Studies of Catalysis by Ribonuclease U_2. Steady-State Kinetics for Transphosphorylation of Oligonucleotide and Synthetic Substrates†

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ABSTRACT: The values of the steady-state kinetic parameters for the RNase U_2 catalyzed transphosphorylation were measured for several di- and trinucleotides such as ApXp (X = A, C, G, or U), ApYpGp, and YpApGp (Y = C or U). The pH dependence of k_{cat}/K_m for ApUp indicated a dependence for catalysis upon an unprotonated group with pK = 3.8 and two proton-associated groups with pK = 4.4 and 5.0. As judged from k_{cat}/K_m values, ApUpGp and ApCpGp are better substrates than the corresponding parent dimers, ApUp and ApCp, respectively. By contrast, the k_{cat}/K_m for UpApGp was about $^1/_{20}$ relative to that of the parent dimer, ApGp. The results were compared for possible thermodynamic and structural information about the chemical consequences. The inhibition of the RNase U_2 catalyzed reaction of ApUp by a series of nucleosides and nucleotides was also studied. Evidence for similarities and dissimilarities in the binding and catalysis by RNase U_2 and RNase T_1 is presented.

Ribonuclease U_2 (RNase U_2)[1] is the ribonucleate 3′-oligonucleotidohydrolase produced from *Ustilago sphaerogena* (EC 3.1.27.4) and catalyzes the depolymerization of RNA in two separable stages, transphosphorylation and hydrolysis (Arima et al., 1968; Minato & Hirai, 1979; Uchida et al., 1980). RNase U_2 is unique among ribonucleases in its specificity: the enzyme is specific for phosphodiester bonds in which the 3′-linked nucleotide is a purine nucleotide and dinucleoside monophosphates such as ApU and GpC or purine nucleoside cyclic 2′,3′-phosphates are substrates (Uchida et al., 1970; Rushizky et al., 1970). RNase U_2 is also known to shown considerable specificity for adenylyl residues under certain conditions (Randerath et al., 1980). For this, interest in this particular RNase has increased considerably during the last few years with the realization that this enzyme, in addition to other RNases such as RNase A and RNase T_1, is indispensable in a new rapid gel sequencing method for RNA, which has been developed recently (Donis-Keller et al., 1977; Simoncsits et al., 1977). However, there appears to be some problems in the use of RNase U_2 that should be considered (Rushizky et al., 1970; Uchida & Machida, 1979).

RNase U_2 is a 12 490 molecular weight single polypeptide composed of 113 amino acids, and the primary structure has been reported (Sato & Uchida, 1975a,b). The amino acid sequence of the active-site portion of RNase U_2 shows an obvious sequence homology with RNase T_1, as judged from the results of chemical modification experiments (Takahashi, 1965; Sato & Uchida, 1975c; Minato & Hirai, 1979). The three-dimensional structure is not known for RNase U_2 and RNase T_1 yet. Nevertheless, on the basis of the sequence homology between RNase T_1 and RNase U_2, it is anticipated that the two enzymes have a similar conformational structure of their active-site region, although RNase T_1 and RNase U_2 exhibit different substrate specificity (RNase T_1 shows strict specificity for the guanylyl group).

Since little information is available concerning the kinetics of RNase U_2 catalyzed transphosphorylation and since no relevant data for the effect of the nearest neighbor base on the enzymatic reaction of adenylyl nucleotides could be found in the literature (Sato & Uchida, 1975d; Uchida & Machida, 1979), in the following, we present the results of steady-state kinetic studies about the transphosphorylation with a series of di- and trinucleotides as substrates. We also report results from kinetic studies about the binding to RNase U_2 of inhibitors such as nucleosides and nucleotides. Evidence is presented that a pair of sequence isomers ApUpGp and UpApGp behave quite differently in the RNase U_2 catalyzed transphosphorylation. These results are discussed in the relation to other work.

Materials and Methods

Materials. Dinucleotides, ApXp where X = A, C, G, and U, and trinucleotides, ApYpGp and YpApGp where Y = C and U, were prepared as described (Aoyagi & Inoue, 1968a,b). Dinucleoside monophosphates, ApX, and trinucleotide diphosphates, ApYpG, were obtained from enzymatic dephosphorylation of ApXp and ApYpGp, respectively, with *Escherichia coli* alkaline phosphatase. Ap(3′)Me and Ap(3′)Bzl were prepared according to the method of Smyth et al. (1958). Ap(3′)Naph and Ap(2′)Naph were also prepared in a similar manner to that described by Kole et al. (1972). d(pAp), d(pApAp), and d(ApA) were prepared by hydrazinolysis of herring sperm DNA followed by column chromatographic separation with DEAE-Sephadex A-25 anion exchanger (Habermann, 1963). Highly purified RNase U_2 prepared by Sankyo Co., Ltd., was a gift of Dr. T. Uchida (Mitsubishi-Kasei Institute of Life Sciences). All other materials were of analytical grade.

