Energetics of Ribonuclease A Catalysis. 1. pH, Ionic Strength, and Solvent Isotope Dependence of the Hydrolysis of Cytidine Cyclic 2′,3′-Phosphate†

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ABSTRACT: The pH, ionic strength, and solvent deuterium isotope dependence of the steady-state kinetics of the ribonuclease A catalyzed hydrolysis of cytidine cyclic 2′,3′-phosphate has been investigated by using, primarily, the technique of flow microcalorimetry to monitor the kinetics. The pH dependence of the Michaelis–Menten parameters has been analyzed by assuming the participation of His-12 and -119 of the enzyme and a third ionizing group, postulated to be on the pyrimidine ring of the substrate, to determine the pH-independent rate constant, k_c, and Michaelis constant, K_m. The reported pH analysis, together with existing NMR data and chemical modification studies, allows an assignment of the functional roles of His-12 and -119 as being those of general acid and general base catalytic residues, respectively. At high pH, the apparent K_m value is found to increase to unity. This drop in affinity between the enzyme and the substrate at high pH indicates that the substrate binds to the enzyme primarily through an electrostatic interaction with the active-site histidine residues, particularly His-12. The apparent absence of an interaction with the riboside portion of the substrate is suggested to be due to the fact that the substrate exists in a syn conformation about its glycosidic bond and thus cannot interact optimally with the enzyme's binding pocket. This will result in a relative destabilization of the enzyme–substrate complex, which can then be relieved upon the formation of the transition state. The ionic strength dependence of ribonuclease activity is shown to be primarily a result of its effect on the pK_a of the histidine residues and a concomitant change in the value of K_m.

Much progress has been made toward understanding the mechanistic details of the enzymatic action of ribonuclease A (RNase A)[1] (Richards & Wyckoff 1971; Roberts et al., 1969b; Witzel, 1963; Findlay et al., 1962; Holmes et al., 1978). However, the problem of accounting for the large magnitude of the rate enhancement ($\sim 10^9$) accomplished by this enzyme has been addressed infrequently (Westheimer, 1962; Richards & Wyckoff, 1971; Flogel et al., 1975). The goal of the present work is to study the energetics of the reactions catalyzed by RNase A in hope of arriving at a molecular thermodynamic explanation for this rate enhancement.

A number of concepts have been advanced to explain the general catalytic efficiency of enzymes. These include (a) the employment of multiple catalytic groups on the enzyme, (b) proximity and orientation effects, (c) geometric or electronic destabilization of the substrate relative to the transition state of the reaction, and (d) other "milieu" effects that provide a stabilizing environment for the transition state (Jencks, 1975; Bruice, 1970; Fersht, 1977). A general way of expressing all of the above arguments is that an enzyme catalyzes a reaction by having a greater affinity for the transition state than for the substrate(s). According to the transition-state theory of enzymatic reactions (Wolfenden, 1972; Lienhard, 1973; Jencks, 1975; Schowen, 1978), the rate enhancement (k_E/k_{NE}) for an enzymatic reaction can be related to the affinity of the enzyme for the substrate (S) and transition state (TS) by

$$k_E/k_{NE} = K_{TS}/K_S \qquad (1)$$

where k_E and k_{NE} are the enzymatic and nonenzymatic reaction rate constants and K_{TS} and K_S are the respective association constants for the transition state and substrate.

This expression provides a means for the evaluation of the magnitude of the rate enhancement for enzymatic reactions. By experimentally determining values of k_E, k_{NE}, and K_S, the association constant for the transition state can be estimated. The problem then becomes one of accounting for the magnitude of K_{TS}. For a typical enzymatic reaction, K_{TS} may be as large as 10^{10}–10^{15} M^{-1}.

The active site of an enzyme must be almost perfectly complementary to the chemical nature of the transition state in order to give rise to such large affinities. The enzyme and transition state will interact with each other at a number of points through hydrogen bonds and Coulombic and van der Waals forces. The enzyme must also interact with the substrate, but the points of interaction with the transition state must be greater in number or more favorable. Understanding the energetics of catalysis involves the identification of the forces acting between the enzyme and substrate and the assessment of how these interactions improve as the transition state is formed.

In the present study, such an approach will be taken in order to evaluate the magnitude of the rate enhancement for the RNase A catalyzed hydrolysis of the cyclic phosphate ester substrate cytidine cyclic 2′,3′-phosphate (cCMP). The interaction between RNase A and various inhibitors and substrate analogues has been extensively studied by X-ray crystallographic, NMR, and equilibrium techniques (Richards & Wyckoff, 1971; Roberts et al., 1969a; Anderson et al., 1968). Calorimetric studies by Flogel et al. (1975) have resulted in a detailed description of the energetics of the interactive forces between the enzyme and the ligands cytidine 3′-phosphate

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[1] Abbreviations: cCMP, cytidine cyclic 2′,3′-phosphate; cUMP, uridine cyclic 2′,3′-phosphate; 3′-CMP, cytidine 3′-phosphate; μ, ionic strength; NMR, nuclear magnetic resonance; RNase A, bovine pancreatic ribonuclease A; Tris, tris(hydroxymethyl)aminomethane.

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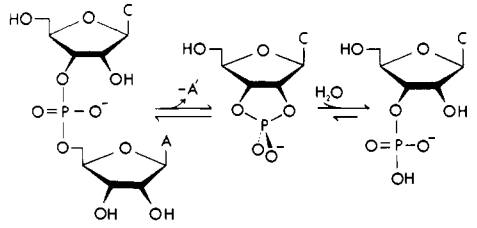


FIGURE 1: Two-step hydrolysis of the dinucleotide cytidylyl(3′→5′)adenosine (CpA). The first step is the transphosphorylation step in which adenosine (A′) is removed to form cytidine cyclic 2′,3′-phosphate (cCMP). The hydrolysis of cCMP follows to form cytidine 3′-phosphate (3′-CMP).

(3′-CMP) and cytidine 2′-phosphate (2′-CMP). These workers found that the binding of these nucleotides is determined by both favorable enthalpic interactions involving the nucleoside moiety and favorable entropic interactions involving the phosphate group. Such studies provide a framework for the evaluation of K_{TS} for the transition state formed during the hydrolysis of the substrate cCMP. The ligands 3′-CMP and 2′-CMP bear a close resemblance to cCMP and are also expected to be chemically very similar (when 3′-CMP and 2′-CMP are in their dianionic state) to the activated intermediate (see below) formed during the catalyzed reaction.

To attempt the above analysis, one first requires a knowledge of k_E (the enzymatic $k_c = V_{max}/[E]$, which for the RNase A catalyzed hydrolysis of cCMP is the rate constant for the actual enzyme–substrate to enzyme–product conversion, since the substrate binding and product dissociation steps are fast; Erman & Hammes, 1966), K_S (the reciprocal of the Michaelis constant, K_m, since the rate constant for substrate dissociation is also fast), and also the appropriate value for k_{NE}. The activity of RNase A varies with pH. In order to obtain the desired pH-independent k_c and K_m values, a complete study of the pH dependence of the hydrolysis of cCMP has been undertaken. Although there have been previous reports of the pH dependence of RNase A activity, the improved kinetic method employed in the present study allows for a more reliable determination of the pertinent parameters defining the system. In addition, studies of the ionic strength and solvent isotope dependence of K_m and k_c have been performed in order to gain insight concerning the possible types of forces involved in the binding of the substrate and transition state to the enzyme.

Brief Review of the Mechanism of RNase A. Before continuing, let us present a brief summary of the important features of the reactions catalyzed by RNase A. RNase A actually catalyzes two reaction steps, as shown in Figure 1. The first step (transphosphorylation step) involves the breaking of a 3′,5′-phosphodiester linkage by intramolecular attack of the neighboring 2′-hydroxyl oxygen to form a cyclic phosphate intermediate. In the second step (hydrolysis step), the cyclic phosphate is hydrolyzed to form a 3′-phosphate ester. Two histidine residues (His-12 and His-119) located at the active site are believed to play important catalytic roles in both steps. Most proposed mechanisms assign a role of general acid catalyst to one of these histidines and general base catalyst to the other. The possible involvement of Lys-41 has frequently been suggested. Studies by Westheimer and co-workers (Westheimer, 1968; Haake & Westheimer, 1961) on the nonenzymatic hydrolysis of cyclic phosphate esters have shown that such reactions proceed via a pentacoordinated cyclic phosphate activated intermediate such as the one represented in Figure 2. It is usually presumed that the enzymatic

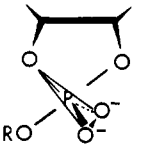


FIGURE 2: Proposed pentacoordinated phosphate intermediate that is formed during the hydrolysis of cCMP.

mechanism will also proceed via a similar pentacoordinated cyclic intermediate. [Note that in our discussions we consider transition states as being the most unstable species on a reaction pathway, in which there is partial bond formation; intermediates are considered to be structures in which bonds are fully formed (Fersht, 1977).]

