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# Subsite Interactions and Ribonuclease $T_1$ Catalysis: Kinetic Studies with ApGpC and ApGpU†

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ABSTRACT: The RNase $T_1$ catalyzed transesterification of ApGpC and ApGpU [to (ApG>p + C) and (ApG>p + U) products, respectively] was studied in steady-state kinetic experiments performed at 25 °C and 0.2 M ionic strength over the pH range 3–9. Values of $k_{cat}/K_m$ and $k_{cat}$ for these substrates were compared, as a function of pH, with those previously determined for GpC and GpU [Osterman, H. L., & Walz, F. G. (1978) *Biochemistry 17*, 4124]. The kinetic properties of ApGpN (N = C or U) substrates differed from those of GpN substrates in the following ways: (1) a dramatic attenuation of the pH dependence of $k_{cat}/K_m$; (2) no discrimination for C or U leaving nucleoside groups; and (3) an opposite pH dependence for $k_{cat}$. These results indicate that the adenosine moiety of the trimeric substrates binds with an enzyme subsite and that catalysis can proceed via three parallel reaction paths which are governed by apparent p$K$ values of 5.2 and 7.7 in the enzyme–substrate complex. These paths are characterized by rate constants that are 130, 280, and 1670 $s^{-1}$, which predominate in the acidic, neutral, and basic pH ranges, respectively. Since catalysis of GpN substrates apparently utilizes a single reaction path [characterized by rate constants of 350 and 38 $s^{-1}$ for GpC and GpU, respectively (Osterman & Walz, 1978)], it is concluded that the mechanism of catalysis for these trimeric and dimeric substrates is different. It is suggested that an active-site carboxylate group serves as a general base catalytic species in all cases except at high pH for ApGpN substrates, where an imidazole residue serves this function.

A complete understanding of substrate recognition and catalysis for depolymerizing enzymes requires studies of their behavior with multimeric substrates. This principle has been documented mainly in studies with proteases and carbohydrases (see Allen & Thoma (1976) for references), where the remote interaction of monomeric substrate units with enzyme subsites has been shown to influence the catalytic process. Similar studies with simple nucleases have been less systematic but have suggested the existence of electrostatic subsites for substrate phosphate groups in staphyllococcal nuclease (Cuatrecasas et al., 1968) and pancreatic RNase[1] A (Li & Walz, 1974; Mitsui et al., 1978). In addition, steady-state kinetic studies of RNase A with minimal RNA substrates (i.e., dinucleoside monophosphates CpN and UpN (N = A, C, G, U)) indicated a base group specific subsite interaction for adenine as the leaving (N) nucleoside group (Richards & Wyckoff, 1971). However, the existence of RNase A subsites for more complicated RNA substrates has not yet been investigated.

We have undertaken a systematic study of RNase $T_1$ with the final aim of understanding the detailed nature of its interaction with native RNA substrates and the relationship of these interactions to its catalytic action. RNase $T_1$ subsites were operationally identified in terms of possible enzyme binding with monomeric nucleoside and phosphate residues for the polymeric sequence: $j$N ... 2N-2p-1N-1p-G-p1-N1-

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[1] Abbreviations used: RNase, ribonuclease; Tris, 2-amino-2-hydroxy-1,3-propanediol; nucleotide designations follow the recommendations of the IUPAC–IUB commission as reported ((1970) *Biochemistry 9*, 4025).

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p2-N2 ... *i*N, where the G (guanosine) moiety interacts at the primary recognition site of the enzyme (Walz & Terenna, 1976). The results of equilibrium binding (Walz & Terenna, 1976; Walz, 1977) and kinetic (Zabinski & Walz, 1976; Osterman & Walz, 1978; Walz et al., 1979) studies indicated the existence of RNase $T_1$ subsites for the 1N, N1, and p1 positions; on the other hand, a subsite for 1p apparently does not exist, unlike the analogous case for RNase A (Li & Walz, 1974; Mitsui et al., 1978).

