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Subsites and Catalytic Mechanism of Ribonuclease T_1: Kinetic Studies Using GpA, GpC, GpG, and GpU as Substrates†

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ABSTRACT: Steady-state kinetic studies of the ribonuclease T_1 catalyzed transesterification of GpA and GpG in 0.2 M buffer and GpC and GpU in 0.02 M buffer were performed at 25 °C in the pH range 2.5-9 and the results were analyzed together with those for GpC and GpU in 0.2 M buffer (Zabinski, M., & Walz, F. G. (1976) *Arch. Biochem. Biophys. 175,* 558). The pH dependences of k_{cat}/K_m for the four dinucleoside monophosphates in 0.2 M buffer were similar and suggested the involvement of two unprotonated groups on the free enzyme having apparent p*K* values of 3.4 and 4.3 and two protonated groups having apparent p*K* values of 7.5 and 8.1. These apparent p*K*s agree with the intrinsic p*K*s found for Glu-58 (Walz, F. G. (1977) *Biochemistry 16,* 4568), His-40 and His-92 (Arata, K., et al. (1976) *Biochem. Biophys. Res. Commun. 37,* 247) and it is concluded that these residues, and an unidentified carboxyl group, take part in binding and/or catalysis at the active site. The effect of the lower ionic strength buffer on the pH dependence of k_{cat}/K_m supports the involvement of Glu-58 and suggests that both active site carboxyl groups are adjacent to cationic species. The pH independent values of k_{cat}/K_m characterizing the four substrates 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. A common mechanism of catalysis for these substrates is proposed in which Glu-58, interacting with His-92, acts as a general base in proton transfer from the Guo-2′-hydroxyl group, and His-40, possibly associated with the unidentified carboxyl residue, serves as a general acid in protonating the 5′ oxygen of the leaving nucleoside group. The different effects of the substrate leaving nucleoside groups on the pH independent values of k_{cat} and K_m are discussed in terms of this mechanism where the intrinsic free energies of binding (Jencks, W. P. (1975) *Adv. Enzymol. 43,* 219) for all substrates are considered to be identical and are differently partitioned into binding or catalytic modes by virtue of coupled interactions of the enzyme with the substrate leaving nucleoside and Guo-2′-hydroxyl groups.

The mechanism of action for RNase[1] T_1 (EC 2.7.7.26) has been studied by a variety of methods; yet the chemical role of essential groups at the active site is still uncertain. Early chemical modification studies clearly indicated the importance of Glu-58 for activity (Takahashi et al., 1967) and led to the proposal of a concerted catalytic mechanism in which Glu-58 and a single histidine residue act as proton transfer agents (Takahashi, 1970). Subsequent pH dependent kinetic studies of the enzyme using GpC and GpU as substrates suggested the participation of at least one carboxylate and two imidazolium groups in binding and/or catalysis (Zabinski & Walz, 1976). This view was supported by more recent chemical modification

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[1] Abbreviations used: RNase, ribonuclease; CM-RNase T_1, γ-carboxymethyl-Glu-58-ribonuclease T_1; NMR, nuclear magnetic resonance; 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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studies which indicated that both His-40 and His-92 are required for activity (Takahashi, 1976b). A role for His-40 in binding phosphate groups has been suggested (Arata et al., 1976; Takahashi, 1976b; Walz, 1977b), whereas, His-92 has been implicated in interacting with Glu-58 (Takahashi, 1976b) which, in turn, was proposed as a direct binding locus for the 2′-hydroxyl group of a complexed guanosine residue (Walz, 1976). This latter role for Glu-58 would be consistent with its proposed catalytic function as a general base species (Takahashi, 1970).

Steady-state kinetic studies on the RNase T_1 catalyzed transesterification of GpC and GpU have demonstrated significant differences between these substrates (Zabinski & Walz, 1976) which have been discussed in terms of possible enzyme subsite interactions of their pyrimidine moieties (Zabinski & Walz, 1976; Walz & Terenna, 1976; Walz, 1977b). The main purpose of the present study is to provide similar kinetic information using GpA and GpG as substrates. In addition, a more rigorous analysis of the pH dependence of the steady-state kinetic parameters for all four dinucleoside monophosphate substrates as well as the effect of ionic strength on these parameters are used to compare the kinetically determined pK_A values of active site carboxyl and imidazole groups with those determined by direct physical methods.

Materials and Methods

RNase T_1 was prepared and its concentration was determined as described previously (Walz & Hooverman, 1973). The dinucleoside monophosphates GpA, GpC, and GpU were obtained from Sigma Chemical Co. and GpG was a product of Calbiochem. 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 conducted at 25 °C using a Radiometer PHM-26 pH meter.

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 sodium lactate–lactic acid replacing sodium acetate–acetic acid was used at pH <4. These buffers had an ionic strength of ~0.2 M: a tenfold dilution of these buffers was used in some experiments which were nominally at ~0.02 M ionic strength.

The molar extinction coefficients at 280 nm for GpA and GpG in the standard buffer at pH 7 and 25 °C were 9760 M^{-1} cm^{-1} and 14 100 M^{-1} cm^{-1}, respectively. these values and those for the difference molar extinction coefficients, $\Delta\epsilon$s, between RNase T_1 substrates and products were determined as described previously (Zabinski & Walz, 1976).