Experimental Procedures

Materials. Salt-free, phosphate-free, lyophilized RNase A was obtained from Worthington Biochemical Co. Cytidine cyclic 2′,3′-phosphate (cCMP) and uridine cyclic 2′,3′-phosphate (cUMP) were obtained from Sigma Chemical Co. The cyclic phosphates were tested for purity by paper chromatography [solvent system of saturated ammonium sulfate–1 M sodium acetate (pH 6.6)–2-propanol, 80:18:2 v/v]. No 2′(3′)-CMP or 2′(3′)-UMP impurities could be detected. The following R_f values were found: cCMP, 0.45; 3′-CMP, 0.7. Imidazole was recrystallized from cyclohexane before use. All other salts and buffers were standard reagent grade. D_2O (99.8% pure) was obtained from Sigma.

RNase concentrations were determined by use of a molar extinction coefficient of 9.8×10^3 cm^{-1} M^{-1} at 277.5 nm. Absorbance measurements were always made on protein solutions below pH 8 to avoid absorbance by ionized tyrosine residues. cCMP concentrations were determined by using an extinction coefficient of 8.5×10^3 cm^{-1} M^{-1} at 268 nm. Sodium acetate, sodium cacodylate, or N-deuterated imidazole were used as buffers when working with deuterated solutions, and the pH was adjusted with a minimal amount of HCl. pH measurements were made with a Radiometer 4b pH meter. For calorimetric assays, the pHs of the solutions to be mixed were matched to at least ±0.01 pH unit. In studies with D_2O as solvent, the "pH" values are given as uncorrected glass electrode meter readings. As discussed by Roberts et al. (1969a), this treatment leads to apparent pK values in D_2O that are approximately equal to those in H_2O for the histidine residues of RNase A. Spectroscopic studies were performed with a Cary 14 instrument. An LKB flow microcalorimeter was used for the calorimetric studies.

Flow Microcalorimetric Assay. The kinetics of the RNase A catalyzed hydrolysis of cCMP were studied by the use of flow microcalorimetry as described in greater detail elsewhere (Eftink et al., 1981a,b; Johnson, 1974). Product inhibition and substrate depletion were included in the determination of V_{max} and K_m by employment of the following integrated Michaelis–Menten equation in the analysis of the data (Segel, 1975):

$$[P]_\tau = \frac{V_{max}\tau}{1 - K_m/K_p} - \frac{K_m(1 + [S]_0/K_p)}{1 - K_m/K_p} \ln \frac{[S]_0}{[S]_0 - [P]_\tau} \quad (2)$$

In this equation, K_p is the product inhibition constant and $[S]_0$ is the initial substrate concentration. $[P]_\tau$ is the concentration of product formed within the calorimetric cell during the residence time, τ, within the cell. The values for $[P]_\tau$ are obtained from the experimentally observed calorimetric signal by dividing by the heat of reaction (ΔH_R), the flow rate, and a calibration constant [see Eftink et al. (1981b), Johnson

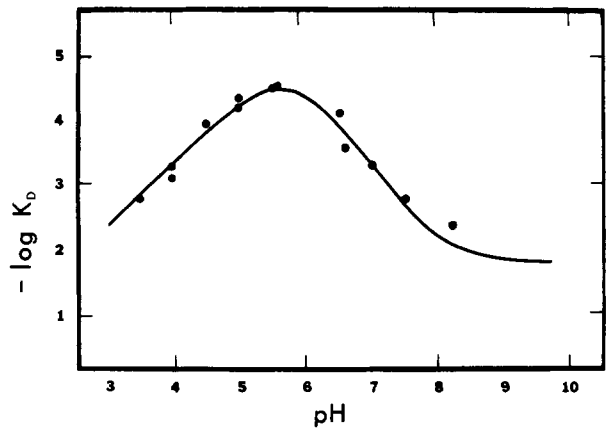


FIGURE 3: pH dependence of the dissociation constant for 3′-CMP and RNase A at an ionic strength of 0.2 M (chloride salt), 25 °C. Data are taken from Anderson et al. (1968), Bolen et al. (1971), and our own unpublished work. The solid line was fitted as described by Flogel & Biltonen (1975), assuming the pK_a of the phosphate of 3′-CMP to be 5.9 and assuming the pK_as of His-12 and His-119 to be shifted from 5.8 to 7.4 and from 6.2 to 8.0, respectively, upon the binding of 3′-CMP (Meadows et al., 1969). From such assumptions, $\Delta G°_0$, the free-energy change for the binding of 3′-CMP to the enzyme in the absence of electrostatic interactions, is found to be −2.4 kcal/mol, and $\Delta G°$, the free-energy change for the binding of the dianionic ligand to the state of the protein having both active-site histidines protonated, is found to be −7.0 kcal/mol.

(1974), and Johnson & Biltonen (1975)].

With the above equation, kinetic data can be analyzed to obtain V_{max}, K_m, and K_p. For such a three-parameter fit to be accurate, however, $[P]_\tau$ must lie within the range of K_p. This condition was not met in all our experiments. Therefore, we have analyzed our data for only V_{max} and K_m, by assuming values for K_p. This is done by plotting $[P]_\tau$ vs. $(1 + [S]_0/K_p)$ ln $[[S]_0/([S]_0 - [P]_\tau)]$. However, the resultant V_{max} and K_m values will be somewhat dependent on the magnitude of the selected K_p value (the greatest dependence on the magnitude of K_p occurs between pH 5 and 6 where K_p is smallest and $[P]_\tau/K_p$ is largest). Because of this dependence, our means of choosing K_p will be briefly discussed.

K_p values for 3′-CMP were gathered from the literature for the conditions 25 °C, ionic strength 0.2 M, chloride salt (see Figure 3). The pH dependence of K_p was then described by the treatment previously employed by Flogel & Biltonen (1975) for the binding of 3′-CMP to RNase A at an ionic strength of 0.05 M. The theoretical fit to the data at an ionic strength of 0.2 M (according to a model in which only the dianionic form of the ligand binds to the protein) is shown as the solid line in Figure 3. The fitting parameters are given in the legend. In our analysis of kinetic data via eq 2 to obtain V_{max} and K_m, K_p values from the theoretical fit were employed. Likewise, for studies at other ionic strengths, a similar dependence of K_p on pH was assumed, and literature information on the ionic strength dependence of K_p (Flogel et al., 1973) and the ionic strength dependence of the pK_a of the active-site histidine residues (Ruterjans & Witzel, 1969) was used to determine the exact features of the curves.

To illustrate how dependent the resultant V_{max} and K_m values are on the selected K_p, both the V_{max} and K_m at pH 5.0 (the most sensitive region) were found to vary by a factor of <1.2 as K_p was varied by a factor of 2 (or $^1/_2$) from the theoretical fit value.

The inclusion of product inhibition in our kinetic analysis leads to certain characteristic improvements in the V_{max} and K_m values, as compared to those values determined when product inhibition is neglected. At high pH, for example, the

Table I: pH Dependence of the Steady-State Kinetic Constants for the Ribonuclease A Catalyzed Hydrolysis of cCMP[a]

pH[c]	K_m (mM)	k_c (s^{-1})	K_p (mM)	$[S]_0/K_m$	ΔH (kcal/mol)
3.25 (F)[e]		3.83[b]	3.1		
3.25 (F)[e]	4.1	0.07	1.8	0.25	
3.98 (A)[e]	0.94	0.15	0.57	3.5	
4.0 (F)[e]	1.94	0.33	0.57	0.44	
4.0 (A)	1.73	0.21	0.57	7	8.1
5.0 (A)	0.92	1.7	0.08	11	8.1
5.0 (A)[e]	0.54	1.4	0.08	2	
5.4 (C)	0.51	2.8	0.05	20	8.4
5.5 (A)[d]	0.61	2.7	0.04	17	8.1
6.0 (C)	1.64	10.2	0.06	6	8.8
6.4 (C)	2.72	18.6	0.14	3.7	9.1
6.5 (C)	3.70	16.2	0.15	2.7	9.2
7.0 (C)	16.9	28.0	0.60	0.6	9.5
7.0 (I)	11.9	20.5	0.60	12	16.8
7.0 (T)	15.5	26.5	0.60	0.7	18.2
7.5 (T)	58	25.0	2.7	0.2	19.1
7.55 (T)	33	28.5	2.9	1.5	19.1
7.55 (T)	28	19.5	2.9	1.9	19.1
8.0 (T)	106	27	7.5	0.35	19.3
8.0 (T)	115	24	7.5	0.31	19.3
8.2 (T)[d]	181	28	9.0	0.37	19.3
8.5 (T)	230	18	13.0	0.15	19.3
9.0 (G)		33[b]			18.7
9.0 (G)[d]		26[b]			18.7
10.0 (G)[d]		1.8[b]		18.7	

[a] Conditions: μ = 0.2 M, 25 °C. [b] Represents k_c/K_m rather than k_c. [c] Letter in parentheses denotes buffer used: F = sodium formate, A = sodium acetate, C = sodium cacodylate, I = imidazole, T = Tris, and G = glycine. [d] Continuous concentration gradient employed. [e] Experiments performed spectrophotometrically by the assay of Crook et al. (1960). The difference in the molar extiction coefficient (M^{-1} cm^{-1}) used at each pH is as follows: $\Delta\epsilon_{300}$ = 1320 at pH 3.25; $\Delta\epsilon_{300}$ = 1250 at pH 3.52; $\Delta\epsilon_{302}$ = 545 at pH 3.98; $\Delta\epsilon_{300}$ = 790 at pH 4.0; $\Delta\epsilon_{295}$ = 760 at pH 5.0.