Determination of Difference Extinction Coefficients. The absorbance difference between the substrate and the products of hydrolysis by RNase U_2 was used for estimating the difference molar extinction coefficients ($\Delta\epsilon$) at the appropriate analytical wavelength where the greatest difference between

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[1] Abbreviations used: RNase, ribonuclease; RNase U_2, ribonuclease from *Ustilago sphaerogena* (EC 3.1.27.4); RNase A, bovine pancreatic ribonuclease A (EC 3.1.27.5); RNase T_1 and RNase T_2, ribonucleases from *Aspergillus oryzae* (EC 3.1.27.3 and EC 3.1.27.1, respectively); Ap(3′)Bzl, adenosine 3′-(benzyl phosphate); Ap(3′)Me, adenosine 3′-(methyl phosphate); Ap(3′)Naph, adenosine 3′-(1-naphthyl phosphate); m^{1}ApU, 1-methyladenylyl(3′→5′)uridine; for other nucleoside and nucleotide abbreviations, see IUPAC–IUB Commission on Biochemical Nomenclature (1970); for example, ApUp, adenylyl(3′→5′)uridine 3′-phosphate.

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Table I: Analytical Wavelengths and the Difference in Molar Extinction Coefficients between the Oligonucleotide Substrates and the Hydrolysis Products Obtained with RNase U_2[a]

substrate	analytical wavelength (nm)	$\Delta\epsilon \times 10^{-3}$
ApAp	262	3.54
ApA	262	4.32
ApCp	267	1.71
ApC	267	1.90
ApGp	267	1.78
ApG	267	1.95
ApUp	260	2.09
ApU	260	2.28
ApCpGp	267	1.49
ApUpGp	260	2.18
CpApGp	267	2.55
UpApGp	267	1.70

[a] All measurements were made at pH 4.5, $I = 0.1$, and 25 °C.

the absorbances was observed for oligonucleotide substrates. The $\Delta\epsilon$ values were determined at pH 4.5 and 25 °C for the di- and trinucleotides used (Table I). For ApUp the $\Delta\epsilon$ at 260 nm was determined as a function of pH: 1240 (pH 3.08), 1440 (pH 3.50); 1780 (pH 3.90), 2090 (pH 4.45), 2190 (pH 5.00), and 2140 (pH 5.81).

Kinetics. The steady-state kinetic measurements were carried out with the apparatus and by methods similar to those described previously (Yasuda & Inoue, 1981). Substrate solution (3 mL) (0.05 M buffer and 0.05 M NaCl) was placed in a 1-cm silica cell in the thermostated cell compartment of a Hitachi 124 spectrophotometer. The ranges of concentrations used for each of the oligonucleotide and chromogenic [Ap(3′)Naph] substrates were 10–80 μM. After temperature equilibration, 50 μL of RNase U_2 solution was added, and recording of the absorbance was initiated. The enzyme concentration was 1.43 nM. In every case, the initial portion of absorbance vs. time plots was linear, and the initial rate of transphosphorylation was determined from the slope of the curve starting at the beginning of the recorder trace. Kinetic measurements for each oligonucleotide substrate were made at least five different concentrations (10–80 μM) with triplicate assays. The rate measurements of transphosphorylation of Ap(3′)Me and Ap(3′)Bzl involved removal of aliquots at appropriate intervals. A 5-mg sample of each substrate dissolved in 500 μL of the buffer is required for each kinetic run, and the enzyme concentration was 15.9 nM for these synthetic nonchromogenic substrates. After addition of 9 mL of 0.12 M HCl and standing for 1 h at 25 °C, the hydrolysis products (Ap) were separated from the unreacted substrate by DEAE-Sephadex A-25 column chromatography with a subsequent quantitation of the hydrolysis products. Less than 10% of substrate was transphosphorylated during the time necessary to evaluate initial rates.

Inhibition constants were measured at varying inhibitor concentrations by use of ApUp as substrate. The concentration of the inhibitors or cosubstrates [Ap(3′)Me, Ap(3′)Bzl, and Ap(3′)Naph] varied from 10 to 70 μM.

