Energetics of Catalysis by Ribonucleases: Fate of the 2′,3′-Cyclic Phosphodiester Intermediate†

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ABSTRACT: Ribonucleases catalyze the hydrolysis of the P–$O^{5'}$ bond in RNA. This reaction occurs in two steps: transphosphorylation of RNA to a 2′,3′-cyclic phosphodiester intermediate and hydrolysis of this intermediate to a 3′-phosphomonoester. ^{31}P NMR spectroscopy was used to monitor the accumulation of the 2′,3′-cyclic phosphodiester intermediate during the transphosphorylation and hydrolysis reactions catalyzed by various ribonucleases and by small molecules. The intermediate was found to accumulate during catalysis by monomeric bovine pancreatic ribonuclease A (RNase A), a dimer and a trimer of RNase A, bovine seminal ribonuclease, RNase T_1, barnase, and RNase I. These enzymes, which are of widely disparate phylogenetic origin, released rather than hydrolyzed most of the intermediate formed by transphosphorylation of RNA. In contrast, the intermediate did not accumulate during catalysis by hydroxide ion or imidazole buffer. In the presence of these small molecules, hydrolysis is faster than transphosphorylation. A trapping experiment was used to assess the throughput of the reaction catalyzed by RNase A. [5,6-^{3}H]Uridylyl-(3′→5′)adenosine was incubated with RNase A in the presence of excess unlabeled uridine 2′,3′-cyclic phosphodiester, which dilutes the specific radioactivity of any released cyclic intermediate. Only 0.1% of the RNA substrate was found to be both transphosphorylated and hydrolyzed without dissociating from the enzyme. These results suggest that ribonucleases have evolved primarily to catalyze RNA transphosphorylation and not RNA hydrolysis.

The RNA produced by cellular transcription is degraded by the action of ribonucleases. These enzymes catalyze the hydrolysis of the P–$O^{5'}$ bond of RNA. This hydrolysis reaction proceeds in two steps: transphosphorylation of RNA to form a 2′,3′-cyclic phosphodiester (N>p) intermediate and hydrolysis of this cyclic intermediate to form a 3′-phosphomonoester (Np) (Markham & Smith, 1952; Brown & Todd, 1953), as shown in Figure 1.

Almost 30 years ago, Westheimer and co-workers determined the relative hydrolysis rates of two simple phosphodiesters, each having P–O bonds that mimic those cleaved by nucleases. These phosphodiesters were dimethyl phosphate (which is the simplest acyclic phosphodiester) and ethylene phosphate (which is a simple cyclic phosphodiester) (Kumamoto et al., 1956). After correction for C–O bond cleavage, P–O bond cleavage was observed to occur 10^8-fold faster in the cyclic diester than in the acyclic diester (Haake & Westheimer, 1961). Westheimer (Westheimer, 1968) and others (Gillespie et al., 1973; Kluger & Taylor, 1990) have attributed this difference to the relief of strain in the trigonal bipyramidal transition state for the hydrolysis of the cyclic diester. Recently, this explanation was challenged by Dejaegere and Karplus (1993), who calculated that most of the rate difference observed by Westheimer resulted from efficient solvation of the transition state for the hydrolysis of ethylene phosphate. Nevertheless, the experimental results of Westheimer are not in dispute.

The instability of ethylene phosphate to hydrolysis suggests that the second step in Figure 1 is also likely to occur quickly. Biochemists often presume that the product of the reaction catalyzed by ribonucleases is a 3′-phosphomonoester, as expected if the second step in Figure 1 were indeed faster than the first. This presumption, which is critical to understanding the mechanism and energetics of catalysis by ribonucleases, may be incorrect (Cuchillo et al., 1993). We report here on the fate of the cyclic intermediate during the hydrolysis of RNA as catalyzed by a variety of ribonucleases. Results from the enzyme-catalyzed reactions are compared to those from the non-enzymatic hydrolysis of RNA. In addition, we have determined the fraction of the cyclic intermediate that remains bound to bovine pancreatic ribonuclease A (RNase A;[1] E.C. 3.1.27.5) through both steps of Figure 1. Together, these results illuminate the energetics of catalysis by ribonucleases.

EXPERIMENTAL PROCEDURES

Materials. Poly(U), UpA, U>p, Up, yeast RNA, and salmon sperm DNA were from Sigma Chemical Co. (St. Louis, MO). Polymeric substrates were precipitated from aqueous ethanol (70%, v/v) before use. UpA was also synthesized by using the methods of Ogilvie (Ogilvie et al., 1978) and Caruthers (Beaucage & Caruthers, 1981). [5,6-^{3}H]UTP was from Amersham (Arlington Heights, IL).