Initial velocities were obtained by recording the absorbance increase at 280 nm with a Cary 118C spectrophotometer using a slit width of 0.2 mm and 0.2 or 1.0 cm pathlength cells. All experiments were conducted using the standard buffer or tenfold dilute standard buffer thermostatted at 25 ± 0.1 °C. Due to its aggregation properties, GpG was dissolved in water at a concentration of ~4×10^{-4} M and heated at 65 °C for 30 min. Aliquots of this solution were added to an appropriate amount of twofold concentrated buffer and the volume was adjusted to give the standard buffer conditions. This solution was allowed to equilibrate at 25 °C for not more than 5 min before adding the enzyme. The ranges of enzyme and substrate concentrations employed for GpG were 7.2×10^{-10} to 1.6×10^{-7} M and 9.6×10^{-6} to 2.0×10^{-4} M, respectively; whereas, for GpA these ranges were 5.6×10^{-9} to 1.3×10^{-7} M and 3.0×10^{-5} to 5.3×10^{-4} M, respectively. Initial velocities were determined in triplicate for at least six substrate concentrations at a given pH value. In experiments at extreme pH values at least 36 initial velocities were determined. The procedures and conditions for initial velocity experiments using GpC and GpU in tenfold dilute standard buffer were the same as those described previously for similar experiments in the standard buffer (Zabinski & Walz, 1976).

Results and Treatment of The Data. The mean value of $\Delta\epsilon$ at 280 nm for GpA was 930 ± 110 M^{-1} cm^{-1} from pH 4 to 9 and this increased slightly at pH 3 to a value of 1190 M^{-1} cm^{-1}. On the other hand, the mean value of $\Delta\epsilon$ at 280 nm for GpG from pH 4 to pH 8 was 1500 ± 105 M^{-1} cm^{-1} which decreased to 880 M^{-1} cm^{-1} at pH 9 and increased to 1610 M^{-1} cm^{-1} at pH 3.

Double-reciprocal plots of the kinetic data were prepared 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 total 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 using a weighted least-squares procedure (Wilkinson, 1961) which was adapted to a Tektronix 31 calculator and was shown to give the same results as those from a similar Fortran program (Cleland, 1967). Values of k_{cat}/K_m and k_{cat} for GpA and GpG obtained from this analysis are plotted as a function of pH in Figure 2. Similar data for GpC and GpU at 0.02 M and 0.2 M ionic strengths are plotted in Figure 3. Most of the data for GpU and GpC at 0.2 M ionic strength was reported previously (Zabinski & Walz, 1976); however, various points have been repeated and experiments were extended to lower pH values. The pH-independent steady-state kinetic parameters and the apparent pK_A values required to fit these data were determined using the KINFIT program for nonlinear curve fitting (Nicely & Dye, 1971).

Discussion

The purpose of the following discussion is to analyze the pH dependence of the steady-state kinetic parameters for the RNase T_1 catalyzed transesterification of GpA, GpC, GpG, and GpU in an effort to determine: (1) the minimum number of enzyme or substrate acid–base groups participating in binding and/or catalysis; (2) the identity of the acid–base groups that are involved; (3) the discrete role played by individual enzyme groups at the active site; and (4) the effect of possible subsite interaction of the leaving nucleotide residue on enzyme binding and/or catalysis. However, conclusions based on the kinetic results alone are necessarily tentative because of inherent limitations in gathering kinetic data at extreme pH values as well as unavoidable theoretical ambiguities in studies of this kind (Cleland, 1977; Knowles, 1976). In view of these problems, wherever it is possible the results in this report will be correlated with information from independent studies regarding the nature, identity, and function of RNase T_1 active site groups.

The Number of Enzyme Acid–Base Groups Involved in Binding and/or Catalysis. If it is assumed that all relevant protonation–deprotonation reactions of the enzyme and its complexes are rapid (i.e., in equilibrium during the course of the reaction) and that the reaction follows a single, horizontal path (i.e., via similarly protonated enzyme species), then the pH dependence of k_{cat}/K_m can reflect ionization of acid–base groups on the free enzyme and/or ligand that are involved in binding and/or catalysis (Peller & Alberty, 1959; Alberty & Bloomfield, 1963). The limited application of these assumptions has recently been emphasized (Knowles, 1976); nevertheless, it appears likely that ionizations in almost all enzyme mechanisms can be properly treated as equilibria (Cornish-Bowden, 1976). In any event, it will be shown below that pK

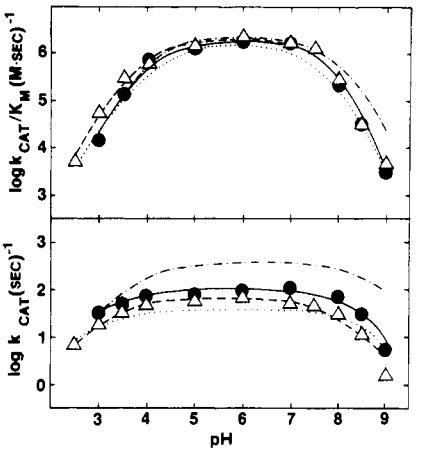

FIGURE 1: Plots of logarithm of k_{cat}/K_m and k_{cat} for the RNase T_1 catalyzed transesterification of GpA and GpG and theoretical curves for all four substrates vs. pH. (●, —) GpA; (- · · · -) GpC; (△, - - -) GpG; (· · ·) GpU. Curves were calculated using the data in Table II and eq 2 and 3. Experiments were conducted in 0.2 M buffer; other details are in Materials and Methods.