Analysis of Steady-State Kinetic Data. The values of initial rate, defined by eq 1, from reactions at varying substrate

$$\frac{E_0}{\nu_i} = \left(\frac{K_m}{k_{cat}}\right)\left(\frac{1}{S_0}\right) + \frac{1}{k_{cat}} \qquad (1)$$

concentration were plotted in the usual double-reciprocal manner to provide k_{cat} and K_m. ν_i, E_0, S_0, K_m, and k_{cat} are the initial rate, the total enzyme concentration, the substrate concentration, the Michaelis constant, and the turnover number, respectively. Initial rates were fitted to a Lineweaver–Burk plot by a least-squares program on the electronic calculator, TI Programmable 58 (Texas Instruments) to determine the steady-state kinetic parameters, k_{cat} and K_m. Values for K_m and k_{cat} obtained with the standard deviation for all 17 substrates are given in Table III under Results and Discussion.

For the nonchromogenic substrates, Ap(3′)Me and Ap(3′)Bzl, the maximal velocity at $S_0 \gg K_{mi}$ was determined by the method of liquid column chromatography. In testing the precision of V_{max} determinations by this method, standard deviations of 15% were obtained for these substrates. K_{mi} values for Ap(3′)Me, Ap(3′)Bzl, and Ap(3′)Naph were determined by the use of these compounds as cosubstrates in the transphosphorylation of ApUp.

The values of K_i and K_{mi} were obtained by graphical analysis of $1/\nu_i$ vs. I plots with varying inhibitor or cosubstrate and fixed substrate (ApUp) concentration using eq 2

$$\frac{E_0}{\nu_i} = \left(\frac{K_m}{k_{cat}}\right)\left(1 + \frac{I}{K_i}\right)\left(\frac{1}{S_0}\right) + \frac{1}{k_{cat}} \qquad (2)$$

where I is the inhibitor of cosubstrate concentration and K_i (or K_{mi}) is the inhibition constant defined as $K_i = k_{-1}'/k_1'$ for

$$E + I \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} EI$$

The K_m values for Ap(3′)Me, Ap(3′)Bzl, and Ap(3′)Naph were obtained by using these substrates as inhibitors of ApUp transphosphorylation. Laidler & Bunting (1973) have demonstrated that the kinetic equation for the velocity of one substrate in the presence of a secondary competing substrate (cosubstrate) has the same form as that for competitive inhibition. It was also shown that the inhibition constant for the second cosubstrate K_i is kinetically equivalent to K_m, the true Michaelis constant. In view of this, the inhibition constant thus obtained is denoted as "K_{mi}" in this paper.

Results and Discussion

pH Dependence of Kinetic Parameters. The transphosphorylation of ApUp was investigated over a pH range, and the pH profiles for k_{cat} and k_{cat}/K_m are illustrated in Figure 1. The pH profiles are bell shaped, similar to results obtained with RNase T_1 (Satoh & Inoue, 1975; Osterman & Walz, 1978) and different from those for RNase T_2 (Yasuda & Inoue, 1981). The bell-shaped curve in a plot of the apparent bimolecular rate constant, k_{cat}/K_m, as a function of pH, showed that the pH optimum region appeared to be around pH 4.1, and it was much narrower than that for RNase T_1. The pH profiles shown in Figure 1 revealed that the ascending and descending limbs were governed by at least one and two ionizable groups, respectively. The ascending limb in Figure 1B for ApUp appears to be determined by the state of ionization of a certain amino acid side chain (pK = 3.8) at the active site of RNase U_2 rather than by the protonation of the substrate base moiety because m^{1}ApU is as reasonably good a substrate as ApU (Uchida et al., 1970). Thus, the simplest mechanistic representation in the systems studied can be summarized by Scheme I. This scheme assumes rapidly equilibrating ionization. K_a, K_b, and K_c are apparent ionization constants characterizing pertinent ionizable groups on the free enzyme and K_a', K_b', and K_c' on the enzyme–substrate complex. Then, the pH dependence of the steady-state kinetic parameters, k_{cat}, K_m, and k_{cat}/K_m can be expressed by a

