedures

Materials. Wild-type, His40Ala, and Glu46Ala RNase T_1 have been previously discussed (Steyaert et al., 1990, 1991a). The Tyr38Phe mutant form of RNase T_1 was constructed by oligonucleotide-directed mutagenesis as described by Stanssens

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[1] Abbreviations: cGMP, 2′,3′-cyclic guanosine monophosphate; EDTA, ethylenediaminetetraacetic acid; 2′-GMP, 2′-guanylic acid; GpN 3′,5′-linked dinucleoside monophosphate compounds (N represents any of the four common nucleosides); Tris,tris(hydroxymethyl)aminomethane.

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et al. (1989) and purified to homogeneity as the above-mentioned RNase T_1 variants. Adenosine deaminase from calf intestine (EC 3.5.4.4) was from Boehringer. The following RNase T_1 substrates, purchased from Sigma, were used: the dinucleoside phosphates GpC, GpA, and GpU and *Torula* Yeast RNA (type VI). Glycerol (Merck), ficoll 400 (Pharmacia), and common reagents were purchased at the highest purity available.

Viscogenic Buffers. Viscogenic buffers had an ionic strength of 0.1 M and contained 0.1 M Tris-HCl, pH 7.5 (in the case of RNA cleavage), or 0.1 M imidazole, pH 6.0 (in the case of GpN transesterification), 2.5 mM EDTA, and the viscogenic agent (glycerol or ficoll 400). All solutions were filtered through a 0.22-μm nylon filter to remove suspended material. The kinematic viscosity η/ρ (where η is the viscosity and ρ is the density) of each assay mixture was determined at 35 °C with a Scott-Geräte AVS 300 viscometer. Densities were measured at 35 °C with an AP Paar DMA 602 density meter. Viscosities of the viscogenic buffers are reported relative to the reference buffer with no added viscogen (η_{rel} or η/η^0).

Kinetic Measurements. All experiments were performed at 35 °C. Ultraviolet absorbance was measured with a Kontron Uvicon 810P spectrophotometer equipped with a thermostated cuvette carriage. RNase T_1 concentrations are based on $A_{278}^{0.1\%} = 1.9$ for a 10-mm light path (Egami et al., 1964). At high substrate concentrations, 1- and 5-mm pathlength cuvettes were used to circumvent the high background absorbances. Kinetic parameters were derived from nonlinear least-squares fitting using the program ENZFITTER (Leatherbarrow, 1987).

(*A*) *GpC Transesterification.* The transesterification of GpN dinucleoside phosphate substrates can be followed by monitoring the absorbance increase at 280 nm (Zabinski & Walz, 1976). The steady-state kinetic parameters K_m and k_{cat} for the Tyr38Phe, His40Ala, Glu46Ala, and wild-type RNase T_1 catalyzed transesterification of GpC were determined from initial rate experiments as described (Steyaert et al., 1991a). The viscosity dependence of the ratio k_{cat}/K_m was determined by recording progress curves at 280 nm, with initial substrate concentrations much lower than K_m ($[S]_0 = 10$ μM); under this condition the GpC transesterification reaction follows first-order kinetics with rate constant V_{max}/K_m (Fersht & Renard, 1974; Steyaert et al., 1990). For each viscogenic buffer, at least three full-time courses were recorded.

(*B*) *GpA Transesterification.* The steady-state kinetic parameters K_m and k_{cat} for GpA transesterification by Tyr38Phe, His40Ala, Glu46Ala, and wild-type RNase T_1 were determined as described (Steyaert et al., 1991a). The viscosity dependence of the second-order rate constant for the conversion of GpA to cGMP and A by wild-type enzyme was measured by a coupled enzyme assay. The release of adenosine was followed with the enzyme adenosine deaminase (Mossakowska et al., 1989); deamination of adenosine is accompanied with a decrease in absorbance at 265 nm. The magnitude of the spectrophotometric signal makes the coupled assay preferable at low substrate concentrations. Full progress curves were recorded at $[GpA]_0 = 4$ μM ($K_m = 78$ μM; see Table I) such that adenosine release by wild-type enzyme follows first-order kinetics with a rate constant equal to V_{max}/K_m. Adenosine deaminase was used at a final concentration of 3×10^{-7} M, resulting in a maximal half-life of adenosine of 0.2 s (k_{cat}/K_m of adenosine deaminase is 1.1×10^7 M^{-1} s^{-1}; Murphy et al., 1969).