TABLE I: Slopes from Log (k_{cat}/K_m) vs. pH Plots.[a]

substrate	slopes[b] pH 2.5–3.5	pH 3.0–4.0	pH 8.0–9.0
GpA		1.68	−1.85
GpC		1.52	−1.58
GpG	1.72	1.11	−1.76
GpU	1.40	1.16	−1.67
GpC (0.02 M)	1.30		−1.22
GpU (0.02 M)	0.96		−1.32

[a] Calculated from plots in Figures 2 and 3. Experiments were conducted in 0.2 M buffer unless indicated otherwise. Other conditions were as described under Materials and Methods. [b] Standard deviations were less than 10%.

values for active site groups on the free enzyme, determined by independent physical methods, correlate very well with those determined from the pH dependence of k_{cat}/K_m. This agreement is taken as prima facie evidence that the above assumptions apply, for the most part, in the current study.

In order to determine the minimal number of ionizable groups on the free enzyme and/or substrate that are involved in RNase T_1 action, analyses of the ascending and descending slopes of the log k_{cat}/K_m vs. pH plots in Figures 1 and 2 were performed (Dixon, 1953) and the results are shown in Table I. It was noted that the absolute values of the ascending and descending slopes for all substrates were significantly greater than one which indicates that at least two ionizable groups on the free enzyme and/or free substrate are required in their dissociated form and at least two other groups are required in their proton-associated form. Since the substrates GpG and GpU do not have acid–base groups that significantly titrate in the pH range investigated (Zabinski & Walz, 1976; Ogasawara & Inoue, 1976), it is likely that the four ionizable groups evidenced from Figures 1 and 2 are on the free enzyme. However, the possible involvement of substrate ionization in the cases of GpA and GpC, whose cytosine and adenine moieties significantly titrate in the pH range investigated, cannot be absolutely ruled out (see below). A reasonable formal mechanism based on these results is shown in Figure 3. This mechanism assumes rapidly equilibrating vertical paths and (as shown) a predominant horizontal path for any finite number of intermediates.

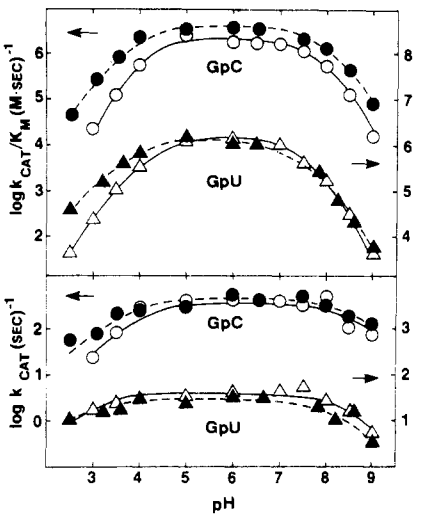

FIGURE 2: Plots of the logarithm of k_{cat}/K_m and k_{cat} for the RNase T_1 catalyzed transesterification of GpC and GpU at two ionic strengths vs. pH. (○, △) Experiments conducted using 0.02 M buffer; (●, ▲) experiments conducted using 0.2 M buffer. Curves were calculated in the same manner as indicated for Figure 1. Most of the data at 0.2 M ionic strength were reported previously (Zabinski & Walz, 1976). Other conditions were as described under Materials and Methods.

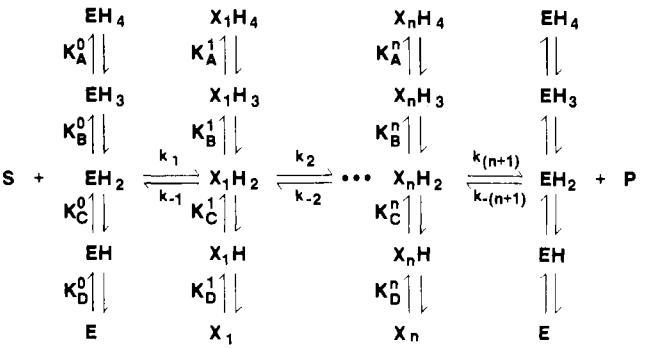

FIGURE 3: A general pH dependent mechanism consistent with the data in Figures 1 and 2 for the RNase T_1 catalyzed transesterification of dinucleoside monophosphate substrates. EH_i, X_jH_i, S, and P represent free enzyme, complex enzyme, substrate (i.e., GpX; X = A, C, G, or U), and product (i.e., Guo-2′:3′-P), respectively. Protons were omitted for clarity. See text for details.