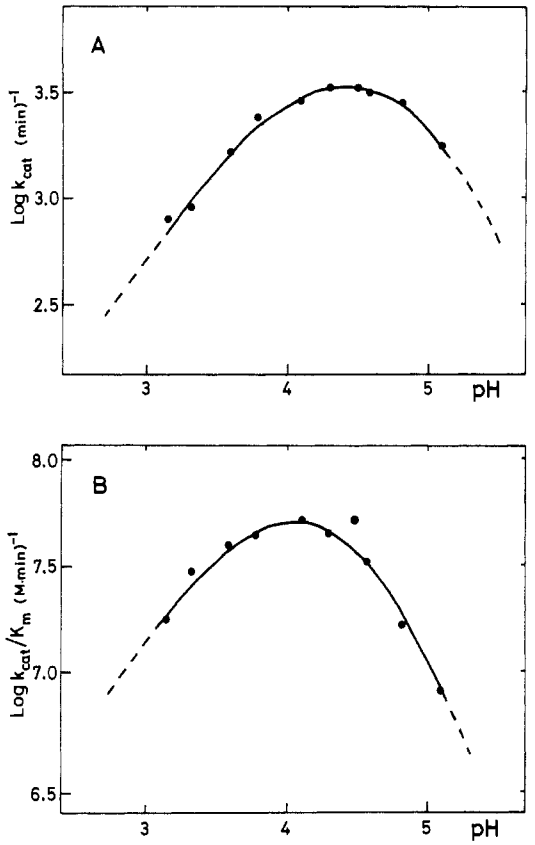
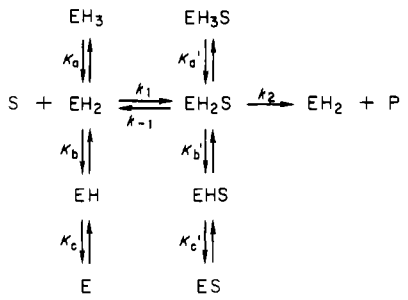


FIGURE 1: pH dependence of (A) k_{cat} and (B) k_{cat}/K_m for ApUp. The lines represent the computer-generated curves which provide the best fit of the data to eq 3 and 5. See text for further details.

Scheme I



combination of rate and equilibrium constants involved in Scheme I:

$$k_{cat} = \frac{k_2}{a_H/K_a' + 1 + K_b'/a_H + K_b'K_c'/(a_H)^2} \quad (3)$$

$$K_m = \frac{\bar{K}_m[a_H/K_a + 1 + K_b/a_H + K_bK_c/(a_H)^2]}{a_H/K_a' + 1 + K_b'/a_H + K_b'K_c'/(a_H)^2} \quad (4)$$

$$k_{cat}/K_m = \frac{k_2/\bar{K}_m}{a_H/K_a + 1 + K_b/a_H + K_bK_c/(a_H)^2} \quad (5)$$

where $\bar{K}_m = (k_{-1} + k_2)/k_1$.

The best fit of the data to eq 3 or 5 was obtained from nonlinear least-squares analysis. The program written in Fortran IV and stored at the Computer Center of the University of Tokyo for a nonlinear iterative least-squares fit to eq 3 and 5 was adopted for the present purpose and run on a HITAC M-200H. The best values for the apparent pK for both free and substrate-associated enzymes and the limiting velocities for their breakdown to form products were obtained and are listed in Table II.

Table II: pH-Independent Rate Constant, k_2, and Michaelis Constant, $\bar{K}_m$, Together with Ionization Constants for Free RNase U_2 and RNase U_2-ApUp Complex at 25 °C and Ionic Strength of 0.1

	free RNase U_2	RNase U_2-ApUp complex
k_2 (min^{-1})	(5.9 ± 0.9) × 10^3	
$\bar{K}_m$ (mM)	0.0573 ± 0.006	
pK_a or pK_a'	3.8 ± 0.1	4.0 ± 0.1
pK_b or pK_b'	4.4 ± 0.1	4.9 ± 0.1
pK_c or pK_c'	5.0 ± 0.1	5.5 ± 0.1

Table III: Steady-State Kinetic Parameters Obtained with RNase U_2 at pH 4.5 and 25 °C[a]