(*C*) *GpU Transesterification.* The steady-state kinetic parameters K_m and k_{cat} were determined essentially as described (Steyaert et al., 1991a). The viscosity dependence of the second-order rate constant for the conversion of GpU to cGMP and U was obtained from full-time course data by fitting the progress curves to the integrated Michaelis–Menten equation. Wild-type enzyme was added at a final concentration of 8.5×10^{-9} M, and $[GpU]_0$ was 92 μM.

(*D*) *RNA Cleavage.* The apparent steady-state kinetic parameters K_m and k_{cat} for the cleavage of *Torula* yeast RNA by wild-type and Glu46Ala RNase T_1 have been reported (Steyaert et al., 1991a). The viscosity dependence of the second-order rate constant for RNA cleavage was studied by monitoring full progress curves at 260 nm. Before use, the RNA was extensively dialyzed (cutoff = 12000) against 100 mM Tris-HCl, pH 7.5, to remove mono- and oligonucleotides. In a control experiment, we observed that the dialysis procedure removes a radiolabeled DNA octamer added to the solution of commercial yeast RNA. The RNA concentration was determined photometrically ($\epsilon_{260} = 8000$ M^{-1} cm^{-1}) and is expressed in mononucleotide equivalents, which equals the concentration of mononucleotides one would obtain after cleavage of all phosphodiester bonds. RNA was added to the reaction mixtures at a final concentration of 130 μM, a concentration that is at least 20 times below the apparent K_m values observed for wild-type and Glu46Ala RNase T_1 (see Table I).

Results and Discussion

Viscosity Variation Method. The rate of a diffusion-limited process is determined by the collision frequency of the reacting species. The variation of the kinetic parameters of an enzyme with solution viscosity can be used to evaluate the extent to which the second-order rate constant for enzymatic turnover (k_{cat}/K_m) is controlled by the encounter between substrate and active site (Brouwer & Kirsch, 1982, and references cited therein). The frequency with which two molecules collide is inversely proportional to the microscopic viscosity of the solution (Kramers, 1940; Caldin, 1964); monomeric polyhydroxylated molecules, such as glycerol, are agents typically used to increase the microviscosity of aqueous solutions. Polymeric species also increase the solution viscosity as measured by conventional methods but do not significantly change the rate at which small molecules diffuse; i.e., polymeric viscogens such as ficoll affect the macroviscosity but not the microviscosity of a solution (Blacklow et al., 1988, and references cited therein).

For an enzyme-catalyzed reaction following steady-state mechanism I, the ratio of the apparent kinetic constants

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (I)$$

k_{cat}/K_m is defined by eq 1. If k_1 and k_{-1} reflect diffusional

$$\frac{k_{cat}}{K_m} = \frac{k_1 k_2}{k_{-1} + k_2} \qquad (1)$$

processes, then $k_1^0/k_1 = \eta/\eta^0$ and $k_{-1}^0/k_{-1} = \eta/\eta^0$, where k_1^0 and k_{-1}^0 are rate constants obtained in buffer without viscogen (η^0) and where k_1 and k_{-1} are the rate constants in viscogenic buffers (η). Combining these relations with eq 1 gives

$$\frac{k_{cat}}{K_m} = \frac{k_1^0}{\eta_{rel} + k_{-1}^0/k_2} \qquad (2)$$

where $\eta_{rel} = \eta/\eta^0$. From eq 2 it is clear that the ratio k_{cat}/K_m depends on the solution viscosity if the partition coefficient of the enzyme–substrate complex ($P = k_{-1}^0/k_2$) is small, i.e., if the enzyme follows Briggs–Haldane kinetics (Briggs & Haldane, 1925). For an enzyme obeying simple Michaelis-