The Identity of Acid–Base Groups Involved in RNase T_1 Action. The mechanism in Figure 3 considers only macroscopic protonic equilibria. (The more authentic treatment of microscopic equilibria would be too complicated and is not warranted by the data.) This mechanism is characterized by

$$k_{cat}/K_m = \frac{\bar{k}_{cat}/\bar{K}_m}{1 + (H)^2/K_A^0K_B^0 + (H)/K_B^0 + K_C^0/(H) + K_C^0K_D^0/(H)^2} \quad (2)$$

and

$$k_{cat} = \frac{\bar{k}_{cat}}{1 + (H)^2/K_A^*K_B^* + (H)/K_B^* + K_C^*/(H) + K_C^*K_D^*/(H)^2} \quad (3)$$

where $\bar{k}_{cat}/\bar{K}_m$ is the ratio of the pH independent value of the turnover number and the Michaelis constant, K_A^0, K_B^0, K_C^0, and K_D^0 are macroscopic acid dissociation constants characterizing pertinent groups on the free enzyme and K_A^*, K_B^*, K_C^*, and K_D^* are apparent dissociation constants that characterize the generalized scheme in Figure 3 (Peller & Alberty, 1959). For the simple case of $n = 2$ and $k_3 \gg (k_2 + k_{-2})$, K_A^*,

TABLE II: Best Fit Kinetic Parameters for the Data in Figures 2 and 3 Using the Mechanism in Figure 4.[a]

parameter	units	substrates[b] GpA	GpC	GpG	GpU	av[b] values	significant differences
$\bar{k}_{cat}$	s^{-1}	96	350 (430)	62	38 (28)		GpC > GpA > GpG > GpU
$\bar{K}_m$	$M \times 10^5$	5.5	16 (12)	2.7	2.2 (2.0)		GpC > GpA > GpG ≥ GpU
$\bar{k}_{cat}/\bar{K}_m$	$(Ms)^{-1} \times 10^{-6}$	1.7	2.2 (3.8)	2.3	1.8 (1.4)		GpC = GpG > GpA = GpU
pK_A^0	M	3.7	4.0 (3.1)	3.2	2.9 (2.1)	3.4 ± 0.5 (2.9 ± 0.3)	GpA > GpU; GpC > GpU, GpG
pK_B^0	M	4.1	4.1 (3.9)	4.3	4.6 (4.0)	4.3 ± 0.2 (3.9 ± 0.1)	none
pK_C^0	M	7.4	7.7 (7.8)	7.7	7.3 (7.2)	7.5 ± 0.3 (7.5 ± 0.4	GpC > GpU
pK_D^0	M	8.2	8.3 (8.9)	7.6	8.1 (8.4)	8.1 ± 0.3 (8.6 ± 0.5)	GpC > GpU
pK_A^*	M	<2.5	<2.5 (<2.5)	<2.5	<2.5 (<2.5)		
pK_B^*	M	3.3	4.0 (3.7)	3.4	3.0 (2.8)	3.4 ± 0.4 (3.3 ±0.7)	GpC > GpA, GpG, GpU
pK_C^*	M	8.2	8.8 (8.4)	7.8	8.3 (8.2)	8.3 ± 0.3 (8.3 ± 0.2)	GPC > GpA, GpG, GpU
pK_D^*	M	>9	>9 (>9)	>9	>9 (>9)		

[a] See text for details of the analysis. [b] Numbers in parentheses for experiments using 0.02 M buffer; all other results for experiments using 0.2 M buffer.

K_B^*, K_C^*, and K_D^* would equal the acid dissociation constants of the initial enzyme-substrate complex, i.e., would be K_A^1, K_B^1, K_C^1, and K_D^1, respectively (see Figure 3). Values for the parameters in eq 2 and 3 were computed using these equations and a nonlinear fitting procedure for the data in Figures 1 and 2 (Nicely & Dye, 1971) and are presented in Table II. Since the pH dependence of k_{cat} (Figures 1 and 2) suggested the apparent involvement of only one acid-base group in its dissociated form and one acid-base group in its proton-associated form, it was arbitrarily assumed that pK_A^* and pK_D^* were beyond the pH range investigated and these are given limit values in Table II. (These limit values could just as well have been assigned to pK_B^* and pK_C^* since the subscript designation is merely a formalism.) Linear estimates of the standard deviation for $\bar{k}_{cat}$, and $\bar{k}_{cat}/\bar{K}_m$ were all less than 10%, whereas standard deviations ranged from an average of 80% for K_A^0 (i.e., ~±0.7 for pK values) to an average of 48% for the remaining dissociation constants (i.e., ~±0.3 for pK values) in Table II. Since it was generally observed that a given pK value determined for one substrate was not significantly different from those for other substrates the average values for pK_A^0, pK_B^0, pK_C^0, and pK_D^0 in Table II can be considered as representative "macroscopic" pK values characterizing the pH dependence of k_{cat}/K_m for all substrates. Considering the large difference (>4 pK units) between the acidic pK values (pK_A^0 and pK_B^0) and the basic pK values (pK_C^0 and pK_D^0), it is reasonable to discuss these as representing independent "titrations".