V_{max} and K_m values will both be larger when product inhibition is included (with V_{max}/K_m remaining about the same). At pH 5–6, inclusion of product inhibition results in K_m values that are smaller and V_{max} values that are about the same (therefore, V_{max}/K_m is larger) as those values determined by neglecting product inhibition.

Our microcalorimetric assays were typically performed in a point by point manner with about five substrate concentrations. In a few cases (as noted in Table I), a continuous concentration gradient of substrate was used (Mountcastle et al., 1976; Eftink et al., 1981b). In the latter experiments, a very large number (~200) of $[P]_\tau$ vs. $[S]_0$ data points were collected, thus allowing an accurate analysis for V_{max} and K_m to be made. Figure 4 illustrates such a study at pH 8.2 plotted as $\tau/[P]_\tau$ vs. $1/[S]_0$ (Lineweaver–Burk fashion, Figure 4a) and as $[P]_\tau$ vs. $(1 + [S]_0/K_p)$ ln $[[S]_0/([S]_0 - [P]_\tau)]$ (according to eq 2, the integrated Michaelis–Menten equation, Figure 4b).

Pertinent information concerning the microcalorimetric assay at each pH (ionic strength 0.2 M) is listed in Table I. Included are the buffer employed, the appropriate heat of reaction (including the heat due to protonation of the buffer), the product inhibition constant, and the highest substrate concentration employed (given as the ratio $[S]_0/K_m$).

Spectroscopic Assay. The assay described by Crook et al. (1960) was used for studies at low pH. A Cary 14 instrument equipped with a 0–0.1 scale and a temperature-regulated cell was used. In most cases, the initial slopes of the progress curves were used to obtain V_{max} and K_m from a double-reciprocal plot (Lineweaver–Burk analysis using weighted linear regression; Wilkinson, 1961). Note that with cUMP there was an inexplicable lag period before the steady-state initial velocity

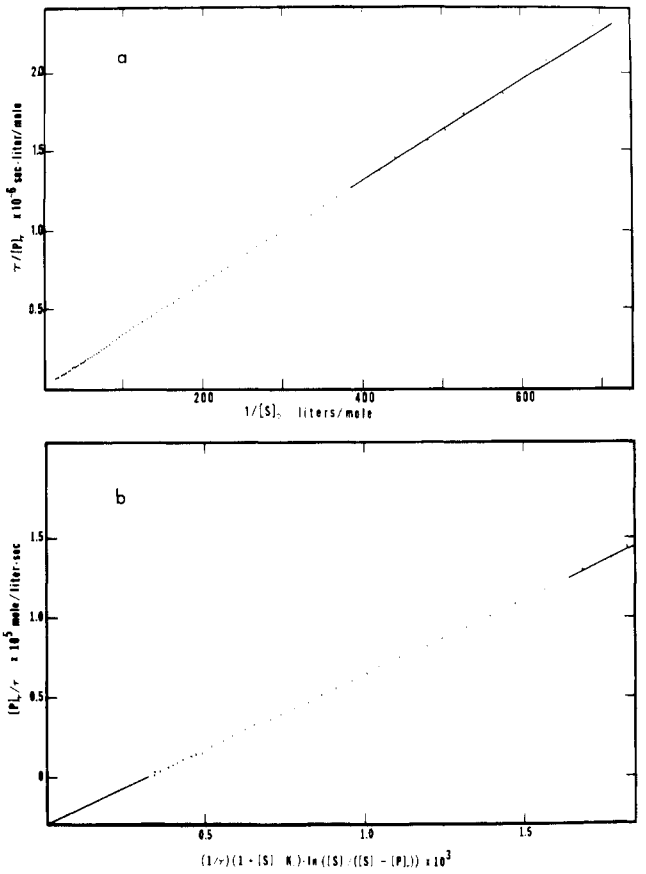


FIGURE 4: (a) Double-reciprocal plot for the RNase A catalyzed hydrolysis of cCMP at pH 8.2, ionic strength 0.2 M, 25 °C, measured by the use of a flow microcalorimeter in conjunction with a concentration gradient generating device. Approximately every fourth data point collected is shown. (b) Integrated Michaelis–Menten plot (eq 2) for the above data, including a product inhibition constant of 9×10^{-3} M. From this plot, $K_m = 0.18$ M and $V_m = 5.5 \times 10^{-5}$ M/s were obtained.

was reached at pH 4 and below. At pH 5, where K_p is smaller and product inhibition is more a concern, eq 2 was again used to analyze the kinetic data by measuring the amount of product formed (measured as the increase in absorbance) in an initial period of 8 min for a series of substrate concentrations.

Results

Values of the rate constant, k_c, Michaelis constant, K_m, and k_c/K_m for the RNase A catalyzed hydrolysis of cCMP at 25 °C and an ionic strength of 0.2 M as a function of pH are listed in Table I and are plotted in logarithmic fashion in Figures 5 and 6. Kinetic data were obtained by both flow microcalorimetric and spectroscopic assays, and analysis of the data was achieved by use of the integrated Michaelis–Menten equation (eq 2) including product inhibition. At the extreme pH values, analysis of the data was limited due to the large K_m values, and only the ratio k_c/K_m is reported.

We present the data in tabular form to allow direct comparison with the previous studies of Herries et al. (1962) which were performed by spectroscopic and potentiometric assays. Our values for k_c/K_m agree reasonably well with those of Herries et al. over most of the pH range, except around pH 5–6 where our values are slightly larger due to our correction for product inhibition. Our values for k_c and K_m also agree reasonable well with those of Herries et al. below pH 7. Above this pH, our k_c and K_m values are consistently higher.

The pattern of the pH dependence of the kinetic parameters can reveal information about the pK of ionizable groups on

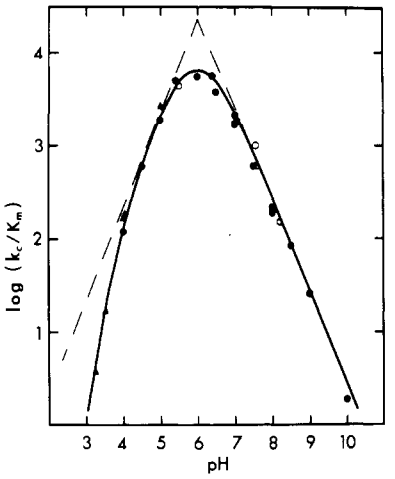


FIGURE 5: pH dependence of the apparent k_c/K_m for the RNase A catalyzed hydrolysis of cCMP, ionic strength 0.2 M, 25 °C. Closed circles represent data from a manual calorimetric assay, open circles represent data from a continuous gradient calorimetric assay, and triangles represent data obtained from a spectroscopic assay. The dashed lines are slope +1 and −1. The solid line is the fit according to eq 3 and the parameters given in Table II.

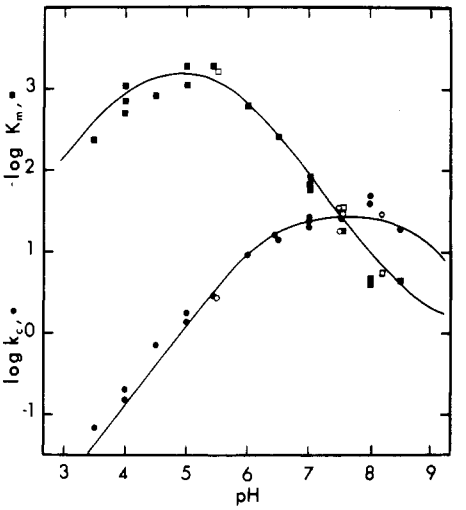


FIGURE 6: pH dependence of the apparent k_c and K_m for the RNase A catalyzed hydrolysis of cCMP, ionic strength 0.2 M, 25 °C. Open symbols represent data from continuous gradient assays. The solid line is the fit according to eq 3 and 4 and the parameters in Table II.

the protein or substrate that are involved in either binding or catalytic steps (Segel, 1975). The plot of log (k_c/K_m) vs. pH (Figure 5) for data taken at an ionic strength of 0.2 M reveals a high-pH limb of unity slope and a low-pH limb that deviates from unity below pH 4. This plot suggests that three ionizable groups participate in the enzymatic reaction. The possible candidates for these three ionizable groups and the protonation scheme describing the reaction will be discussed in the following section. For now, let us simply state that the pH dependence of the kinetic parameters was fitted by the following equations:

$$\frac{k_c}{K_m}(\mathrm{pH}) = \frac{k_c}{K_m(1 + K_a/[\mathrm{H}] + [\mathrm{H}]/K_b + K_a/K_b)(1 + [\mathrm{H}]/K_c)} \quad (3)$$

$$k_c(\mathrm{pH}) = \frac{k_c}{1 + K_{a,s}/[\mathrm{H}] + [\mathrm{H}]/K_{b,s} + K_{a,s}/K_{b,s}} \quad (4)$$