no.	substrate	$10^{-1}k_{cat} \pm \sigma$ (min^{-1})	10^6K_m or $10^6K_{mi} \pm \sigma$ (M)	$10^{-5}k_{cat}/K_m \pm \sigma$ (min^{-1} M^{-1})
1	ApAp	146 ± 12	74.7 ± 11	195 ± 17
2	ApCp	577 ± 50	77.4 ± 12	745 ± 65
3	ApGp	223 ± 15	41.1 ± 6	543 ± 50
4	ApUp	330 ± 25	62.0 ± 9	532 ± 45
5	ApA	20 ± 2	234 ± 35	8.5 ± 0.8
6	ApC	143 ± 11	424 ± 70	33.7 ± 3
7	ApG	38 ± 3	300 ± 40	12.7 ± 1.1
8	ApU	44 ± 4	319 ± 50	13.8 ± 1.2
9	ApCpGp	1372 ± 100	129 ± 20	1064 ± 100
10	ApUpGp	1694 ± 130	209 ± 30	811 ± 70
11	CpApGp	357 ± 25	141 ± 20	253 ± 20
12	UpApGp	89 ± 7	357 ± 55	25 ± 3
13	ApCpG	847 ± 70	100 ± 13	847 ± 80
14	ApUpG	893 ± 70	130 ± 20	687 ± 60
15	Ap(3')Naph	0.511 ± 0.08	114 ± 9	0.448 ± 0.04
16	Ap(3')Bzl	0.059 ± 0.009	124 ± 10	0.048 ± 0.004
17	Ap(3')Me	0.019 ± 0.003	126 ± 10	0.015 ± 0.001

[a] The enzyme had a specific activity (T. Uchida) of 2.2 × 10^3 units/mg; enzyme concentration was 1.79 × 10^{-5} mg/mL for the oligonucleotide substrates 1–14 and 1.99 × 10^{-4} mg/mL for the synthetic substrates 15–17; concentrations of substrates 1–14 were 10 to ~80 μM and those of substrates 15–17 were 16 mM; concentration of the synthetic substrates as cosubstrates was 10 to ~70 μM.

According to the results based on chemical modification studies, two amino acid residues, Glu-61 and either His-40 or His-100, have been implicated as critical components of the active site of RNase U_2 (Sato & Uchida, 1975c; Uchida, 1975; Minato & Hirai, 1979). The pK governing the ascending limb is now tentatively assigned to the active-site Glu-61. The descending limb of the pH profile for k_{cat}/K_m depended on the ionization of two amino acids with pK = 4.4 and 5.0. The simplest picture of the catalytic site which is in accordance with our results as well as others' is one in which a carboxyl group of Glu-61 is a base and at least one imidazole (His-40 or His-100) is in the form of the conjugate acid. It should be noted that the peptide sequences near the amino acids located in the active site of RNase T_1 were shown to have a close similarity with the corresponding sequences for RNase U_2 when they aligned as below (Sato & Uchida, 1975a):

RNase U_2:
...-Tyr-Pro-*His*^{40}-Glu-Tyr-...-*Glu*^{61}-Phe-Pro-Leu-Val-...

RNase T_1:
...-Tyr-Pro-*His*^{40}-Lys-Tyr-...-*Glu*^{58}-Trp-Pro-Ile-Leu-...

RNase U_2:
...-Asp-*Arg*^{84}-Val-Ile-Tyr-...-Val-Thr-*His*^{100}-Thr-Gly-Ala-...

RNase T_1:
...-Asp-*Arg*^{77}-Val-Val-Phe-...-Ile-Thr-*His*^{92}-Thr-Gly-Ala-...

The presence of such homologous regions usually implies that the corresponding amino acids in RNase U_2 are obvious candidates for the "active-site amino acid residues" concerned with binding and catalysis. The present work would also suggest that some additional ionizable group on the enzyme has to be protonated in order that the catalysis can take place.

Determination of k_{cat} and K_m Values for RNase U_2 Catalyzed Reaction with Oligonucleotide and Synthetic Substrates. Results of study of the kinetics of RNase U_2 catalyzed transphosphorylation of 17 substrates from ApAp to Ap(3′)Me are summarized in Table III. The values of k_{cat} and K_m for four dinucleoside monophosphates, ApX (X = A, C, G, and U), are qualitatively similar to the reported values (Uchida & Machida, 1979; Minato & Hirai, 1979), but the K_m values for ApC and ApG are not in agreement with those reported by Uchida & Machida (1979) (standard deviations were not specified in their report).

Osterman & Walz (1979) have shown with a couple of trinucleoside diphosphates that the length of the nucleotide chains does affect the catalytic mode of RNase T_1. These authors have also explained their kinetic results on RNase T_1 catalysis in terms of a defined model of subsite interaction between di- and trinucleotide substrates and the enzyme (Osterman & Walz, 1978, 1979). It has also been previously reported that RNase A has shown a chain-length dependency for activity toward the oligonucleotide substrates as determined by White et al. (1977a). Work with synthetic nucleotide inhibitors has also shown that the rate of Staphylococcal nuclease is dependent on nucleotide chain length (Cuatrecasas et al., 1969). We shall now discuss the results for RNase U_2.