On the basis of chemical modification experiments (Takahashi, 1976b) as well as NMR studies of the enzyme in the presence of inhibitors (Rüterjans et al., 1969; Arata et al., 1976), it was concluded that His-40 and His-92 are present at the active site of RNase T_1. Proton NMR studies of the enzyme in D_2O containing 0.2 M NaCl have led to the unambiguous assignment of the C-2 hydrogen of His-40 (Arata et al., 1976) and its pK value was found to be 7.9 (Arata et al., 1976) or 7.75 (Markley, J. L., Walz, F. G., & Westler, F. M., unpublished experiments). Furthermore, the C-2 proton resonance of His-92 has been tentatively assigned (Arata et al., 1976) and its pK value is 7.48 (Markley, J. L., Walz, F. G., & Westler, F. M., unpublished experiments). Because these "microscopic" pK values are nearly the same, it is not valid to identify them with particular "macroscopic" pK values in Table II. However, if the titrations of His-40 and His-92 are independent, which seems to be the case from the above NMR studies, then it is simple to show that the sum of the "macroscopic" pK values in the present study (i.e., pK_C^0 and pK_D^0) should equal the sum of the "microscopic" pK values (i.e., should equal $pK_{His\text{-}40} + pK_{His\text{-}92} = 15.3 \pm 0.2$) if they represent the same titrating groups on the free enzyme. The sums of pK_C^0 and pK_D^0 for GpA, GpC, GpG, and GpU, calculated from the data in Table II, were 15.6 ± 0.4, 16.0 ± 0.5, 15.3 ± 0.6, and 15.4 ± 0.2, respectively, which substantiates the conclusion that the pH dependence of k_{cat}/K_m reflects the titration of His-40 and His-92 on the free enzyme.

The pK value for Glu-58 has recently been determined to be 4.3 ± 0.1 under the same conditions as those used in the present study (Walz, 1977a) which is consistent with its being reflected in the "macroscopic" constants, pK_A^0 and pK_B^0, in Table I. The nature and identity of the second ionization at low pH are currently unknown; however, its pK value, pK_X, can be calculated using

$$pK_X = pK_A^0 + pK_B^0 - pK_{Glu\text{-}58}$$

if it is assumed that Glu-58 represents the other group which independently titrates in the same pH range. The values calculated for pK_X using the data in Table I for GpA, GpC, GpG, and GpU were 3.5 ± 0.4, 3.8 ± 0.4, 3.2 ± 0.4, and 3.2 ± 0.7, respectively (average $pK_X = 3.4$). In order to substantiate the identification of Glu-58 as a group whose titration is reflected in pK_A^0 and pK_B^0, experiments were conducted in tenfold dilute standard buffer (i.e., ~0.02 M ionic strength). This condition was tested since the pK value of Glu-58 dramatically changes from 4.3 in the standard buffer to 3.6 in the dilute buffer (Walz, 1977a) which represents a change in ~−0.7 pK unit. The only significant changes in the macroscopic pK values in Table II at low ionic strength were in pK_A^0 for GpC and in pK_B^0 for GpU. The average change in the mean values of pK_A^0 and pK_B^0 at low ionic strength was ca. −0.6 pK unit which corroborates the view that the ionization of Glu-58 is reflected in these "macroscopic" pKs. Using 3.60 as the value of $pK_{Glu\text{-}58}$ at ~0.02 M ionic strength, values for pK_X were calculated under these conditions using eq 4 with the data for GpC and GpU and gave pK_Xs of 3.4 ± 0.4, and 2.5 ± 0.7 (average $pK_X = 2.9$), respectively. In this case the average change in going to low ionic strength was ca. −0.5 pK unit which is comparable to that found for Glu-58.

The Role of Acid-Base Groups at the Active Site. Early NMR studies of RNase T_1 and CM-RNase T_1 indicated that one active site His residue interacts with the γ-carboxyl group of Glu-58 and the other with the newly introduced carboxyl group of CM-RNase T_1 (Rüterjans & Pongs, 1971): in light of the recent assignments for His C-2 proton resonances (Arata et al., 1976), these would represent His-92 and His-40, respectively. This proposal is consistent with the results of chemical modification studies which suggested that His-92

interacts with Glu-58 (Takahashi, 1976b). A specific role for Glu-58 in ribose recognition has recently been deduced from binding studies of RNase T_1 and CM-RNase T_1 with guanosine and deoxyguanosine which suggested that the γ-carboxylate group of Glu-58, associated with an imidazolium side chain (presumably that of His-92 (see above)), functions as a binding locus for the 2′-hydroxyl group of the complexed guanosine (Walz, 1977a).[2] Furthermore, this specific interaction of the enzyme persists in the binding of guanosine nucleotides whose phosphate groups also take part in the binding process at the active site (Walz, 1977b). Since the involvement of Glu-58 and His-92 in substrate binding and/or catalysis is consistent with the results of the present kinetic study, it is possible that these groups provide a binding locus for the guanosine 2′-hydroxyl group of guanylyl-(3′–5′)-nucleoside substrates which is similar to that proposed for binding guanosine (Walz, 1977a). This view is supported by the fact that the average apparent free energy change for binding (i.e., $\Delta G^\circ = RT \ln K_m$) of GpA, GpC, GpG, and GpU is ~1.3 kcal/mol more negative than that for binding the corresponding deoxydinucleoside monophosphate substrate analogues (Walz & Terenna, 1976).