The pertinent finding in these studies, which led to the present investigation, was that ligand interaction at the 1N subsite appeared to be specific for an adenine moiety (i.e., as compared with uracil, cytosine, and thymine groups on dinucleotide and dinucleoside monophosphate substrate analogues (Walz & Terenna, 1976)). On this basis, it was decided to test the functional significance of this putative subsite interaction in the present study by using ApGpC and ApGpU as substrates for the RNase $T_1$ catalyzed transesterification reaction (i.e., ApGpC(U) → ApG>p + C(U)). The groundwork for assessing the affect of the Ap moiety in these trimers was established in comparable kinetic studies with GpC and GpU (Zabinski & Walz, 1976; Osterman & Walz, 1978). It will be demonstrated that the presence of the Ap moiety in ApGpN (N = C, U) substrates dramatically alters the pH-dependent kinetic behavior observed with simpler GpN substrates which suggests that the mechanism for RNase $T_1$ catalyzed transesterification can be different for these trimeric and dimeric homologues.

## Materials and Methods

RNase $T_1$ was prepared and its concentration determined as described previously (Walz & Hooverman, 1973). Nucleotides and ApG were products of Sigma; bovine pancreatic RNase A (RAF) and *Escherichia coli* alkaline phosphatase (BAPC) were obtained from Worthington; RNase $T_2$ was from Calbiochem; and *Micrococcus luteus* "primer-dependent" polynucleotide phosphorylase (0431) was from P-L Biochemicals. All other chemicals were reagent grade and deionized water having a specific conductance of 0.5 μmho was used in all solutions. pH measurements were performed at 25 °C by using a Radiometer PHM-26 pH meter.

ApGpNs (N = C, U) were prepared according to the method of Borer et al. (1975). ApG (0.1 g), $Na_2NDP$ (1.02 g), RNase A (5 mg), and 25 units of polynucleotide phosphorylase were reacted in 0.2 M Tris-HCl, pH 8.2 (11 mL), containing 10 mM $MgCl_2$ and 0.4 M NaCl, for 19 h at 37 °C. The mixture was heated for 2 min at 90 °C and filtered, and 5 mg of alkaline phosphatase ($(NH_4)_2SO_4$ suspension) was added and allowed to react at 37 °C for 8.5 h. Solid urea was then added to a concentration of 8 M and the mixture was extracted three times with chloroform/isoamyl alcohol (3:1 v/v) to remove protein. The aqueuous phase was diluted with water to 200 mL and applied to a DEAE-Sephadex A-25 column (2.6 × 47 cm) which was preequilibrated with 0.05 M $NH_4HCO_3$. Product was eluted by using a linear gradient from 0.1 to 0.6 M triethylammonium bicarbonate (pH 7.5) at 4 °C with a flow rate of 1.7 mL/min. ApGpNs appeared as single isolated peaks at 0.43 M buffer. Buffer was stripped with methanol until no triethylamine odor remained and the material was finally lyophilized yielding ~110 mg. Both ApGpC and ApGpU were analyzed on cellulose thin-layer sheets (Eastman) by using three solvent systems: 0.3 M $NH_4HCO_3$; saturated $(NH_4)_2SO_4$:1 M ammonium acetate: isopropyl alcohol (80:18:2); isobutyric acid:water:concentrated $NH_4OH$ (66:33:1). In all solvent systems, single spots were observed for ApGpC and ApGpU and digestion with RNase $T_1$ + alkaline phosphatase and RNase $T_2$ gave the expected products ((ApG + C(U)) and (Ap + Gp + C(U)), respectively).

The standard buffer used was 0.05 M Tris, 0.1 M potassium chloride, and 0.05 M sodium acetate titrated to the desired pH with acetic acid. The same buffer with lactate–lactic acid replacing acetate–acetic acid was used at pH <4.

The concentrations of ApGpC and ApGpU were determined spectrophotometrically by using molar extinction coefficients of $1.49 \times 10^4$ and $1.28 \times 10^4$ $M^{-1}$ $cm^{-1}$, respectively, at 280 nm, pH 7.0 and 25 °C. These values were determined by measuring the absorbance before and after complete reaction with RNase $T_2$ and using known extinction coefficients for the digestion products (P-L Biochemicals circulars OR-10 and 24). The difference molar extinction coefficients for ApGpC and ApGpU and their RNase $T_1$ digestion products were determined in the standard buffer at 25 °C by measuring the absorbance increase at 280 nm after equilibrium was achieved: these were essentially constant from pH 3 to pH 9 and had mean values of $2 \times 10^3$ and $0.6 \times 10^3$ $M^{-1}$ $cm^{-1}$ for ApGpC and ApGpU, respectively.