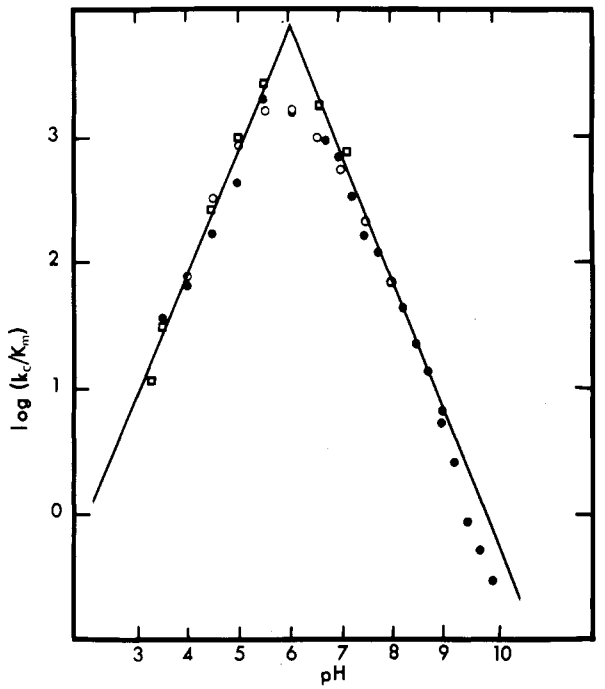


FIGURE 7: pH dependence of the apparent k_c/K_m for the RNase A catalyzed hydrolysis of cUMP, ionic strength 0.2 M, 25 °C. Our data (□); data of del Rosario & Hammes (1969) (○); data of Machuga & Klapper (1977) (●).

where [H] is the hydrogen ion concentration, $k_{\bar{c}}$ and $K_{\bar{m}}$ are the pH-independent rate constant and Michaelis constant, respectively, for the enzymatic hydrolysis of cCMP, K_a and K_b are the apparent microscopic acid dissociation constants of two residues (a and b) on the protein, and $K_{a,s}$ and $K_{b,s}$ are the acid dissociation constants for the same two residues in the enzyme–substrate complex. The two residues a and b are assumed to be functional when protonated (for a) and unprotonated (for b), respectively. The term $1 + [H]/K_c$ in eq 3 is due to the third ionizable group (group c) that must be unprotonated for maximal activity. As we will discuss later, we have tentatively assigned this group as the 3-imino group of the pyrimidine ring which has a pK of around 4 [pK = 4.05 by the spectroscopic titration method of Anderson et al. (1968)]. In fitting the above equations to the data, we used a value for K_c of 10^{-4} M.

Equations 3 and 4 can be transformed into the following linear relationships:

$$\frac{1}{1 + [H] \times 10^4}\frac{K_m}{k_c}(pH) = \frac{K_{\bar{m}}}{k_{\bar{c}}K_b}\left([H] + \frac{[H]_m^2}{[H]}\right) + \frac{K_{\bar{m}}}{k_{\bar{c}}}\left(1 + \frac{[H]_m^2}{K_b^2}\right) \quad (5a)$$

$$\frac{1}{k_c}(pH) = \frac{1}{k_cK_{b,s}}\left([H] + \frac{[H]_{m,s}^2}{[H]}\right) + \frac{1}{k_{\bar{c}}}\left(1 + \frac{[H]_{m,s}^2}{K_{b,s}^2}\right) \quad (5b)$$

where $[H]_m = (K_aK_b)^{1/2}$ and $[H]_{m,s} = (K_{a,s}K_{b,s})^{1/2}$ are the hydrogen ion activities at which there is a maximum in the values of k_c/K_m (pH) and k_c(pH), respectively. These equations were fitted to the data in Figures 5 and 6 by weighted linear regression.

Analysis of the k_c/K_m (pH) data does not provide a unique fit (since microscopic acid dissociation constants are employed) but yields two equivalent sets of $k_{\bar{c}}/K_{\bar{m}}$, K_a, and K_b values.

Table II: Parameters Describing the pH Dependence of the Ribonuclease A Catalyzed Hydrolysis of cCMP According to Scheme I [a,b]

solution set	base		acid			
	pK_b	p$K_{b,s}$	pK_a	p$K_{a,s}$	$k_{\bar{c}}$(s^{-1})	$K_{\bar{m}}$ (mM)
1	5.85	6.25	6.15	9.0	28	1.5
2	6.15	6.25	5.85	9.0	28	0.6
3	5.85	9.0	6.15	6.25	28 000	1.5
4	6.15	9.0	5.85	6.25	28 000	0.6

[a] Conditions: μ = 0.2 M, T = 25 °C. [b] The error limits for the parameters in solution set 2 are the following: $k_{\bar{c}}$, ±2 s^{-1}; $K_{\bar{m}}$, ±0.2 mM; pK_a, ±0.25; pK_b, ±0.25; p$K_{a,s}$, ±0.5; p$K_{b,s}$, ±0.2. Although the quality of the data defining $k_{\bar{c}}/K_{\bar{m}}$, pK_a, and pK_b is good (see Figure 5), the closeness of pK_a and pK_b leads to a high correlation between the fitting parameters.

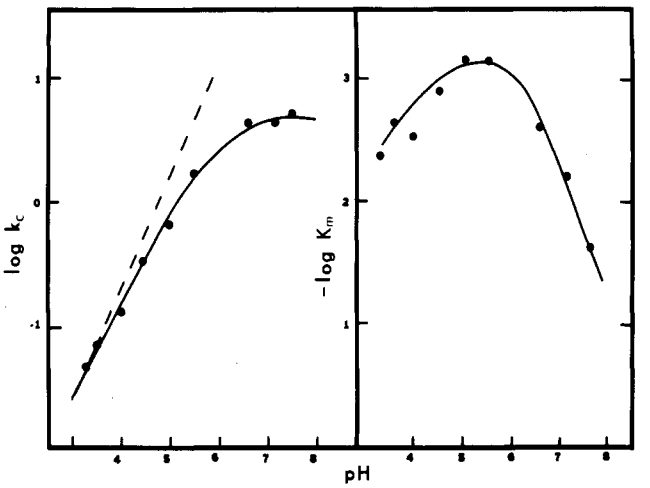


FIGURE 8: pH dependence of the apparent k_c and K_m for the RNase A catalyzed hydrolysis of cUMP.

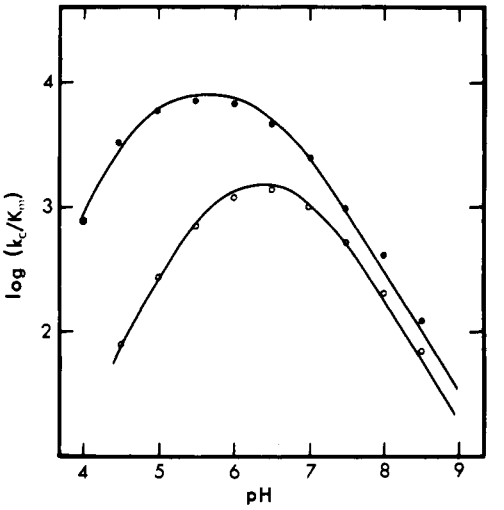


FIGURE 9: pH dependence of the apparent k_c/K_m for the hydrolysis of cCMP at ionic strengths of 0.05 (●) and 1.0 M (○).

These two sets are $k_{\bar{c}}/K_{\bar{m}} = 1.9 \times 10^4$ s^{-1} M^{-1}, pK_a = 6.15, and pK_b = 5.85 or $k_{\bar{c}}/K_{\bar{m}} = 4.6 \times 10^4$ s^{-1} M^{-1}, pK_a = 5.85, and pK_b = 6.15. Likewise, for the k_c(pH) data, two solutions are obtained. Combining these results, we find four possible combinations of parameters that give the same fit to the data shown in Figures 5 and 6. These four solution sets are listed in Table II.

The pH dependence of the hydrolysis of cUMP was also studied. These data are presented in Figures 7 and 8. Similar studies of the pH dependence of the activity of RNase toward

Table III: Ionic Strength Dependence of the Ribonuclease A Catalyzed Hydrolysis of cCMP[a,b]

ionic strength (M)	solution set	base		acid			
		pK_b	$pK_{b,s}$	pK_a	$pK_{a,s}$	$k_{\bar{c}}$ (s^{-1})	$K_{\bar{m}}$ (mM)
0.05	1	5.2	5.8	6.0	8.7	21 ± 5	0.58
	2	6.0	5.8	5.2	8.7	21 ± 5	0.09
0.20	1	5.85	6.25	6.15	9.0	28 ± 2	1.5
	2	6.15	6.25	5.85	9.0	28 ± 2	0.6
0.308	1	5.9	6.2	6.5	9.3	22 ± 3	2.4
	2	6.5	6.2	5.9	9.3	22 ± 3	0.6
1.0	1	6.0	6.6	6.7	8.9	25 ± 6	6.8
	2	6.7	6.6	6.0	8.9	25 ± 6	1.4

[a] Conditions: 25 °C. [b] The error limits of pK_a, pK_b, and $pK_{b,s}$ are all approximately ±0.3 (except pK_a and pK_b for the 0.05 M ionic strength data which are ±0.6). The error limits for $pK_{a,s}$ range from ±0.6 to ±0.9.