The general acid–base mechanism previously suggested for the RNase T_1 catalyzed transesterification reaction proposed that the γ-carboxylate group of Glu-58 acted alone as a general base species in abstracting a proton from the substrate Guo-2′-OH group (Takahashi, 1970). The extension of this mechanism to include the proposed Glu-58–His-92 pair is chemically reasonable since an adjacent imidazolium residue could stabilize the γ-carboxylate group of Glu-58 in a geometry that facilitates a more efficient proton transfer from the substrate 2′-OH group, even though the intrinsic basicity of this group would be expected to be lower. Furthermore, a tandem arrangement of hydrogen bonded imidazolium–carboxylate–2′-hydroxyl groups (Walz, 1977a) would decrease the electrostatic repulsion of negative charges generated during the course of the reaction. Moreover, the idea that proton transfer groups have a precise orientation at the active site is precedented in the cases of chymotrypsin (Blow, 1971) and RNase A: in the latter case the presumed general base species, His-12, is stabilized by a hydrogen bond of its *pro-S*-NH group with the peptide carbonyl of Thr-45 (Van Batenburg et al., 1977; Richards & Wyckoff, 1971).

The function of His-40 could involve, in part, the binding of the substrate phosphate group since NMR experiments suggested a direct interaction of its imidazolium group with the 3′-phosphate of Guo-3′-P (Arata et al., 1976). Even though it may be fortuitous, it is interesting that the pK of 8.6 found for His-40 in the RNase T_1–Guo-3′-P complex (Arata et al., 1976) agrees with the average value of 8.3 ± 0.3 found for pK_C* (see Table II) which, in turn, could represent a His residue in the RNase T_1–guanylyl-(3′–5′)-nucleoside complex (see above). In any event, the binding of substrate-like phosphodiester groups at the active site of RNase T_1 also appears to involve a His imidazolium group (Walz, 1977b). The proximity of His-40 with the substrate phosphate group would be consistent with its possible role as a general-acid species for proton transfer to the leaving nucleoside group (Takahashi, 1970).

The remaining acid–base group evidenced in the present study is apparently required in its ionized form and has an average pK of 3.4 (i.e., pK_X; see above) which is consistent with its representing a carboxyl group. In addition, the calculated value of pK_X appears to decrease significantly at low ionic strength which suggests that this putative carboxyl group might be adjacent to a cationic species at the active site. Considering that both Glu-58 and the unidentified carboxyl residue are apparently required as anionic species and that substrate transesterification most probably proceeds via a dianionic pentavalent phosphorous intermediate (or transition state; Eckstein et al., 1972), it is not likely that this unidentified group participates directly in substrate proton transfer. However, the unidentified carboxyl group could interact with His-40 and thereby promote an efficient orientation for this residue in its proposed role as a general-acid species in catalysis. Evidence in support of this proposal includes the observations that His-40 is associated with an unidentified carboxyl group on the free enzyme (Arata et al., 1976; Markley, J. L., Walz, F. G., & Westler, F. M., unpublished experiments) and that phosphate group binding of guanine nucleotides appears to require an ionized carboxyl group (Walz, 1977b). Nevertheless, it is also possible that this carboxyl group participates in binding the guanine residue at the primary recognition site of the enzyme since the involvement of a carboxylate group has been evidenced for this interaction (Walz, 1977b; Walz, F. G., in preparation).

Since the current investigation can only detect participating groups at the active site that ionize in the pH range investigated, there is no basis for speculating on the role of Arg-77 which also appears to be at or adjacent to the active site (Takahashi, 1976a); however, its possible significance regarding the properties and functions of other active site groups cannot be discounted at this time.

The Effect of Leaving Nucleoside Groups on Binding and/or Catalysis. Inspection of Figures 2 and 3 suggests a general similarity in the pH dependence of the steady-state kinetic parameters for the various substrates; however, some significant differences were observed. For example, the slopes of log k_{cat}/K_m vs. pH in Figures 1 and 2 (see Table I) for GpA and GpC in the pH range 3 to 4 are significantly greater than those for GpG and GpU. Furthermore, the analysis of the data in terms of the mechanism in Figure 3 showed that pK_A^0 for GpC was significantly different from those for GpG and GpU but was indistinguishable from that for GpA (see Table II). These similar characteristics for GpA and GpC could result from ionizations of their free substrate species since the pK value for the adenine moiety of GpA is ~3.7 and that for the cytosine moiety of GpC is ~4.3 (Ogasawara & Inoue, 1976). Considering this different pH dependence of k_{cat}/K_m for GpA and GpC vis-a-vis GpG and GpU it is possible that GpA and GpC are somewhat less efficient substrates when their respective adenine and cytosine groups are protonated. However, the current data cannot clearly discriminate for this possibility and experiments are planned using substrate analogues of protonated GpA and GpC having methyl groups at N-1 of adenine and N-3 of cytosine, respectively.