Initial velocities were obtained by recording the absorbance increase at 280 nm with a Cary 118C spectrophotometer by using a slit width of 0.2 mm and 0.2- and 1.0-cm pathlength cells. All experiments were conducted by using the standard buffer at 25 ± 0.1 °C. The ranges of enzyme and substrate concentrations employed were $4 \times 10^{-10}$ to $5 \times 10^{-9}$ M and $1.7 \times 10^{-5}$ to $6.3 \times 10^{-4}$ M, respectively. Initial velocities were determined in triplicate for at least six substrate concentrations at a given pH.

*Results and Treatment of the Data.* Thin-layer chromatography of the products resulting from 2-h, 25 °C incubations of RNase $T_1$ ($10^{-8}$ M) with either ApGpC ($10^{-4}$ M) or ApGpU ($10^{-4}$ M) in the standard buffer at pH 3, 6, and 9 indicated, in all cases, that catalytic cleavage occurred in the expected manner only at guanylyl residues. Care was taken to demonstrate this point since RNase $T_1$ has recently been shown to catalytically cleave ApN (N = A, C, G, U) substrates with a low efficiency (Walz et al., 1979).

Double-reciprocal plots of the initial velocity data were prepared by using

$$[E]_0/v = (K_m/k_{cat})(1/[S]_0) + 1/k_{cat} \quad (1)$$

where $[E]_0$ and $[S]_0$ are the total enzyme and substrate concentrations, respectively; $v$ is the initial velocity; $K_m$ is the Michaelis constant; and $k_{cat}$ is the turnover number. The data were analyzed by using the weighted least-square procedure of Wilkinson (1961) as described previously (Osterman & Walz, 1978).

Values of log ($k_{cat}/K_m$) for ApGpC and ApGpU are plotted as a function of pH in Figure 1 along with best-fit theoretical curves representing data for GpC and GpU (Osterman & Walz, 1978). From pH 4.5 to 7.5, $k_{cat}/K_m$ values for homologous trimeric and dimeric substrates are essentially the same. On the other hand, the pH dependence of this parameter was dramatically attenuated for ApGpN substrates such that, at pH 3 and 9, $k_{cat}/K_m$ values are one to two orders of magnitude greater than those for corresponding GpN substrates. Another striking difference between ApGpNs and GpNs is apparent in $k_{cat}$ values which are presented as a function of pH in Figure 2. In this case, there is a marked qualitative difference in the pH dependence such that $k_{cat}$ is unchanging at low pH and increases in two steps toward high pH for ApGpNs, whereas $k_{cat}$ values for GpNs significantly decrease at both pH extremes (Osterman & Walz, 1978). It is also obvious from Figures 1 and 2 that the significant

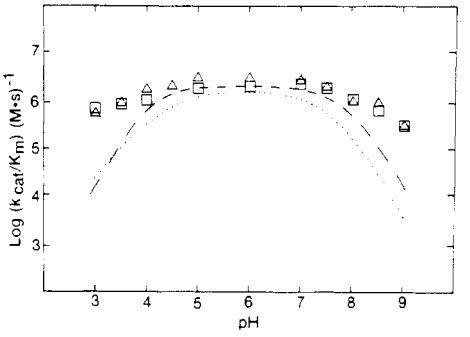



FIGURE 1: Plots of the logarithm of $k_{cat}/K_m$ for the RNase $T_1$ catalyzed transesterification of ApGpC and ApGpU and theoretical curves for GpC and GpU vs. pH. (□) ApGpC; (△) ApGpU; (---) GpC; (···) GpU; theoretical curves are from Osterman & Walz (1978). All experiments were conducted at 25 °C in 0.2 M standard buffer; other details are under Materials and Methods.