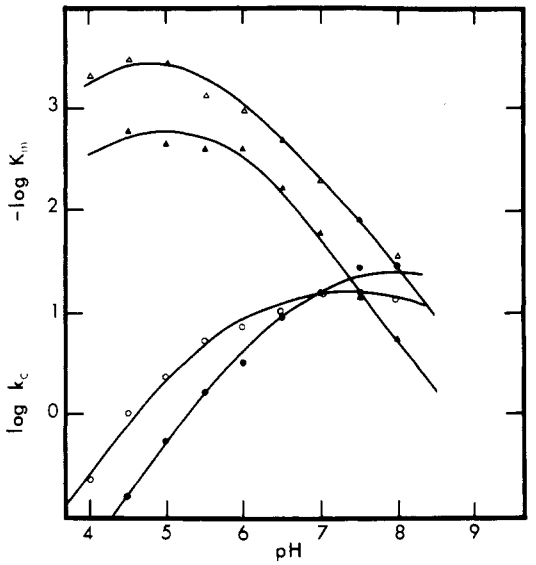


FIGURE 10: pH dependence of the apparent k_c and K_m at ionic strengths of 0.05 (○ and △, respectively) and 1.0 M (● and ▲, respectively).

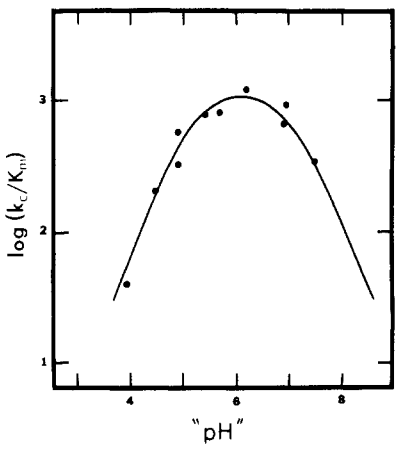


FIGURE 11: "pH" dependence of the apparent k_c/K_m for the RNase A catalyzed hydrolysis of cCMP in D_2O. All data obtained by using a spectroscopic assay. "pH" refers to uncorrected glass electrode measurements.

cCMP were performed at ionic strengths of 0.05, 0.308, and 1.0 M. Plots of log k_c, log (k_c/K_m), and log K_m vs. pH under these various conditions are shown in Figures 9 and 10. The parameters describing the pH dependence of the activity under these various conditions are given in Table III. (Only the solutions corresponding to solution sets 1 and 2 of Table II are given for reasons to be discussed later.) The values for $K_{a,s}$ and $K_{b,s}$ have large uncertainties in these cases due to the fact that the pH optimum of k_c could not be well-defined from the experimental data. For the 0.05 M ionic strength studies, the concentration of substrate (a sodium salt) that could be used was limiting. At 1.0 M ionic strength, K_m becomes so large at high pH that it is not possible to accurately determine V_{max} and K_m.

The "pH" dependence of k_c/K_m for the hydrolysis of cCMP in D_2O (ionic strength 0.2 M) is shown in Figure 11. The data can be fit by $k_{\bar{c}}/K_{\bar{m}} = 3.5 \times 10^3\ s^{-1}\ M^{-1}$, $pK_a = 6.2$, and $pK_b = 5.7$ (corresponding to solution set 1 in Table II), or $k_{\bar{c}}/K_{\bar{m}} = 1.1 \times 10^4\ s^{-1}\ M^{-1}$, $pK_a = 5.7$, and $pK_b = 6.2$ (corresponding to solution set 2). The apparent K_m for cCMP in the plateau region of pH 4.5–5.5 was determined to be approximately 6×10^{-4} M.

Discussion

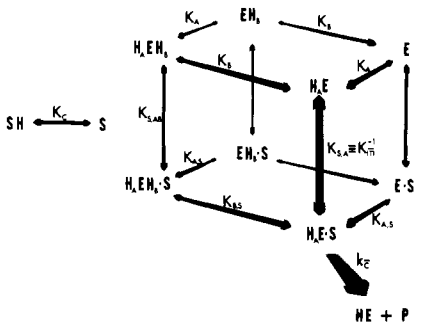
Interpretation of pH Dependence: General Features of the Mechanism. Three ionizing groups control the rate of the RNase A catalyzed hydrolysis of cCMP as evidenced by Figure 5. Possible candidates for these ionizing groups include (a) the 3-imino group on the pyrimidine ring, with a pK of 4.05, (b) the two active-site histidine residues, His-12 and His-119, each having a pK of about 6, (c) either of two carboxylic amino acid residues that are located near the active site, (d) lysine-41 which is also near the active site and reportedly has a pK of 8.8 (Murdock et al., 1966), and (e) any other ionizable group (i.e., His-48) whose protonation might possibly alter the structure and reactivity of RNase A.

Of these candidates, His-12 and -119 have been implicated most frequently as having essential functional roles. Base largely on the early steady-state kinetic studies of Findlay and co-workers (Findlay et al., 1962), it has been presumed that one of these histidine residues acts in the unprotonated state as a general base catalyst, the other histidine functioning in the protonated state, possibly as a general acid. The involvement of these two residues alone can account for the major features of the pH dependence.

To explain the inflections seen in the log (k_c/K_m) and -log K_m data at low pH, another ionizing group must be invoked. Comparison of the plots of log (k_c/K_m) vs. pH for cCMP with that of cUMP (compare Figures 5 and 7; note the absence of the low-pH inflection for cUMP) suggests that this third ionizing group may be the 3-imino group of the pyrimidine ring of cCMP.

In Scheme I is presented a mechanism for the RNase A catalyzed hydrolysis of cCMP. The substrate (S) is assumed to bind to various protonated states of the enzyme only when its pyrimidine ring is neutral.[2] The enzyme is considered to

Scheme I



have two catalytically essential residues, a and b (A and B in Scheme I) (the active-site histidine residues). Furthermore, catalysis is proposed to take place only when residue a is protonated and b is unprotonated, with the active complex, H_aES, reacting with rate constant k_c and breaking down with dissociation constant $K_{\bar{m}}$. The horizontal reactions involving proton addition and dissociation are assumed to be at equilibrium. The rate constants for the dissociation of enzyme–substrate and enzyme–product complexes are assumed to be much larger than k_c, so that rapid equilibrium conditions exist. Relaxation studies by Cathou & Hammes (1965) and Erman & Hammes (1966) support these assumptions. According to Scheme I and the above conditions, the pH dependence of k_c/K_m and k_c is given by eq 3 and 4. In the mechanism in Scheme I, we do not consider the existence of rapidly interconverting enzyme–substrate complexes that have been observed in the stopped-flow temperature jump studies of Erman & Hammes (1966). Thus, the pK values and substrate dissociation/association constants we obtain must be considered apparent values.

By labeling the essential residues as a and b, we have considered *microscopic* rather than *macroscopic* proton dissociation constants (thus the multiple solution sets). The more complicated treatment is desired in the present studies to allow comparison with pK values of His-12 and -119 determined from NMR studies.

In this scheme, we have interpreted the low-pH behavior [inflection in the log (k_c/K_m) vs. pH plot] as being entirely due to the ionization of the pyrimidine ring of the substrate cCMP. We must note, however, that the system may actually be more complicated at low pH than we have presented. del Rosario & Hammes (1969) have suggested that there exists an additional low-pH reaction pathway for the enzymatic hydrolysis of cUMP (i.e., that the complex H_aEH_bS in Scheme I has a low activity) based on their observation of a slope of <1 for log k_c vs. pH at a pH below 5. Our cursory studies with cUMP (see Figure 8) also show a similar, but less pronounced, anomaly in log k_c at low pH. At the same time, however, a plot of log (k_c/K_m) vs. pH for cUMP for the data of del Rosario & Hammes (1969) and Machuga & Klapper (1977), as well as our own, shows no significant deviation form a slope of +1 down to pH 3.5 as shown in Figure 7 (recall that experimental k_c/K_m values are more reliable than k_c values). In order for there to exist an active low-pH pathway and in order to be consistent with the linear slope of +1 for log (k_c/K_m) vs. pH for cUMP, one must postulate yet another ionizable group (necessarily on the protein, possibly one of the acidic amino acids near the active site) which when protonated decreases the affinity of the substrate. In fact, a plot of $-\log K_m$ vs. pH for cUMP does show a drop at low pH. In light of the complicated behavior at low pH, we recognize that it may be an oversimplification to explain the low-pH inflection (for cCMP) in terms of a single ionizing group. Nevertheless, we find that for cCMP as substrate our data for k_c, K_m, and k_c/M_m as a function of pH can be well fitted by including the ionization of a group (group c) on the free substrate (as in Scheme I) or the free enzyme having a pK of 4.0. Since the 3-imino group of the pyrimidine ring of cCMP has a pK of 4.05 and since it is reasonable to assume that only the neutral form of the ring will bind to the enzyme, we tentatively assign group c as this 3-imino group of cCMP.