(*a*) *Dinucleotide and Dinucleoside Monophosphates.* ApCp appears to be the best RNase U_2 substrate, as judged from its k_{cat}/K_m value. With RNase T_1 (Irie, 1968) and RNase T_2 (Imazawa et al., 1968), GpC and ApC were respectively transphosphorylated at faster rates than other dinucleoside monophosphates. The low reactivity of the -ApA- sequence in RNA demonstrated for RNase U_2 (Rushizky et al., 1970; Adams et al., 1969). The present results on ApAp and ApA confirm these previous observations. The binding of the adenylyl moiety at the 3′ end of the molecule ApAp could result in a nonproductive displacement for catalysis or competitive inhibition. This might explain why the -ApA- sequence functioned as poor substrates.

An examination was made of the steady-state kinetics of the action of RNase U_2 on ApA, according to

$$SE \overset{K_i}{\rightleftharpoons} E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

where ES is the Michaelis complex and SE is the nonproductive complex. Kinetic relationships for this mechanism are $K_m = (k_{-1} + k_2)/k_1$, $K_i = (SE)/(S)(E)$, $K_{m(app)} = K_m/(1 + K_iK_m)$, and $V_{max(app)} = V_{max}/(1 + K_iK_m)$. The kinetic consequences of nonproductive binding of ApA would provide low estimates for K_m and V_{max}. However, because $V_{max(app)}/K_{m(app)} = V_{max}/K_m$, the causes of the decreased relative reactivity must not be a possible formation of the nonproductive complex. It should be noted that the k_{cat}/K_m data for the dinucleotide substrates correlate inversely with the extent of intramolecular base-stacking association of these substrates (Inoue & Satoh, 1969; Brahms et al., 1969; see also the $\Delta\epsilon$ values in Table I). It may be safely said that the stronger the base-stacking interaction in the dimer substrates the poorer the reactivity toward RNase U_2. A roughly reverse correlation was previously noticed by comparison of catalytic constants for different dinucleoside monophosphates for RNase T_1 (Whitfield & Witzel, 1963; Irie, 1968; cf. Osterman & Walz, 1978) and RNase A (Richards & Wyckoff, 1971). In this respect the catalytic configuration of RNase U_2 appears to be somewhat different from those of RNase T_1 and RNase A, though a comparison of the active-site regions of RNase T_1 and RNase U_2 has revealed a high degree of homology (Sato & Uchida, 1975a). With regard to substrate binding, changes in the nature of the leaving group, X, in Ap(3′)X do not much affect the Michaelis constant K_m.

(*b*) *Trinucleotide Substrates.* Although the studies on the trinucleotide substrates were made on only two pairs of sequence isomers, ApUpGp–UpApGp and ApCpGp–CpApGp, the results invite some speculation. An introduction of a guanylyl residue to the 3′ end of ApCp and ApUp contributes to a favorable kinetic constant, k_{cat}, for ApCpGp and ApUpGp relative to the corresponding parent dimers, leading to a 2.4-fold and a 5-fold increase in this parameter. It appears, however, that the trinucleotide substrates ApCpGp and ApUpGp may bind slightly weaker than the corresponding parent dinucleotides ApCp and ApUp. The internal pyrimidine nucleoside groups in ApCpGp and ApUpGp tend to exert the "destacking effect" (Inoue et al., 1967; Brahms et al., 1969), thereby allowing the next neighboring base–base stacking interaction (Lee & Tinoco, 1980; Hartel et al., 1981). Consequently, decreased binding and enhanced catalytic activity of ApCpGp and ApUpGp relative to ApCp and ApUp could be accounted for by the possible interaction between next nearest neighbor purine bases. If this explanation is correct, the results of a recent study by Lee & Tinoco (1980) on trinucleoside diphosphates should enable some predictions to be made concerning the susceptibility of RNase U_2 catalyzed transphosphorylation.

An introduction of a pyrimidine nucleotide to the 5′ end of ApGp has a rate-retarding effect, 0.47-fold for CpApGp and 0.05-fold for UpApGp. The k_{cat} for ApUpGp is about 20 times that of the sequence isomer UpApGp while the ratio for the corresponding parent dinucleotides is only about 1.5. Thus the -UpApGp- sequence is the poorest substrate for RNase U_2 and is quite resistant to this enzyme, which is in agreement with the previous observations by Rushizky et al. (1970). The present steady-state kinetic experiments with the trinucleotide substrates therefore provide an indication for uniqueness of molecular conformation and thereby reactivity of this particular sequence, -UpApGp-, which is incidentally equivalent to the termination code sequence.