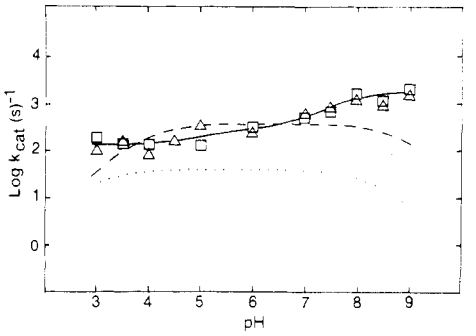



FIGURE 2: Plots of the logarithm of $k_{cat}$ for the RNase $T_1$ catalyzed transesterification of ApGpC and ApGpU and theoretical curves for GpC and GpU vs. pH. (□) ApGpC; (△) ApGpU; (---) GpC; (···) GpU; theoretical curves for GpC are from Osterman & Walz (1978). Theoretical curve for ApGpC and ApGpU was calculated by using eq 2 with the constants from Table I. Conditions were as described in Figure 1.

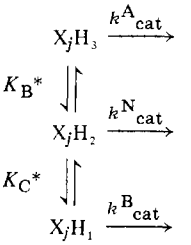
differences observed between GpC and GpU (Zabinski & Walz, 1976; Osterman & Walz, 1978) are not observed with ApGpC and ApGpU which are kinetically indistinguishable.

## Discussion

Previous kinetic studies of RNase $T_1$ with GpN (N = A, C, G, U) substrates (Osterman & Walz, 1978) were undertaken, in part, to provide the conceptual basis for interpreting the results of the present work using more complicated oligomers. The results from these studies led to the proposal of a concerted, general acid–base mechanism for the enzyme-catalyzed transesterification of GpN substrates having the γ-carboxylate group of Glu-58 (interacting with the imidazolium group of His-92) act as a general base for proton abstraction from the guanosine 2′-hydroxyl group and the imidazolium group of His-40 (possibly interacting with an unidentified carboxylate group) act as a general acid in protonating the 5′-oxygen of the leaving nucleoside group.

Differences in the kinetic properties of RNase $T_1$ with ApGpC and ApGpU, compared with GpC and GpU, can be summarized as follows: (1) a dramatic attenuation of the pH dependence for $k_{cat}/K_m$; (2) no kinetic discrimination for the leaving nucleoside groups; and (3) a qualitatively different pH dependence for $k_{cat}$. It is not likely that these differences are due to the effect of the adenylyl group on substrate conformation for the following reasons: (1) the conformation of ApGpC is considerably different from that of ApGpU as determined from circular dichroism measurements (Brahms et al., 1969) whereas the kinetic properties of these substrates with RNase $T_1$ are the same within experimental error; and

Scheme I




(2) substrate conformational effects should not be reflected in $k_{cat}$; yet this parameter is clearly distinct for ApGpN and GpN substrates.

The effect of subsite interactions in attenuating the pH dependence of $k_{cat}/K_m$ has already been observed in studies of RNase A comparing pU>p and U>p substrates (Li & Walz, 1974): in this example, the 5′-phosphate group of pU>p interacts with the $P_0$ subsite (Mitsui et al., 1978). In the case of GpN substrates with RNase $T_1$, binding and catalysis appear to be significant only with species of the enzyme having two unprotonated active site carboxyl groups and two protonated active site imidazole groups as deduced from the pH dependence of $k_{cat}/K_m$ (Osterman & Walz, 1978). A simple rationale for the attenuated pH dependence of $k_{cat}/K_m$ in the present study of ApGpN substrates is that their adenosine moieties favorably interact with an enzyme subsite such that binding and catalysis can significantly occur with enzyme species having active site groups in *several* ionization states. This view is consistent with the observation that the pH dependence for this parameter is equivalently attenuated in the acidic and basic pH ranges (see Figure 1). The existence of a specific subsite interaction of RNase $T_1$ with an adenosine moiety in the 1N position was previously indicated from equilibrium binding studies of the enzyme with dinucleotides and dinucleoside monophosphates (Walz & Terenna, 1976). Furthermore, an adenosine specific site, adjacent to the primary recognition site, has also been evidenced from kinetic studies of RNase $T_1$ using ApN substrates (Walz et al., 1979).