In the case of GpC, the equivalence of $(pK_C^0 + pK_D^0)$ with the sum of microscopic pK values for His-40 and His-92 (see above) is of borderline significance which could mean that the general mechanism proposed in Figure 3 is not entirely appropriate for this substrate. In addition, the macroscopic pK values for GpC were generally greater than those of some other substrates (see Table II). These idiosyncrasies for GpC are

[2] Additional evidence for the involvement of an imidazolium carboxylate ion pair in interacting with the guanosine 2′-hydroxyl group was obtained from binding studies of RNase T_1 with Guo and dGuo in tenfold dilute standard buffer in which it was found that the maximum binding constants for Guo from pH 5 through pH 7 significantly increased by ~60% when compared with those found in the standard buffer (Walz, 1976), whereas the binding constants for dGuo remained the same (Walz, F. G., unpublished experiments).

particularly interesting in contrast with GpU since these substrates show the greatest differences in their kinetic characteristics (see Table II), yet they are chemically very similar.

The substrate GpG could conceivably bind in a nonproductive complex with the enzyme having the 5′ terminal Guo residue at the primary recognition site and the 3′ terminal Guo residue at the 1N subsite (Walz & Terenna, 1976). If binding in this mode occurred then the steady-state rate law would be

$$V = \bar{k}_{cat}^{app}(E)_0(S)_0/(\bar{K}_m^{app} + (S)_0) \quad (4)$$

where

$$\bar{k}_{cat}^{app} = \bar{k}_{cat}/(1 + \bar{K}_m/K) \quad (5)$$

$$\bar{K}_m^{app} = \bar{K}_m/(1 + \bar{K}_m/K) \quad (6)$$

where K is the dissociation constant characterizing nonproductive binding. Therefore, it is possible that $\bar{k}_{cat}$ and $\bar{K}_m$ listed in Table II for GpG are apparent values, whereas $\bar{k}_{cat}/\bar{K}_m$ is authentic. An attempt has been made to examine whether nonproductive binding of GpG occurs in binding studies of the enzyme with dGprG which presumably would bind mainly in the nonproductive mode; however, the results of these experiments have not yet resolved this ambiguity (Walz, F. G., unpublished experiments).

A previous consideration of the binding free energy contributions of various substrate groups indicated that a complementary binding of guanine, Guo-2′-OH, and Guo-3′-phosphodiester moieties of guanosine nucleotides occurs at the active site and, based on the assumption that K_m values determined in the present study represent thermodynamic dissociation constants, it was estimated that binding of the X nucleoside moiety of GpX (X = A, C, G, and U) substrates could contribute up to ~1 kcal/mol toward the binding free energy (Walz, 1977b). Furthermore, it appears that the binding of the X nucleoside residue with the enzyme depends on a complementary enzyme interaction with the substrate's Guo-2′-OH group since the association constants characterizing the binding of deoxydinucleoside substrate analogues (i.e., dGpdX; X = A, C, G, and T) with RNase T_1 are the same and indistinguishable from that for RNase T_1 binding with the methyl ester of dGuo-3′-P which only involves enzyme interactions with guanine and 3′-phosphodiester groups (Walz & Terenna, 1976; Walz, 1977b).

The observation that $\bar{k}_{cat}/\bar{K}_m$ is virtually identical for GpA, GpC, GpG, and GpU (see Table II) suggests that they have the same intrinsic free energy for RNase T_1 binding (Jencks, 1975). However, the individual values of $\bar{k}_{cat}$ and $\bar{K}_m$ range about an order of magnitude for these substrates (see Table II). As discussed above, the bindings of the substrate X nucleoside and Guo-2′-OH groups are apparently coupled; therefore, the unique partitioning of the same intrinsic binding free energy for each substrate, to affect either binding (K_m) or catalysis (k_{cat}), could ultimately depend on the nature of the enzyme's interaction with the Guo-2′-OH group since this group is involved in substrate binding (Walz, 1976, 1977b) and necessarily in catalysis. For example, in the case of GpU (lowest K_m, lowest k_{cat}; see Table II) a hydrogen bond of Glu-58 with the Guo-2′-OH group could complement the binding of other substrate groups (i.e., guanine and Guo-3′-phosphate groups (Walz, 1977b)); whereas, for GpC (highest K_m, highest k_{cat}; see Table II) a stronger hydrogen bond of the enzyme with the Guo-2′-OH group may be formed which could facilitate proton transfer but antagonize binding interactions at other substrate loci. The strength of this putative hydrogen bond should depend on the basicity of Glu-58 in the enzyme-substrate complex which, in turn, could be reflected in the values of pK_B^* (see above). In this regard, it is interesting that the values of pK_B^* in Table II are virtually in the same order as those for $\bar{k}_{cat}$, i.e., GpC > GpA = GpG ≥ GpU. Since the γ-carboxylate of Glu-58 most likely interacts with the imidazolium residue of His-92 (see above), it is possible that this imidazolium group mediates the effect of the bound X nucleoside residue on the enzyme's interaction with the bound Guo-2′-OH by modulating the effective basicity of the Glu-58 carboxyl group. Even though these speculations are consistent with the current data, other proposals (e.g., involving rapid conformational equilibria of the enzyme–substrate complex) could also explain the characteristic $\bar{k}_{cat}$ and $\bar{K}_m$ values observed for the different substrates. A more complete understanding of this phenomenon will require a detailed knowledge of the dynamics of the enzyme–substrate complex. For this purpose, relaxation kinetic studies are planned on the interaction of RNase T_1 with the dinucleoside monophosphonate substrate analogues, GpcC and GpcU, which possess all dinucleoside monophosphate substrate groups that are known to interact with the enzyme (i.e., guanine, Guo-2′-OH, Guo-3′-phosphate, and the leaving nucleoside residue) (Walz, 1977b).