As an alternative model to rationalize the observed results, the binding sites of RNase U_2 could be considered to constitute a series of subsites which are capable of binding several nucleotide residues at either end of the nucleotide chain. However, the results obtained for the system of YpApGp and ApGp suggest that the causes of the decreased reactivity of YpApGp as compared to ApGp are not elucidated by subsite interactions. For the other system, ApYpGp and ApYp, the enhanced activity of the enzyme toward ApYpGp could be explained on the basis of the subsite interaction model. Nevertheless, final evaluation of the mechanism of RNase U_2 catalysis of any oligonucleotide must await elucidation of position in the tertiary structure of the enzyme.

(*c*) *Synthetic Substrates.* Ap(3′)Naph, Ap(3′)Bzl, and Ap(3′)Me are all shaped as substrates for RNase U_2, but they are poorer substrates than natural ones such as ApAp to ApUpG. Surprisingly, no transphosphorylation of Ap(3′)-Naph, which has a good leaving group because the departure of the naphtholate anion will proceed without any electrophilic assistance as compared to the case for the poor leaving groups

Table IV: Relative Rates (k_{cat}/K_m) of Transphosphorylation of the Synthetic Substrates for RNase U_2, RNase T_1, and RNase T_2[a]

enzyme	natural substrate	k_{cat}/K_m	K_m (M)	synthetic substrate	k_{cat}/K_m	K_m (M)
RNase U_2	ApU	100	3.2×10^{-4}	Ap(3')Bzl	0.35	1.2×10^{-4}
RNase T_1[b]	GpU	100	4.0×10^{-5}	Gp(3')Bzl	0.048	8.2×10^{-5}
RNase T_2	ApU	100	2.1×10^{-5}	Ap(3')Bzl	94	1.9×10^{-5}

[a] The dinucleoside monophosphate substrates, ApU and GpU, are taken as standard = 100; Michaelis constants (K_m) are also shown (at pH 4.5 and 25 °C). [b] Data for GpU (at pH 5.0) were taken from Walz et al. (1979), and those for Gp(3')Bzl are unpublished results by K. Satoh and Y. Inoue.

such as alkyloxy anion, by RNase U_2 could be observed under the same conditions as were used for the RNase T_2 reaction (Yasuda & Inoue, 1981) or even at higher enzyme concentrations. Thus, if Ap(3')Naph is an RNase U_2 substrate at all, it is a very poor substrate. In fact Ap(3')Naph is by no means enzymatically inert, and k_{cat} was determined to be 5.11 min^{-1} by the column chromatographic method; k_{cat} for ApU under the same conditions is 440 min^{-1} and $K_m = 3.19 \times 10^{-4}$ M (Table III). Thus Ap(3')Naph is bound even more tightly to RNase U_2 than ApU, and the major difference in reactivity between Ap(3')Naph and ApU is that k_{cat} for the latter is nearly 1000 times greater than that for Ap(3')Naph. The rate ratios for synthetic substrates and natural substrates may furnish a new type of evidence on the RNase catalytic mechanism. Toward the synthetic substrates, Ap(3')Bzl and Ap(3')Me, we found that while these were all bound to RNase U_2 as tightly as the natural substrates, they were transphosphorylated much more slowly than the natural substrates (Table IV).

Table IV shows values of the kinetic constants of the reaction catalyzed by RNase U_2 and related RNases with the corresponding natural and synthetic substrates, where relative values of k_{cat}/K_m, based on the figure 100 for the former, are given. The results in Table IV indicate that nucleoside groups in Ap(3')X must be needed as the leaving group, X, for high reactivity in the substrates for RNases U_2 and T_1. The synthetic substrates proved to have extremely low reactivity. By contrast, in the RNase T_2 reaction, Ap(3')Bzl undergoes transphosphorylation as rapidly as ApU. Thus the observed low ability to transphosphorylate the synthetic substrates, Ap(3')Bzl and Gp(3')Bzl, is common for RNases U_2 and T_1 and is definitely not due to the less favorable electronic effect of the leaving group. As a preliminary assessment, the relative reactivities of the natural and synthetic substrates presented in Table III and IV, in view of the high binding affinity of the synthetic substrates to RNases and much higher reactivity of Ap(3')Naph over ApU in nonenzymatic base-catalyzed hydrolysis, all point strongly to a unique reactive conformation about the central phosphorus atom in the natural substrates. However, until information about the three-dimensional structure of the enzyme–substrate complexes becomes available, any explanation remains speculative.