Table I: Steady-State Kinetic Parameters of Wild-Type, Tyr38Phe, His40Ala, and Glu46Ala RNase T_1 for Transesterification of GpC, GpA, and GpU and Cleavage of *Torula* Yeast RNA

	GpC[a]	GpA[a]	GpU[a]	RNA[b]
		wild type		
K_m (μM)	216 ± 29	78 ± 8	33 ± 3	2865 ± 377
k_{cat} (s^{-1})	429 ± 15	100 ± 3	36 ± 1	1296 ± 57
k_{cat}/K_m (mM^{-1} s^{-1})	1986	1282	1091	452
		Tyr38Phe		
K_m (μM)	32 ± 3	33 ± 7	40 ± 7	ND
k_{cat} (s^{-1})	5.3 ± 0.1	1.3 ± 0.1	0.66 ± 0.03	ND
k_{cat}/K_m (mM^{-1} s^{-1})	166	39	16.5	ND
		His40Ala		
K_m (μM)	354 ± 65	427 ± 53	394 ± 54	ND
k_{cat} (s^{-1})	0.35 ± 0.03	0.12 ± 0.01	0.11 ± 0.01	ND
k_{cat}/K_m (mM^{-1} s^{-1})	0.99	0.28	0.28	ND
		Glu46Ala[c]		
K_m (μM)	>5000	>5000	>5000	57117 ± 16055
k_{cat} (s^{-1})				725 ± 148
k_{cat}/K_m (mM^{-1} s^{-1})	5.1	3.5	2.05	12.7

[a] Measurements were performed in imidazole, pH 6.0, 35 °C, and 0.1 M ionic strength. The transesterification kinetics of GpG have not been investigated. This substrate could conceivably bind in a nonproductive complex with the enzyme (Walz & Terenna, 1976). [b] Measurements were performed in Tris-HCl, pH 7.5, 35 °C, and 0.1 M ionic strength. The RNA concentration is arbitrarily expressed in mononucleotide equivalents, which equals the concentration of mononucleotides one would obtain after cleavage of all phosphodiester bonds. ND, not determined. [c] For the Glu46Ala RNase T_1 catalyzed GpN transesterification, the ratio k_{cat}/K_m is the only determinable parameter since the initial velocities are linearly proportional to $[S_0]$ (≤ 2 mM); a lower limit for K_m has been estimated [see Steyaert et al. (1991a) for more details].

Menten kinetics ($P \gg 1$) the second-order rate constant should be independent of the microviscosity. The reciprocal of eq 2 (eq 3) shows that a plot of K_m/k_{cat} vs η_{rel} gives (a) the rate

$$\frac{K_m}{k_{cat}} = \frac{1}{k_1^0}\eta_{rel} + \frac{k_{-1}^0}{k_1^0 k_2} \qquad (3)$$

constant for association k_1^0 as the reciprocal of the slope and (b) the partition ratio as k_1^0 multiplied by the ordinate intercept.