Note that Scheme I does not include the ionization of a group on the enzyme with p$K \approx 9$ (i.e., Lys-41). The linearity of the log (k_c/K_m) data at high pH with cCMP as substrate argues against the absolute essentiality of such a lysine residue in either the binding or the catalytic steps. (A slight deviation at high pH is seen for cUMP as substrate, but it should be noted that this substrate undergoes a proton dissociation in this pH range.) This finding is consistent with the chemical modification studies of Walter & Wold (1976) which led these workers to conclude that either (a) the activity of the enzyme does not require the participation of a protonated lysine residue or (b) a protonated lysine is required for activity but a lysine residue other than Lys-41 may fulfill this requirement. Studies reported by Jentoft and co-workers (Jentoft et al., 1981) add further insight concerning the role of Lys-41. Using a combination of chemical modification and ^{13}C magnetic resonance studies, these workers found Lys-41 to have a pK_a in the free enzyme of 9.0, in agreement with Murdock et al. (1966). The pK_a of Lys-41 increases by only about 0.1–0.3 pK_a unit upon binding cytidine monophosphate inhibitors. The Gibbs energy for the electrostatic interaction between Lys-41 and the cytidine monophosphate inhibitors is thus quite small ($-\Delta G_{elec} \leq 0.4$ kcal/mol for Lys-41, vide infra) compared to the electrostatic interaction between the inhibitors and the active-site histidine residues. In fact, Jentoff and co-workers found that cytidine produces the same slight increase in the pK_a of Lys-41 as the anionic inhibitors, while 2′-deoxycytidine produces a slight lowering of this pK_a. The location of the side chain of Lys-41 has been difficult to determine in X-ray diffraction studies (Carlisle et al., 1974; Wlodawer & Sjolin, 1983). In a recent refinement of X-ray and neutron diffraction data on this protein, Wlodawer & Sjolin (1983) have argued that the amino group of this residue can be found close enough to the phosphate binding site to form a salt link. However, in view of the present kinetic studies and the above-mentioned chemical modification and thermodynamic studies (all being solution studies), we feel that the functional role of Lys-41 is uncertain and that this residue is apparently less important for ligand binding and catalysis than the histidine residues.

Assignment of the Roles of His-12 and -119 and Determination of k_c and $K_{\bar{m}}$. For the hydrolysis of cCMP at an ionic strength of 0.2 M, four sets of parameters listed in Table II are obtained that provide the same fit to the data. In order to arrive at the desired k_c and $K_{\bar{m}}$ values, we must decide which of the solution sets provides the correct description of the system.

[2] The proposal that only the neutral state of the pyrimidine ring of cCMP binds to the enzyme is in apparent contrast to the report of Anderson et al. (1968) that both the neutral and protonated states of the pyrimidine ring of 3′-CMP bind equally well to RNase A. It is difficult to rationalize the results of Anderson et al. in view of the known binding of the pyrimidine ring of 3′-CMP deep into the specificity pocket of the protein. Also, as will be pointed out in the subsequent discussion, the conflict between their results and our Scheme I is tempered by our proposal that the pyrimidine ring of cCMP interacts differenty with the enzyme than does that of 3′-CMP.

In solution set 1, the pK values of the histidine residues in the free enzyme are such as to maximize the probability of finding the two groups in the proper state of protonation for catalysis (H_aE). With the substrate bound, the pK of both residues is increased. Residue a, however, undergoes the larger pK shift, thus further increasing the probability of the residues being in their functional state (state H_aES). In solution set 2, the pK values of the free enzyme are reversed ($pK_b > pK_a$). This diminishes the probability of finding the free enzyme in the functional state of protonation. The binding of substrate, however, corrects this situation by selectively shifting the pK of residue a from 5.85 to 9.0, without significantly affecting residue b. Solution sets 3 and 4 call for $pK_{b,s}$ to be greater than $pK_{a,s}$, a situation that would greatly reduce the population of the active state of the enzyme–substrate complex (H_aES) and that would necessitate a very large k_c. In our further discussions, we will neglect solution sets 3 and 4.

To decide between solution sets 1 and 2, we will have to invoke additional information. From the combined knowledge of the pKs of the active-site histidine residues and their functional roles (i.e., which functions when protonated and which functions when unprotonated), the correct solution can be obtained.

The pKs of His-12 and His-119 in RNase A have been determined by NMR titrations of the imidazole C-2 hydrogens (Meadows et al., 1969; Roberts et al., 1969a; Ruterjans & Witzel, 1969). The assignment of the C-2 hydrogen resonances originally made by Meadows et al. (1969) has been corrected by two independent groups (Markley, 1975; Patel et al., 1975). The approximate pK values at an ionic strength of 0.2 M, 25 °C, are pK(His-12) = 5.8 and pK(His-119) = 6.2.

To identify the role of each histidine residue, let us consider some relevant X-ray crystallographic and chemical studies. The X-ray structure of the complex between RNase A and UpcA (a dinucleotide pseudosubstrate analogue of UpA having a methylene bridge between the phosphate and the adenosine) shows His-12 to be in a position to interact with the 2′-OH of the pyrimidine nucleoside and His-119 to interact with the phosphate moiety (Richards & Wyckoff, 1971). It has thus been proposed that for the transphosphorylation step His-12 acts as a general base by promoting the attack of the 2′-oxygen on the phosphate. Further, His-119 appears to be properly located to donate a proton to the leaving 3′-oxygen of the adenosine moiety (i.e., to act as a general acid). The roles played by the two histidine residues thus appear to be consistent with the so called "in-line" mechanism of RNase catalysis, at least for the transphosphorylation step (Findlay et al., 1962; Roberts et al., 1969b).

For the hydrolysis step, the one considered in the present studies, X-ray studies cannot provide a clear-cut assignment of the roles of His-12 and His-119. For this step, two distinct molecular mechanisms must be considered: the in-line mechanism (essentially the reverse of step 1) and the "adjacent" mechanism. If the second step is in-line like the first, His-119 would function as a general base in promoting the attack of water on the cyclic phosphate ring. His-12 would then function in the protonated state, possibly by donating a proton to the 2′-oxygen leaving group or by stabilizing the formation of the pentacoordinated transition state.

The alternate adjacent mechanism (Usher, 1969; Hammes, 1968) involves a process called pseudorotation. There is evidence that pseudorotation occurs in the acid-catalyzed hydrolysis of cyclic phosphate esters (Haake & Westheimer, 1961), and this mechanism has been given much consideration in connection with RNase A. If the adjacent mechanism were to occur, the general base would be His-12 and the acidic residue would be His-119.

The two proposed mechanisms give rise to an interesting question: Does the enzyme utilize the same mechanism for each step (i.e., are both steps 1 and 2 in-line), or do the mechanisms differ for the two steps? In the first case, the role of each histidine would not be the same for the two septs. In the second case, each histidine would have the same functional role in both steps. What is conserved between the two steps, the mechanism or the role of the catalytic residues?

Studies by Usher et al. (1970) using a phosphorothioate analogue of cUMP have failed to obtain any evidence for the pseudorotation process in the hydrolysis step of RNase A. This would seem to mitigate against the adjacent mechanism for step 2.

If the hydrolysis step does proceed via the in-line mechanism, His-119 must have a general base role (and therefore be group b in Scheme I), and His-12 must therefore be the acid residue (group a). From this role assignment, and the NMR pK values given above, it is clear that solution set 2 in Table II provides the correct description of the pH dependence of RNase A catalysis. Therefore, $k_c = 28\ s^{-1}$ and $K_m = 6 \times 10^{-4}$ M.

In all previous studies of the pH dependence of RNase A activity, only solution set 1 has been reported since workers considered macroscopic dissociation constants for the ionizable groups (Herries et al., 1962; del Rosario & Hammes, 1969; Machuga & Klapper, 1977). If the above assignment of the role of each histidine is correct,[3] and, likewise, solution set 2 is correct, this study demonstrates the necessity of considering microscopic dissociation constants when studying the pH dependence of the activity of an enzyme, particularly when two ionizable groups have similar pKs.

Ionic Strength and Solvent Isotope Effects. The dependence of the activity of RNase A on ionic strength noted previously by earlier workers (Irie, 1965; Winstead & Wold, 1965) is here shown to be predominantly due to two effects: a shift in the pK of the active-site histidines in the free enzyme and a change in the affinity of the substrate for the enzyme.

Ruterjans & Witzel (1969) have demonstrated by NMR titrations that the pKs of His-12 and -119 are shifted upward with ionic strength. At low ionic strength, the pKs of these residues are low due to the presence of a cluster of positively charged residues near the active site. Added salt shields the positive charges from one another, thus normalizing the pKs. In the present studies, the pKs of groups a and b in the free enzyme show a similar ionic strength dependence.

At low pH, where both active-site histidines are protonated, the interaction between the anionic substrate and the enzyme is optimal. An ionic strength dependence is observed in terms

[3] At this point, we must add a word of caution concerning our assignment of solution set 2. This assignment is based largely on the chemical studies of Usher et al. (1970) that seemingly prove the second step to proceed by an "in-line" mechanism. However, in view of the fact that the reported X-ray structures (Coulter, 1973; Saenger & Eckstein, 1970) of the substrate cCMP and the substrate analogue uridine cyclic 2′,3′-phosphorothioate [cUMP(S)] used by Usher et al. show significant differences, we have some reservations concerning the assignment of the mechanism of the hydrolysis step. In particular, cCMP and cUMP(S) differ in the orientation of the pyrimidine ring with respect to the ribose ring [cCMP is syn, cUMP(S) is anti] and differ in the orientation of the phosphorus with respect to the plane of the ribose ring. Nevertheless, at this time, we conclude that while solutions 1 and 2 in Table II are both possible, the current literature favors solution 2, and we will hereafter consider only this solution. If in time experimental results appear that alter the above assignment, the general points made under Discussion will not be drastically changed.

of the low-pH plateau K_m values as shown in Figure 10. A similar ionic strength effect has been observed for the binding of 3′-CMP to RNase A (Bolen et al., 1971; Eftink et al., 1983). Such a dependence on ionic strength is consistent with the existence of electrostatic interactions between the protonated histidine residues at the active site and the phosphate groups of the substrate or ligand.