The substantial differences in the steady-state kinetic parameters for RNase $T_1$ with GpC and GpU (Osterman & Walz, 1978; see the theoretical curves for these substrates in Figures 1 and 2) were explained, in part, by postulating a selective subsite interaction of the leaving cytidine group with the enzyme which primarily affects $k_{cat}$ (Walz et al., 1979). Furthermore, this effect on $k_{cat}$ is probably mediated by the Glu-58-His-92 pair which interacts with the guanosine 2′-hydroxyl group (Osterman & Walz, 1978; Walz, 1976). Since the kinetic behavior of ApGpC and ApGpU is identical, it is reasonable to presume that the interaction of the leaving nucleoside group with the Glu-58-His-92 pair has been supplanted by that of the substrate adenosine group.

The pH dependence of $k_{cat}$ for GpN substrates is in the form of a plateau curve which significantly declines at the acid and alkaline extremes (Osterman & Walz, 1978; see Figure 2). This behavior was interpreted in terms of a single reaction path that reflects the apparent ionization of one carboxyl group and one imidazole group at the active site in the enzyme–substrate complex (the remaining carboxyl and imidazole groups participating in catalysis at the active site were presumed to be ionized at all pH values tested, having p$K$ values beyond the pH range investigated (Osterman & Walz, 1978)).

The pH dependence of $k_{cat}$ for ApGpN substrates, which is apparently biphasic and shows no decline at extreme pH values, can be fit by the formal model in Scheme I, where $X_jH_i$'s are species of the enzyme–substrate complex that

Table I: Theoretical Constants Characterizing the Rate-Limiting Step for the RNase $T_1$ Catalyzed Transesterification of ApGpC and ApGpU Substrates[a]

| constant | units | value |
|---|---|---|
| $k^{A}_{cat}$ | $s^{-1}$ | 130 ± 13 |
| $k^{N}_{cat}$ | $s^{-1}$ | 280 ± 70 |
| $k^{B}_{cat}$ | $s^{-1}$ | 1670 ± 560 |
| $K_B$* | M | 5.2 ± 0.7 |
| $K_C$* | M | 7.7 ± 0.6 |

[a] Calculated from the data in Figure 2 according to Scheme I by using eq 2; see text for additional details.

immediately precede the rate-limiting step; $K_B$* and $K_C$* are apparent acid dissociation constants that could characterize groups in the enzyme–substrate complex; $k_{cat}^{A}$, $k_{cat}^{N}$, and $k_{cat}^{B}$ are catalytic rate constants for predominant reactions in the acidic, neutral, and basic pH ranges, respectively. The equation describing this model is

$$k_{cat} = \frac{k_{cat}^{A}[H]^2 + k_{cat}^{N}K_B^*[H] + k_{cat}^{B}K_B^*K_C^*}{[H]^2 + K_B^*[H] + K_B^*K_C^*} \quad (2)$$

where [H] is the hydrogen ion concentration. Since the kinetic data for ApGpC and ApGpU were the same, within experimental error (see Figures 1 and 2), values of $k_{cat}$ for both substrates (and corresponding values of [H]) were used with eq 2 to obtain the best fit values for the constants in this equation. The KINFIT program for nonlinear curve fitting (Nicely & Dye, 1971) was employed and yielded the values which are listed in Table I. Simpler models for the pH dependence of $k_{cat}$, having a single proton dissociation, did not fit the data as well. The theoretical curve for ApGpN substrates in Figure 2 was calculated by using these constants with eq 2.

At this point, it is reasonable to conclude that the adenosine moiety of ApGpN substrates binds with the enzyme (possibly interacting with the Glu-58-His-92 pair) and that catalysis proceeds via three parallel paths which are governed by apparent p$K$ values of 5.2 and 7.7 in the enzyme–substrate complex. A plausible chemical mechanism for the RNase $T_1$ catalyzed transesterification of ApGpN substrates, based on these results, is illustrated in Figure 3. This mechanism is a reasonable extension of that already proposed for GpN substrates (Osterman & Walz, 1978) since it employs the same active site groups which are involved in a concerted general acid–base catalysis. It is proposed that the kinetic differences observed with ApGpN substrates vis-à-vis GpN substrates can be accounted for by the direct interaction of the substrate adenosine group with the Glu-58-His-92 pair.