The applicability of these relations is straightforward as long as the viscogenic agent does not alter the intrinsic kinetic properties of the enzyme. We decided to measure the viscosity dependencies of the kinetics of Glu46Ala and of His40Ala RNase T_1 as controls for any effect, other than the viscosity change, that may accompany the addition of the viscogenic agent. The steady-state kinetic parameters of His40Ala, Glu46Ala, and wild-type enzyme for a variety of substrates are listed in Table I. His40 is a key electrostatic catalyst, and replacing it by alanine results in an at least 100-fold decrease of k_{cat} for GpN transesterification (Steyaert et al., 1990). Glu46 contributes about 3.8 kcal/mol to guanine binding (Steyaert et al., 1991a), and the Glu46 to Ala mutation most probably increases k_{-1} by orders of magnitude. Both mutants must kinetically be limited by chemical rather than by diffusive processes because the mutations dramatically increase the partition coefficient; the second-order rate constants for GpN transesterification are reduced at least 100-fold. The k_{cat}/K_m values of these "slow" mutants should not vary with viscosity. The use of sluggish mutants as control enzymes in viscosity variation experiments has previously been introduced by Blacklow and co-workers (1988).

Steady-State Mechanism for GpN Transesterification. Figure 1 compares the viscosity dependence of the second-order rate constant for the wild-type RNase T_1 catalyzed transesterification of GpC with the control curves we obtained for Glu46Ala and His40Ala RNase T_1. Since the values of k_{cat}/K_m for the various enzymes differ by orders of magnitude, the data have been normalized according to eq 4 derived from eq 3 (Brouwer & Kirsch, 1982)

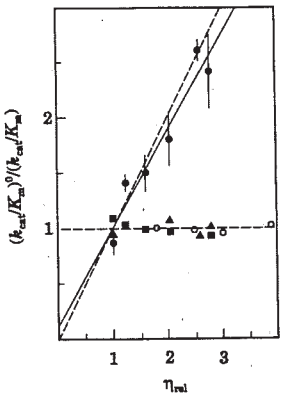

FIGURE 1: Plots of $(k_{cat}/K_m)^0/(k_{cat}/K_m)$ vs η_{rel} for the transesterification of GpC by wild-type (●, ○), His40Ala (▲), and Glu46Ala (■) RNase T_1 in glycerol-containing (filled symbols) and ficoll-containing (open symbols) buffers (100 mM imidazole, pH 6.0, 35 °C). The points represent the average of at least three independent measurements. For the viscosity-independent reactions, error bars have been omitted for clarity. The solid line through the wild-type/GpC data was drawn according to eq 4. The dashed lines are drawn for reaction rates that are entirely controlled by diffusive (slope = 1) and chemical (slope = 0) processes.

$$\frac{(k_{cat}/K_m)^0}{k_{cat}/K_m} = \frac{P}{1+P} + \frac{1}{1+P}\eta_{rel} \qquad (4)$$

where $(k_{cat}/K_m)^0$ equals the least-squares value of k_{cat}/K_m from eq 3 with $\eta_{rel} = 1$. For the wild-type RNase T_1 catalyzed conversion of GpC to cGMP and C, the normalized k_{cat}/K_m values are found to be inversely proportional to the relative viscosity when the microviscogenic agent glycerol is used. Linear least-squares analysis of K_m/k_{cat} vs η_{rel} according to eq 3 gives $k_1^0 = 2.3$ μM^{-1} s^{-1} and a partition ratio $k_{-1}^0/k_2 = 0.14$. That glycerol does not affect the intrinsic kinetic parameters of RNase T_1 is supported by the observation that the second-order rate constants of both slow mutants are, within experimental error, insensitive to the solution microviscosity (see Figure 1). Consistent with the fact that polymeric viscogens do not alter the rate at which small molecules diffuse, addition of ficoll 400 to the reaction mixture does not influence the second-order rate constant for GpC transesterification (see Figure 1). These data show that the bimolecular reaction rate (k_{cat}/K_m) for the conversion of GpC to cGMP and C is ~90% encounter-controlled.