The pH-independent rate constant, (k_c), however, does not show a significant dependence on ionic strength. Such an observation came somewhat as a surprise in view of the expected importance of electrostatic interactions in promoting the formation of the transition state in the catalytic process (Flogel et al., 1975; Roberts et al., 1969b). However, a number of studies with RNase A and other enzymes have also revealed a relative insensitivity of k_c to solution conditions (Fink, 1973; Findlay et al., 1962; Goldstein, 1972). These findings argue that once a substrate is bound to (or within) an enzyme's active site it is relatively impervious to the ionic strength and dielectric constant of the surrounding solution.

When D_2O is used as the solvent, the activity of RNase A is reduced as shown in Figure 11. Analysis reveals that k_c/K_m in D_2O is approximately one-fourth the value in H_2O. If it is assumed that K_m is the same in both solvents [which seems reasonable since the K_m in the pH ("pH") range of 5–5.5 is the same, $\sim 6 \times 10^{-4}$ M, in both], $k_c(D_2O)$ is one-fourth the value of $k_c(H_2O)$. A solvent isotope effect of this magnitude is consistent with the action of the active-site histidines as general acid/general base catalysts.

Nature of the Enzyme–Substrate and Enzyme–Transition-State Complexes. Having obtained values of k_c and K_m and having studied the effect of various solution perturbations on the enzymatic reaction, we can now begin to address the problem of explaining the rate enhancement produced by the enzyme. As discussed in the introduction, this involves the description of the interactive forces that give rise to the much greater affinity of the enzyme for the transition state than for the substrate and the visualization of the way in which these intermolecular interactions between the enzyme surface and the substrate develop as the transition state is formed. Although a meaningful discussion of the energetic details of the catalytic process must await presentation of the work in the following papers, (Eftink & Biltonen, 1983a,b), at this point let us discuss our view of the nature of the enzyme–substrate and enzyme–transition-state complexes based on the present and previous studies with RNase A.

(*a*) *Enzyme–Substrate Complex.* Direct spectroscopic or X-ray studies of Michaelis–Menten states are not feasible under ordinary conditions. Information about the nature of the enzyme–substrate interactions can, however, be provided through K_m determinations [since K_m is a true dissociation constant in this case (Erman & Hammes, 1966)]. In the present work, we have studied the manner in which the affinity of the enzyme for the substrate is perturbed by the pH, the ionic strength, and the solvent isotope effect. These studies reveal some interesting aspects about the enzyme–substrate interactions.

K_m for cCMP is optimal at pH $\sim$5, where the active-site histidine residues are protonated and are thus capable of interacting electrostatically with the anionic substrate. When His-119 is deprotonated, however, the affinity does not diminish significantly [compare $K_{s,ab} = K_b/(K_{b,s}K_m) = 2.1 \times 10^3$ M^{-1}, the association constant of the substrate to the state of the protein having both histidines protonated, to $K_{s,a} = 1/K_m = 1.7 \times 10^3$ M^{-1}, the association constant to the state of the enzyme having only His-12 protonated]. This indicates that most of the electrostatic interaction occurs between His-12 and the phosphate. The substrate must therefore bind in such a fashion that the phosphate group is close to His-12. The ΔpK for His-12 upon the binding of substrate is approximately 3 at an ionic strength of 0.2 M. The strength of the electrostatic interaction, ΔG_{elec}, between the phosphate group of cCMP and His-12 is thus equal to (standard state taken as 1 M) $\Delta G_{elec} = -2.3(RT)\Delta pK = -4.1$ kcal/mol.

As the pH is increased, the K_m increases, consistent with the importance of electrostatic bonding to His-12, and approaches a very large value at high pH (K_m approaches 0.9 M at high pH according to our fit in Figure 6). This behavior is quite unlike that for the binding of 3′-CMP to RNase A (Flogel & Biltonen, 1975). For 3′-CMP, the dissociation constant also increases as the pH is increased but approaches a limiting value of $\sim 10^{-2}$ M at high pH. This limiting dissociation constant for 3′-CMP reflects the affinity of the ligand for the enzyme in the absence of electrostatic interactions and is approximately the same as that for the binding of cytidine to RNase A (Flogel et al., 1975). The fact that the K_m for cCMP fails to approach a limiting value of $\sim 10^{-2}$ M at high pH suggests that the riboside portion of the substrate does not interact well with the enzyme's binding site as it does in the case of 3′-CMP.

Another way of expressing this observation is that the ΔG_{elec} calculated from the ΔpK of the histidines is approximately equal to the total Gibbs energy change for the binding of cCMP to the fully protonated enzyme ($\Delta G_{s,ab} = RT \ln K_{s,ab}$). This again indicates that almost all of the affinity between the substrate and the enzyme must be due to electrostatic interactions between the histidines (particularly His-12) and the cyclic phosphate portion of the substrate.

These results suggest that the nucleoside moiety of the substrate does not interact well with the enzyme's binding pocket. Studies of the temperature dependence of K_m (Eftink & Biltonen, 1983b) further substantiate this point. This observation may at first seem somewhat surprising since RNase A is known to be specific for pyrimidine substrates and this specificity must in some way reflect a recognition by the protein of the nucleoside moiety. However, upon close consideration of the studies by Gassen & Witzel (1967) on the specificity of RNase A catalysis, one finds that the selectivity resides primarily in k_c rather than K_m. A number of cyclic phosphate substrates having chemically altered pyrimidine rings show approximately the same K_m value but show a much larger variation in k_c. This would be expected if the substrates all bind to the enzyme predominantly via electrostatic interactions between the cyclic phosphate and the histidine residues. Specificity must therefore result from the interactions between the enzyme and the transition state.

What structural–chemical property of the substrate could be responsible for the apparent weakness of binding of the pyrimidine ring to the enzyme in the Michaelis–Menten complex? A molecular explanation can be found by consideration of studies on the structure of cCMP. In the crystal state, this compound appears to be rigidly locked into a unique syn conformation about the glycosidic bond (Coulter, 1973). NMR studies suggest that the solution structure of cCMP is also syn (Lavallee & Coulter, 1973). In the syn conformation, the 2-carbonyl of the pyrimidine ring lies directly above the ribose ring. The glycosidic bonds of most other pyrimidine nucleosides and nucleotides enjoy relatively free rotation, resulting in a mixture of syn and anti conformations, with the latter often being perferred (in the anti conformation, the 6-methylene group is positioned above the ribose ring and the

2-carbonyl protrudes away from the ribose ring) (Sundaralingam, 1969; Lavallee & Coulter, 1973).

Richards & Wyckoff (1971) have concluded from X-ray studies that 3′-CMP binds to RNase A in an anti conformation with the formation of hydrogen bonds between the 2-carbonyl, 3-imino, and 4-amino groups of the base and complementary polar groups lining the binding site. For cCMP, these interactions would not be immediately possible due to the improper disposition of the pyrimidine ring in the syn conformation.

There are two possible consequences of the apparently poor interaction between the enzyme and the pyrimidine ring of cCMP. One is that upon binding, the substrate will be distorted into the anti conformation [the favorable Gibbs energy available for the interaction of the pyrimidine ring with the enzyme being responsible for the unfavorable syn → anti distortion (Jencks, 1975)]. If the Gibbs energy change for the distortion cancels most of the Gibbs energy change gained from interactions with the protein, the result would be an apparent poor binding of the nucleoside portion of the substrate. This possibility seems unlikely, however, due to the fact that the K_m for a variety of substrate analogues is approximately the same (Gassen & Witzel, 1967). If distortion about the glycosidic bond were to occur in forming the Michaelis–Menten complex, a greater dependence on the chemical structure of the pyrimidine ring would be expected. Stopped-flow, temperature jump studies of Erman & Hammes (1966) show the "on" rate constant for cCMP binding to RNase A to be a factor of 10 lower than the on rate constant for 3′-CMP. If rotation of the glycosidic bond of cCMP were to occur upon binding, one might have anticipated a much lower on rate constant. Also, Erman and Hammes observed an isomerization of the RNase A–cCMP complex on the time scale of ~0.1 ms which could be identified with an induced syn → anti rotation of the bound substrate. However, relaxation processes on a similar time scale were found for the RNase–3′-CMP complex, and since the syn → anti transition of 3′-CMP is much more facile, it seems unlikely to us that the observed isomerizations involve rotation about the glycosidic bond of the bound ligand.

The other possibility, which is thought to be more likely the case, is that in the Michaelis–Menten complex the polar groups on the pyrimidine ring, as well as the complementary polar groups of the binding site, are not satisfactorily hydrogen bonded to each other. Improvement of the interactions between the enzyme and the pyrimidine ring could, however, be an important driving force for the formation of the transition state.

(*b*) *Enzyme–Transition-State Complex.* Direct information concerning the structure of the fleeting enzyme–transition-state complex cannot be obtained. To gain insight about the nature of this complex, one must piece together the available evidence concerning the structure of the transition state for the analogous noncatalyzed reaction along with insight gained about the enzymatic reaction from the response of k_c to various perturbations.