Under pH conditions where the pathway characterized by $K_{cat}^{N}$ would predominate (i.e., pH 6–6.5), $k_{cat}$ and $k_{cat}/K_m$ for ApGpN substrates are virtually identical with those for GpC (see Figure 1 and 2) and the proposed mechanism (i.e., for the $X_jH_2$ species in Figure 3) is essentially the same as that already postulated for GpC (Osterman & Walz, 1978). The fact that $k_{cat}^{N}$ for ApGpN substrates (280 $s^{-1}$; see Table I) is comparable to $k_{cat}$ for GpC (350 $s^{-1}$; Osterman & Walz, 1978) is consistent with the view that the interaction of the adenosine moiety with the enzyme is similar, in some regards, to that proposed for the leaving cytidine residue of GpC (Osterman & Walz, 1978; Walz et al., 1979). The predominant pathway at low pH characterized by $k_{cat}^{A}$ is proposed to result from protonation of the bound adenine moiety. The minor effect of this apparent protonation (i.e., $k_{cat}^{A}$ is about one-half $k_{cat}^{N}$; see Table I) suggests that it does not directly involve a catalytic acid–base group; on the other hand, protonation of an adjacent adenine group could decrease the p$K$ of Glu-58 which, in turn, would result in a decreased catalytic rate. In this event, the p$K$ of the bound adenine residue would be expected to be increased compared with that for the free substrate; this is in agreement with the present data since the p$K$ for the adenine group of Ap is 3.7 (T'so, 1974) and p$K_B$* is 5.2 ± 0.7. Furthermore, a similar effect resulting from cytosine protonation of GpC was suggested from the pH dependent kinetic properties of this substrate with RNase $T_1$ (Osterman & Walz, 1978), which again indicates that adenine (of ApGpNs) and cytosine (of GpC) groups interact at a similar loci on the enzyme. At high pH, under conditions where $k_{cat}^{B}$ predominates, the proposed role of the adenosine group is to stabilize the neutral imidazole residue of His-92 so that it displaces the γ-carboxylate group of Glu-58 and assumes its role as a general base species for catalysis. This speculation is consistent with the following facts: (1) $k_{cat}$ increases to a plateau at high pH reflecting an apparent p$K$ of 7.7 ± 0.6; (2) $K_{cat}^{B}$ is ca. six times greater than $k_{cat}^{N}$ which would be reasonable if a more basic imidazole group substituted for a less basic carboxylate group as a general base catalytic species; and (3) the value of $k_{cat}^{B}$ approaches the maximal value of $k_{cat}$ determined for dinucleoside monophosphate substrates of RNase A, where an imidazole group most likely serves as a general base catalytic species (Richards & Wyckoff, 1971). Since the adenosine 2′-hydroxyl group