The second-order rate constants for the RNase T_1 catalyzed transesterification of the four GpN dinucleoside phosphates are virtually identical, while the individual values for k_{cat} and K_m range within an order of magnitude of each other and follow the sequence GpC > GpA > GpG > GpU (Osterman & Walz, 1978; see also Table I). A comparative study of the viscosity dependence of the second-order rate constants for GpC, GpA, and GpU transesterification (see Figure 2) indicates that the partition coefficient for decomposition of the enzyme–substrate complex largely depends on the leaving nucleoside. The second-order rate constant for GpC transesterification reflects predominantly the enzyme–substrate association rate ($P = 0.14$) leading to $K_m > K_s$ (K_s is the equilibrium dissociation constant k_{-1}/k_1). The K_s value can be calculated from the relation $K_m = K_s + k_2/k_1$, derived from eq 1, and equals 29.5 μM. The second-order rate constant for GpU transesterification is independent of the microviscosity

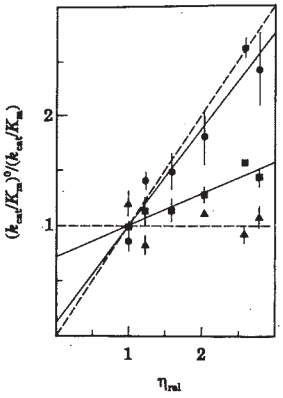

FIGURE 2: Plots of $(k_{cat}/K_m)^0/(k_{cat}/K_m)$ vs η_{rel} for the wild-type-catalyzed transesterification of GpC (●), GpA (■), and GpU (▲) in glycerol-containing buffers (100 mM imidazole, pH 6.0, 35 °C). The points represent the average of at least three independent measurements. Solid lines were drawn according to eq 4. Dashed lines represent the relative rates of reactions limited by diffusive and chemical processes.

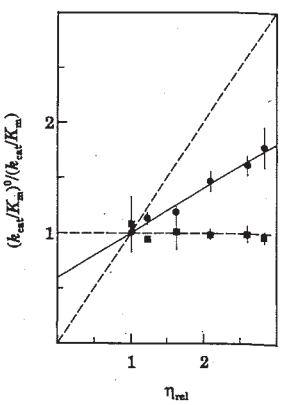

FIGURE 3: Plots of $(k_{cat}/K_m)^0/(k_{cat}/K_m)$ vs η_{rel} for the cleavge of *Torula* yeast RNA by wild-type (●) and Glu46Ala (■) RNase T_1 in glycerol-containing buffers (100 mM Tris-HCl, pH 7.5, 35 °C). The solid line was drawn according to eq 4. Dashed lines have the same meaning as in Figures 1 and 2.

of the reaction mixture, indicating that chemical rather than diffusional processes are rate-determining. This implicates that $K_m = K_s = 33$ μM if GpU is used as the substrate. Linear least-squares analysis of the data on GpA transesterification according to eq 3 yields $k_1^0 = 4.5 \times 10^6$ M^{-1} s^{-1} and a partition coefficient equal to 2.49, indicating that GpA transesterification is only in part diffusion-limited. The calculated K_s value for the latter substrate equals 56 μM.

The above calculations suggest that the equilibrium dissociation constants for the different GpN dinucleoside phosphates are comparable. The transesterification kinetics of GpC, GpA, and GpU by two mutant enzymes, Tyr38Phe and His40Ala RNase T_1, further illustrate that ground-state binding of the GpN substrates does not depend on the leaving nucleoside N. Similar to the above-discussed His40 to Ala mutation, removal of the Tyr38 hydroxyl group increases the partition coefficient considerably because of a dramatic reduction of the turnover number (see Table I), leading to K_m values equal to K_s. The observed Michaelis constants characterizing Tyr38Phe and His40Ala RNase T_1 are almost independent of the leaving nucleoside and equal ~35 μM and ~400 μM, respectively. The K_m values observed for Tyr38Phe RNase T_1 are very similar to the K_s values calculated for the wild-type enzyme, indicating that the Tyr38 hydroxyl group contributes considerably to catalysis (~2.6 kcal/mol) but not to ground-state binding. Crystallographic data (Arni et al., 1988; Koepke et al., 1989) suggest that this contribution results from a hydrogen bond between the phenolic hydroxyl group and one of the phosphate oxygens [for a further discussion see Steyaert et al. (1991b)]. The His40Ala substitution, however, clearly affects ground-state binding to a greater extent than would appear from the effect on the Michaelis constant for GpA and GpC; the true contribution is appreciated only when GpU is used as the substrate. In total, the present results demonstrate that the nature of the leaving nucleoside N of GpN substrates affects the value of k_{cat} but not the value of K_s. This strongly suggests the existence of a subsite with a preference for cytidine; interactions at this subsite are reflected in chemical turnover rather than in ground-state binding.