Inhibition of RNase U_2 by Nucleosides and Nucleotides. Prior to the onset of this study, little information could be obtained from the literature concerning the effects of substrate analogues upon the initial rates of RNase U_2 catalysis, although the binding affinity of various nucleosides and mononucleotides was studied by the method of gel filtration (Sato & Uchida, 1975d). The complex formation of RNase T_1 with a number of competitive inhibitors has been previously studied (Walz & Hooverman, 1973; Walz, 1977). Values for K_i and $\Delta G°$ ($= -RT \ln K_i$), obtained for RNase U_2 at pH 4.5 and 25 °C, and ionic strength 0.1 are compiled in Table V (values of K_{mi} and $\Delta G°$ for cosubstrates, Ap(3')Me, Ap(3')Bzl, and Ap(3')Naph, are also included for comparison). The inhibitory power decreased in the order 2'-AMP > 3'-AMP > 5'-AMP ~ 5'-dAMP > Ado > dAdo. A similar trend was also demonstrated by Walz & Hooverman (1973) for RNase T_1: 2'-GMP > 3'-GMP > 5'-GMP ~5'-dGMP > Guo > dGuo. The 2'-phosphate group has a large binding-enhancing effect, but Ap(2')Naph and Ap(2'→5')A inhibit less strongly the RNase U_2 catalyzed reaction of ApUp as compared with 2'-AMP. This is also in qualitative agreement with general experience of the behavior of RNase T_1 (White et al., 1977b) and RNase A (White et al., 1977a), and the results shown in Table V indicate that the proton-associated form of the secondary phosphate must be important for high binding affinity. The inhibitory power of 3'-phosphomono- and 3'-phosphodiester groups was found to be almost identical.

Table V: Inhibition Constants (Shown as $1/K_i$ or $1/K_{mi}$) and Standard Free Energy of Binding ($\Delta G°$) of Some Inhibitors and Cosubstrates for RNase U_2 at pH 4.5 and 25 °C

compd	$1/10K_i$ or $1/10K_{mi}$ (M^{-1})	$-\Delta G°$ (kcal/mol)
dAdo	150 ± 12	4.3 ± 0.17
d(pA)	410 ± 30	4.9 ± 0.20
d(pAp)	1590 ± 120	5.7 ± 0.20
d(ApA)	780 ± 60	5.3 ± 0.20
d(pApAp)	1430 ± 100	5.7 ± 0.22
Ado	220 ± 18	4.6 ± 0.20
Ap(2')	8300 ± 650	6.7 ± 0.25
Ap(3')	620 ± 50	5.2 ± 0.20
pA	370 ± 30	4.9 ± 0.20
Ap(2')Naph	390 ± 30	4.9 ± 0.20
Ap(2'→5')A	600 ± 50	5.2 ± 0.20
Ap(3')Me	790 ± 60	5.3 ± 0.20
Ap(3')Bzl	810 ± 60	5.3 ± 0.20
Ap(3')Naph	470 ± 30	5.0 ± 0.20

Table V shows that Ado is a better inhibitor than dAdo, and the 2'-hydroxyl group contributes to favorable binding free energy by 0.24 kcal/mol ($\Delta\Delta G°$) for Ado relative to dAdo. Similar and even a more marked effect ($\Delta\Delta G° = 1.11$ kcal/mol) was reported for the inhibitors (Guo vs. dGuo) toward RNase T_1 reaction (Walz & Hooverman, 1973). The differences in $\Delta G°$ observed for 3'-AMP/Ado, 5'-AMP/Ado, d(pAp)/5'-dAMP, and 5'-dAMP/dAdo are 0.60, 0.30, 0.81, and 0.59, respectively, showing that $\Delta\Delta G°$s gained by the addition of a phosphate group are slightly but definitely greater for deoxy compounds than for ribo compounds. This is again explained by the fact that the lack of the 2'-hydroxyl group of deoxyribonucleotides would not restrict the possible change of the binding configuration at the enzyme active site on going from 5'-dAMP to d(pAp) or from dAdo to 5'-dAMP, thus allowing the best fit in the inhibitor–enzyme interaction at the binding locus. A similar inhibitory behavior has also been demonstrated earlier for RNase T_1 by Walz & Hooverman (1973), suggesting RNase U_2 and RNase T_1 are qualitatively quite similar from the standpoint of the mode of inhibitor binding.

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