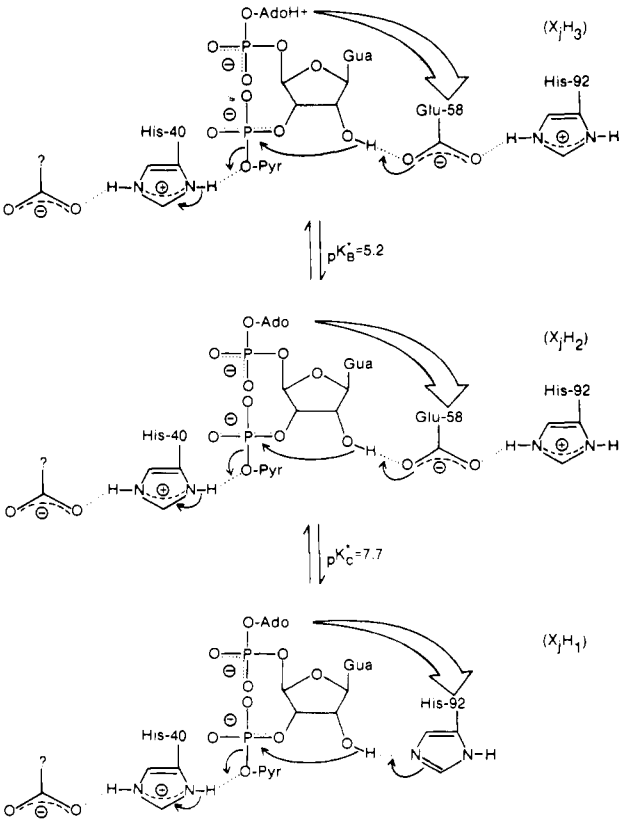



FIGURE 3: Possible mechanism for the RNase $T_1$ catalyzed transesterification of ApGpC and ApGpU. The species ($X_jH_i$s) and acid dissociation constants represent those designated in Scheme I. The p$K$ values for Glu-58, His-40, and the unknown (?) carboxyl group are assumed to be beyond the pH range investigated. p$K_B$* is identified with protonation of the adenine group at nitrogen-1. p$K_C$* is identified with protonation of His-92, and deprotonation of this group is coupled with a conformational change yielding species $X_jH_1$. The broad arrows from the substrate adenine group schematically illustrate a direct interaction with the Glu-58-His-92 pair. Electron flows are indicated by the small arrows for a concerted general acid–base catalysis which could involve a pentavalent phosphorus transition state or intermediate (Ekstein et al., 1972). See text for further details.

of ApG apparently binds with the enzyme[2] and ApNs are substrates (albeit poor ones) for RNase $T_1$ catalyzed transesterification (Walz et al., 1979), it is possible that the $\gamma$-carboxylate group of Glu-58 can bind to some extent with the adenosine 2′-hydroxyl group and this interaction could facilitate the conformational change that would necessarily accompany the proton dissociation characterized by $K_C$* in Figure 3. Steady-state kinetic studies with dAprGprN substrates are planned which could elucidate this point.

It is noteworthy that the interactions illustrated schematically in Figure 3 are sterically possible when tested by using space-filling models of ApGpN and relevant H-bonded active site groups; e.g., the substrate adenine group can directly contact the Glu-58-His-92 pair (as proposed for species $X_jH_3$ and $X_jH_2$ in Figure 3) and "stack" with the neutral imidazole residue of His-92 when it is H-bonded with the guanosine 2′-hydroxyl group (as proposed for species $X_jH_1$ in Figure 3). However, an ultimate demonstration for these proposed interactions will require more direct evidence. In the absence of X-ray crystallographic results for RNase $T_1$, we are currently preparing several substrate analogues having 8-azidoadenosine (or guanosine) groups and photoaffinity labeling experiments with the enzyme are planned which could elucidate the nature of specific enzyme–substrate contacts.

Although the proposed mechanism is speculative, it does satisfy all of the information currently available on RNase $T_1$ and provides a basis for future investigations. In any event, if the chemical mechanism for enzyme catalysis is defined in terms of the identity and chemical role of enzyme groups that are directly involved in the process, then, at least at high pH values, two mechanisms appear to occur with RNase $T_1$ which are dependent on whether ApGpN or GpN substrates are employed. Considering the *opposite* pH dependence of $k_{cat}$ at high pH for these substrates, this conclusion is phenomenologically obvious and does not depend on the validity of the heuristic mechanism proposed in Figure 3. It has been tacitly assumed that ribonuclease mechanisms, postulated on the basis of kinetic studies with minimal RNA (dinucleoside monophosphate) substrates, can be extrapolated to polymeric substrates; considering the present results, this assumption does not appear to be valid, at least for RNase $T_1$. On the other hand, kinetic studies of RNase $T_1$ with GpCpC (H. L. Osterman and F. G. Walz, in preparation) indicate that the "chemical mechanism" is apparently the same as that for GpC even though some interesting differences in the kinetic parameters were observed. Therefore, it seems that a systematic investigation by using increasingly more complex oligomeric substrates will ultimately be required to fully understand the nature of RNase $T_1$ action.

The adenosine subsite interaction indicated from the present study could be biologically purposeful in decreasing the pH dependence of $k_{cat}/K_m$ (see Figure 1) for native substrates. That is, enzyme action would be less subject to ambient pH changes, particularly at low substrate concentrations (i.e., $[S]_0 \ll K_m$), where the rate of catalysis is directly proportional to $k_{cat}/K_m$. Since RNase $T_1$ is an extracellular enzyme elaborated by *Aspergillus oryzae*, the role for this particular subsite could have evolved to accommodate maximum enzymatic function in a variety of extracellular environments.

[2] RNase $T_1$ binding with dAprG is characterized by a $\Delta G°$ value that is 0.7 kcal/mol less negative than that for rAprG (Walz, unpublished results).

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