Dramatic effects of subsite interactions of RNase T_1 with other RNA nucleoside residues, beyond the primary recognition site, have been observed in kinetic studies of the enzyme with ApGpC, ApGpU and GpCpC (Osterman & Walz, 1977, and in preparation). The present study provides a necessary basis for highlighting the effects of these additional subsite interactions; however, the generalization of a plausible catalytic mechanism, based on the kinetic behavior of minimal RNA substrates (i.e., dinucleoside monophosphates), must be viewed cautiously since interactions of more complicated substrates at additional enzyme binding loci can either promote more efficient catalytic pathways that are parallel to the central pathway in Figure 3 or affect the arrangement of groups at the active site (Osterman & Walz, in preparation).

Summary

Some tentative conclusions of the present study can be summarized as follows: (1) two carboxyl groups including that of Glu-58 as well as His-40 and His-92 are involved in substrate binding and/or catalysis at the active site of RNase T_1; (2) the γ-carboxylate group of Glu-58 interacting with the imidazolium group of His-92 could serve as a general base in proton transfer from the substrate Guo-2′-OH group and the imidazolium group of His-40, possibly associated with the unidentified carboxylate group, could serve as a general acid for proton transfer to the 5′-O of the leaving nucleoside in a concerted acid–base catalytic mechanism of transesterification; (3) the leaving nucleoside residues are implemented via differently induced interactions of the enzyme with the substrate Guo-2-OH group.

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5′-Nucleotide Phosphodiesterase: Isolation of Covalently Bound 5′-Adenosine Monophosphate, an Intermediate in the Catalytic Mechanism†

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ABSTRACT: 5′-Nucleotide phosphodiesterase was purified from bovine intestine, employing L-histidyldiazobenzylphosphonic acid–agarose (an affinity resin) to remove copurifying alkaline phosphatase. Stoichiometric amounts of enzyme were incubated with the substrate 3′,5′-[^{3}H]cAMP, quenched and analyzed for bound [^{3}H]AMP. Incubations were quenched with liquified phenol, which quantitatively extracted the protein into the phenol phase as shown by experiments with ^{125}I-labeled enzyme, while unbound substrate and product were removed by multiple aqueous washes. Active phosphodiesterase incorporated [^{3}H]AMP in a form that could not be removed by extensive washing, and migrated with the protein on gel filtration under unfolding conditions. The amount of radioactivity incorporated per milligram of protein was proportional to the specific activity of the enzyme and increased as the pH was lowered or the incubation period was shortened. Labeling was drastically diminished when the usual incubation mixture contained a competitive inhibitor. One-hour incubation time, which permitted complete hydrolysis of substrate, abolished the incorporation of label. These observations suggest the formation of a covalent enzyme–substrate intermediate in the hydrolysis of substrate. The bond between enzyme and the [^{3}H]AMP intermediate was acid stable but labile at pH 13. Addition of active phosphodisterase to labeled (denatured) enzyme also released label from the protein. The pH stability data and susceptibility of the label to phosphodiesterase cleavage suggest that the [^{3}H]AMP is bound to phosphodiesterase through a phosphoester bond to either a serine or threonine residue.

The enzyme 5′-nucleotide phosphodiesterase (EC 3.1.4.1) from bovine intestine catalyzes the hydrolysis of a wide range of phosphoesters. In addition to the hydrolysis of nucleic acids at the 3′ end to liberate 5′-nucleotides, the enzyme cleaves NAD,[1] ADP, ATP, and cyclic 3′,5′-AMP (cAMP) to 5′-AMP (Kelly et al., 1975). Also substrates are phosphonate esters such as 4-nitrophenyl phenylphosphonate, which provides a convenient and specific assay for this enzymatic activity (Kelly and Butler, 1975).

Bovine intestine is a rich source of this enzyme (Dardinger, 1974). It has been estimated that the small intestine of one yearling contains more than 1 g of 5′-nucleotide phosphodiesterase. Previous reports from this laboratory described purification and characterization of this enzyme (Kelly et al., 1975). 5′-Nucleotide phosphodiesterase was reported to have a molecular weight of 108 000, to contain 21% carbohydrate, and to consist of identical subunits with a dimeric structure likely (Kelly et al., 1975).

Kelly and Butler (1977) have investigated the mechanism of 5′-nucleotide phosphodiesterase by determining kinetic constants of systematically varied substrates and by pre-

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[1] Abbreviations used: NAD, nicotinamide adenine dinucleotide; cAMP, cyclic 3′,5′-adenosine monophosphate; DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; $NaDodSO_4$, sodium dodecyl sulfate.

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