RNase A (type X-A) was from Sigma Chemical Co. A dimer and a trimer of RNase A were prepared by lyophilization of RNase A from aqueous acetic acid (50%, v/v) and purified

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[1] Abbreviations: NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; PEI, poly(ethylenimine); poly(A), polyadenylic acid; poly(U), polyuridylic acid; RNase A, bovine pancreatic ribonuclease A; TLC, thin-layer chromatography; UpA, uridylyl(3′→5′)adenosine; U>p, uridine 2′,3′-cyclic phosphate; Up, uridine 3′-phosphate.

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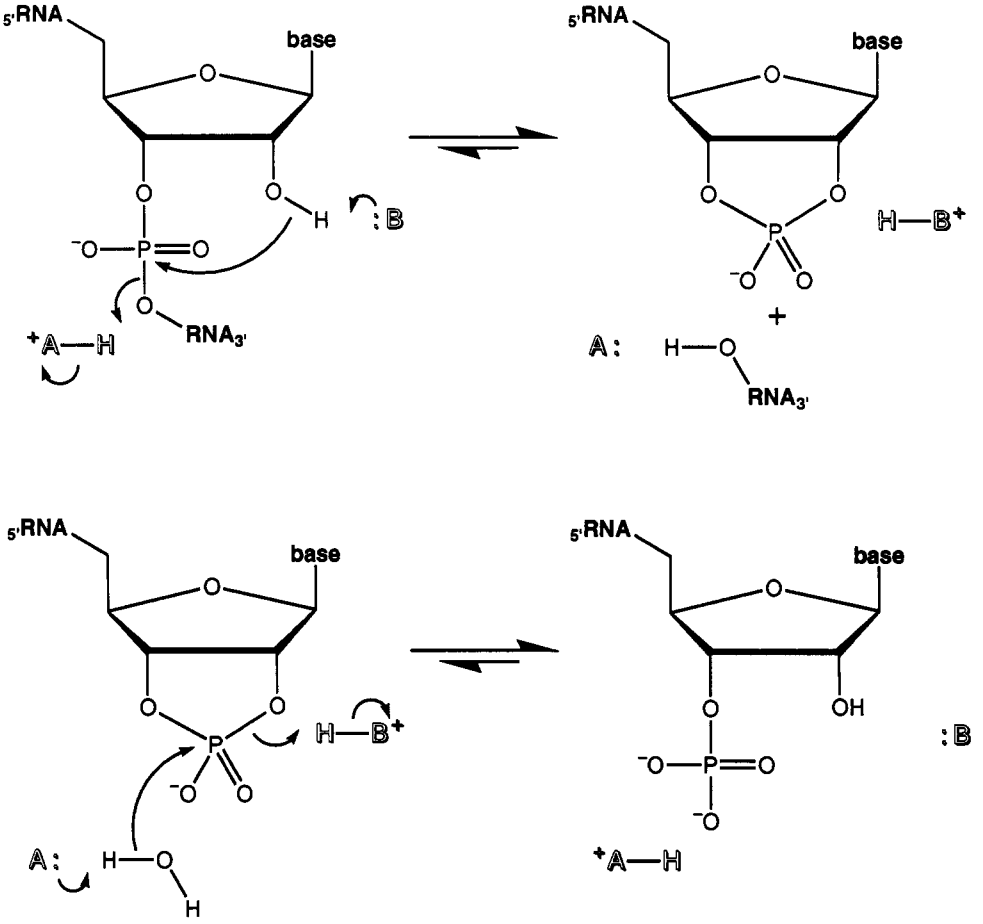


FIGURE 1: Transphosphorylation (top) and hydrolysis (bottom) reactions catalyzed by ribonucleases. In RNase A, B is His12, and A is His119.

by gel filtration through Sephadex G-75 resin (Crestfield et al., 1962; Kim & Raines, 1993). Dimeric bovine seminal ribonuclease was prepared from bovine seminal plasma (Tamburrini et al., 1986; Kim & Raines, 1993). Barnase (Hartley, 1993) and staphylococcal nuclease (Serpersu et al., 1986) were prepared from *Escherichia coli* cells expressing genes that code for these enzymes. RNase T_1 was from Boehringer Mannheim (Indianapolis, IN). RNase I and *Xho*I restriction endonuclease were from Promega (Madison, WI). RNase U_2 and alkaline phosphatase were from United States Biochemical (Cleveland, OH).