The hydrolysis of cyclic phosphate esters is thought to involve the formation of a pentacoordinated cyclic phosphate intermediate having a trigonal-bipyramidal structure about the phosphorus as shown in Figure 2 (Westheimer, 1968). The transition state must bear some resemblance to this intermediate. The deuterium solvent isotope effect reported here is consistent with the operation of general base catalysis in the enzymatic reaction. From the arguments made above, the general base is His-119 for the hydrolytic reaction. The enzyme–transition-state complex must be a species having partial proton transfer between His-119 and the water molecule attacking the phosphorus.

A positively charged His-12 must also interact with the transition state. It is not clear whether or not the interaction between this residue and the transition state is simply electrostatic in nature or whether there is a partial proton transfer from His-12 to the 2′-oxygen.

Improved interactions between the pyrimidine ring and the enzyme must be an important feature of the enzyme–transition-state complex. From studies of the binding of 3′-CMP, cytidine, and cytosine to RNase A, it appears that optimal binding occurs with the pyrimidine ring in an anti conformation (Flogel et al., 1975; M. R. Eftink and R. L. Biltonen, unpublished results). In order to gain all the favorable interactions possible, it is expected that the transition state would also have an anti-like conformation. If this is the case, rotation about the glycosidic bond must be coupled to the attack of water on the phosphate ester. It should be noted that the rotation of the glycosidic bond must proceed concomitant with the attack of water. Rotation of the pyrimidine ring after the formation of the transition state would afford no catalytic advantage.

The question that must be asked is how rotation about the glycosidic bond can affect the susceptibility of the phosphorus to attack by water. For rotation about the glycosidic bond of a pyrimidine nucleoside to occur, there must be a simultaneous flexing of the ribose ring in order to avoid contact between the 2-carbonyl group of the pyrimidine ring and the 2′-hydrogen (Haschemeyer & Rich, 1967; Sundaralingam, 1969; Yathindra & Sundaralingam, 1974). In the substrate, cCMP, the five-membered phosphate ring bridges together the 2′- and 3′-carbons, and, in doing so, also forces the ribose ring into a relatively rigid and almost planar configuration (Coulter, 1973). For the pyrimidine ring to rotate, there must be some flexing of the ribose ring. This is illustrated by the semiempirical potential energy calculations of Yathindra & Sundaralingam (1974) which show that the preferred conformation of 2′,3′-cyclic nucleotides goes from syn ($\chi \simeq 240°$) for the O(1′)-endo conformer of the ribose ring (the conformer found for cCMP in X-ray studies) to a mixture of syn ($\chi \simeq 260°$) and anti ($\chi \simeq 20°$) for the planar conformer of the ribose ring to an anti conformation ($\chi \simeq 350°$) for the O(1′)-exo ribose conformer. Flexing of the ribose ring would undoubtedly cause some distortion of the five-membered cyclic phosphate ring. Distortion of the O(2′)–P–O(3′) bond angle could cause the phosphorus to become susceptible to attact by water. Again, we emphasize that the rotation of the glycosidic bond and the attack of water must be virtually simultaneous for any catalytic advantage to be gained.

Note that we are using the terms syn and anti very loosely in describing the conformation of the substrate and transition state. These terms have specific meaning with respect to the range of the torsional angles (χ) of the glycosidic bond [$\chi \simeq 50°$ for anti and $\chi \simeq 230°$ for syn conformations (Donahue & Trueblood, 1960; Sundaralingam, 1969)]. In our use of these terms, we are concerned not so much with the actual value of the torsional angle but with the extent to which favorable interactions occur with the enzyme's specificity pocket. We realize that for the bound substrate or transitional state the glycosidic torsional angle may be outside of the normal ranges referred to as syn or anti. Also, note that if our proposal, that interactions between the enzyme and the pyrimidine ring improve upon formation of the transition state as a result of rotation about the glycosidic bond, is valid, then a similar mechanism should occur with other substrates of RNase A

besides cCMP. We are unaware of any X-ray data concerning whether cUMP is in the syn or anti conformation. Based on the similarity of proton NMR chemical shift and coupling constant data for cCMP and cUMP and on conformation studies involving lanthanide-induced NMR chemical shifts, Lavallee and co-workers (Lavallee & Coulter, 1973; Lavallee & Myers, 1978) concluded that cUMP is also syn. In addition, the chemical shifts of the ribose hydrogen atoms of cUMP (Lavallee & Coulter, 1973; Lapper & Smith, 1973) are consistent with the pattern found by Schweizer et al. (1973) for other syn pyrimidine nucleosides. On the other hand, X-ray studies with uridine cyclic 2′,3′-phosphorothioate show this cUMP analogue (which also is a substrate for RNase A) to be anti (Saenger & Eckstein, 1970). The point we propose is that improved interactions with the pyrimidine ring as a result of rotation about the glycosidic bond can play a role in RNase A catalysis. This improvement in interactions with the enzyme may involve rotations about the glycosidic bond in a slightly different manner for different substrates. In the case of cCMP, we propose that the mechanism of RNase A catalysis involves the rotation of the pyrimidine ring from a syn-like orientation in the substrate to an anti-like orientation in the enzyme-bound transition state.

Support for this type of mechanism comes from studies by Nagyvary & Provenzale (1971) showing that O^2,5′-cyclouridine cyclic 2′,3′-phosphate, a compound having a covalent bridge between the 2-position of the pyrimidine ring and the 5′-position of the ribose ring (thus preventing rotation about the glycosidic bond), is not susceptible to hydrolysis by RNase A.

Conclusions

For the RNase A catalyzed hydrolysis of cCMP, the enzymatic rate constant is $k_E = 28\ s^{-1}$, and the association constant for the substrate is $K_S = 1.7 \times 10^3\ M^{-1}$. The substrate interacts with the protein almost entirely through an electrostatic interaction between its anionic phosphate and a histidine residue at the active site (probably His-12). Interaction is minimal between the substrate and the other active-site histidine (His-119). Also, the interaction between the pyrimidine ring of the substrate and the enzyme is apparently very weak. Favorable interactions with His-119 are generated in forming the transition state. In addition, we propose that there is at least a partial rotation about the glycosidic bond in the formation of the transition state, thus permitting interactions between the pyrimidine ring and the enzyme to take place.

Registry No. RNase, 9001-99-4; cyclic 2′,3′-CMP, 633-90-9; cyclic 2′,3′-UMP, 606-02-0; 3′-CMP, 84-52-6; L-histidine, 71-00-1.

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Energetics of Ribonuclease A Catalysis. 2. Nonenzymatic Hydrolysis of Cytidine Cyclic 2′,3′-Phosphate†

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ABSTRACT: Various kinetic aspects of the nonenzymatic hydrolysis of cytidine cyclic 2′,3′-phosphate and uridine cyclic 2′,3′-phosphate have been studied in order to provide a basis for comparison with the ribonuclease A catalyzed hydrolysis reaction. Studies of the pH dependence of the nonenzymatic reaction reveal mechanisms that are first order in hydroxide concentration and second order in hydrogen ion concentration, in addition to a "water" reaction. The rate constant for the water reaction was found to be very small, approximately equal to 2.5×10^{-6} min^{-1}. General base catalyzed hydrolysis reactions were also studied with imidazole as the catalyst. At pH values in which both the protonated and neutral forms of imidazole are present, a kinetic mechanism was observed that appears to be second order in total imidazole concentration, thus suggesting that bifunctional catalysis occurs. The activation enthalpy for the hydroxide, hydrogen ion, water, and imidazole catalyzed reactions was determined.

The hydrolysis of the cyclic 2′,3′-phosphate esters of pyrimidine nucleotides is catalyzed by the enzyme ribonuclease A (RNase A).[1] Much is understood about the mechanism of this catalyzed reaction (Richards & Wyckoff, 1971; Roberts et al., 1969), but a complete explanation for the forces responsible for the catalytic power of this enzyme remains to be established, as is the case for enzymes in general. Analysis of the action of enzymes in terms of the so-called transition-state theory of enzyme catalysis has become popular in recent years (Wolfenden, 1973; Lienhard, 1973; Jencks, 1975; Schowen, 1978). The strategy of this approach is to relate the rate constants for the enzymatic and nonenzymatic reactions to the association constant, K_{TS}, for the reaction transition state to the enzyme. In the accompanying papers of this series (Eftink & Biltonen, 1983a,b), we employ the transition-state approach to analyze the energetics of RNase A catalysis.

A key point in the transition-state analysis is the choice of the appropriate nonenzymatic reaction to be used as a reference. In order to discern the nature of the forces responsible for the interaction of the transition state with the enzyme, it is necessary to have as much insight as possible concerning the chemical structure of the transition state of the reference reaction. The reference reaction transition state and the enzymatic transition state may be quite similar in structure. On the other hand, it is possible that the transition state, when bound to the enzyme's active site, will be significantly different

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[1] Abbreviations: cCMP, cytidine cyclic 2′,3′-phosphate; cUMP, uridine cyclic 2′,3′-phosphate; RNase A, bovine pancreatic ribonuclease A; k_H, second-order rate constant for hydrogen ion catalyzed hydrolysis reaction; k_{OH}, rate constant for hyroxide reaction; k_w, rate constant for "water" hydrolysis reaction; k_{Im}, rate constant for imidazole-catalyzed reaction; k_{Im^+}, rate constant for imidazolium-catalyzed reaction; k_{Im^2}, second-order rate constant for imidazole-catalyzed reaction; K_{Im}, acid dissociation constant of imidazole.

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