Steady-State Mechanism for RNA Cleavage. We addressed the question of to what extent the rate of cleavage of natural polymeric substrates is limited by the rate of protein–substrate association. Therefore, we analyzed the dependence of the second-order rate constant for *Torula* yeast RNA cleavage on the solution viscosity. From initial rate experiments (measuring the absorbance decrease at 298.5 nm; Oshima et al., 1976) we previously determined apparent values for the steady-state kinetic parameters k_{cat} and K_m for both wild-type and Glu46Ala RNase T_1 (see Table I; Steyaert et al., 1991a). Although the absolute values of these parameters are meaningless because of the arbitrary definition of the RNA concentration (see Experimental Procedures), they are useful to evaluate the effect of the mutation. Also, the second-order rate constant, derived from the ratio of k_{cat} over K_m, is reliable because the arbitrary substrate concentration units cancel out. Progress curve recordings at substrate concentrations much below K_m provide an alternative means to determine k_{cat}/K_m; this approach is independent of the absolute value of $[S]_0$. The absorbance increase at 260 nm for $[S]_0 \ll K_m$ was observed to obey pseudo-first-order kinetics. The derived k_{cat}/K_m values (data not shown) agree quite well with the ones obtained from initial rate experiments. The observation that the progress curves at $[S]_0 \ll K_m$ fit reasonably well to a monoexponential rather than a multiphasic function suggests that the second order rate constants for the cleavage of the various GpN sequences in single-stranded RNA are quasiindependent of N, as observed for the various GpN dinucleoside phosphates (see Table I). Figure 3 shows that the second-order rate constant for wild-type enzyme, unlike that of the slow Glu46Ala mutant (see Table I), depends on solution viscosity. For wild-type enzyme, k_1^0 and the partition coefficient were calculated to be 1.42 μM^{-1} s^{-1} and 1.45, respectively. The data indicate that the bimolecular rate constant for the RNase T_1 catalyzed cleavage of *Torula* yeast RNA contains a diffusion-controlled component.

Conclusions

RNase T_1 is an optimal catalyst for the transesterification of the dinucleoside phosphate GpC; the magnitude of k_{cat}/K_m is in large part limited by the rate constant for diffusion-controlled encounter between substrate and active site. The kinetics of GpU transesterification are limited by chemical processes. The second-order rate constant for GpA transesterification exhibits intermediate behavior and is partially diffusion-limited ($k_{-1}^0 \approx 2.5k_2$). The equilibrium dissociation constants for GpC, GpA, and GpU are similar (about 40 μM). The variation of the k_{cat} values indicates that RNase T_1 possesses a binding site for the leaving nucleoside preferring C > A > U. Interactions at this subsite appear to contribute to catalysis.

The second-order rate constant for the cleavage of *Torula* yeast RNA is partially diffusion-controlled (~40%), indicating

that RNase T_1 is a near-optimal catalyst. Further improvements in the efficiency of the chemical transformation step (k_{cat}) would lead to only small increases in the rate of RNA cleavage (maximally 60%) at low substrate concentrations. Since the RNA content of most natural environments may be expected to be low, the extracellular RNase T_1 enzyme seems to have been perfected catalytically from an evolutionary point of view (Albery & Knowles, 1976).

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