General Methods. Ultraviolet absorbance measurements were made on a Cary Model 3 spectrophotometer equipped with a Cary temperature controller. Substrate concentrations were determined by ultraviolet absorption using ϵ_{260} = 24 600 M^{-1} cm^{-1} at pH 7.0 for UpA (Warshaw & Tinoco, 1966), ϵ_{261} = 9430 M^{-1} cm^{-1} at pH 7.5 for poly(U) (Yakovlev et al., 1992), ϵ_{260} = 9090 M^{-1} cm^{-1} for yeast RNA, and ϵ_{260} = 10 290 M^{-1} cm^{-1} for DNA at pH 7.0 (Ausubel et al., 1989) and are given in terms of the molarity of P–$O^{5'}$ bonds.

DNA oligonucleotiodes were synthesized on an Applied Biosystems Model 392 DNA/RNA synthesizer by using the β-cyanoethylphosphoramidite method (Sinha et al., 1984) and reagents from Applied Biosystems (Foster City, CA), except for acetonitrile, which was from Bater Healthcare (McGaw Park, IL). Run-off transcriptions were performed with the Mega Shortscript Kit (Ambion, Austin, TX). The PCR and other manipulations of DNA were performed as described (Ausubel et al., 1989).

Reactions during preparation and use of [5,6-^{3}H]UpA were monitored by TLC on PEI–cellulose plates (Alltech, Waukegan, IL). Mobile phases were 0.5 M LiCl, system A; 0.5 M ammonium bicarbonate, system B; or saturated ammonium sulfate, pH 3.5, system C (Bochner & Ames, 1982). Elution profiles from TLC were constructed by detection of the ^{3}H using a Bioscan System 200 imaging scanner (Bioscan, Washington, DC). Samples for radiochemical analysis were dissolved in Biosafe scintillation counting fluid (Research Products International, Mount Prospect, IL) and counted with a TriCarb 1900CA liquid scintillation analyzer (Packard, Meridan, CT). HPLC was performed with a Waters instrument equipped with a Novapak C-8 reverse-phase column.

^{31}P NMR Analysis. ^{31}P NMR spectroscopy was used to assess the kinetics of the hydrolysis of RNA as catalyzed by various ribonucleases, staphylococcal nuclease, hydroxide ion, or imidazole buffer. In each reaction, the concentration of P–$O^{5'}$ bonds was 3 mM [for poly(U) or UpA] or 10 mM (for yeast RNA).

Reactions catalyzed by ribonucleases were performed at 25 °C in 0.10 M imidazole·HCl buffer, pH 7.0, containing NaCl (0.20 M). These reactions had poly(U) as the substrate for RNase A, dimeric RNase A, trimeric RNase A, or dimeric bovine seminal ribonuclease; yeast RNA as the substrate for RNase T_1, barnase, or RNase I; and UpA as the substrate for RNase A. The hydrolysis of poly(U) by staphylococcal nuclease was performed in 0.10 M imidazole·HCl buffer, pH 7.0, containing NaCl (0.20 M) and $CaCl_2$ (0.10 M). In each reaction, the concentration of enzyme was such that essentially all of the substrate was transphosphorylated in 1 h.

Reactions catalyzed by small molecules were performed as follows. The hydrolysis of poly(U) by hydroxide ion was performed for 1 h at 25 °C in 0.2 M NaOH. As a control, DNA was also incubated under these conditions. The hydrolysis of poly(U) by imidazole buffer was performed for

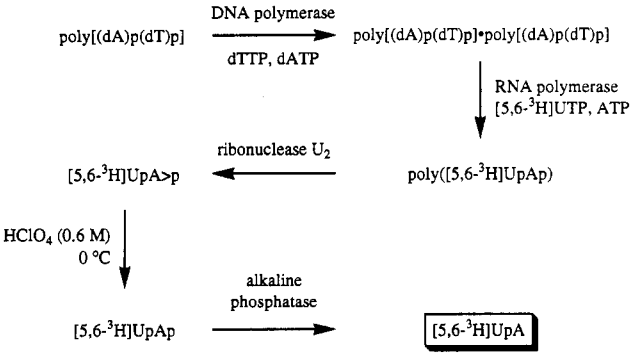


FIGURE 2: Scheme for the preparation of [5,6-^{3}H]UpA.

1 h at 70 °C in 2.5 M imidazole·HCl buffer, pH 7.0. [Imidazole buffer has been shown to catalyze the transphosphorylation of RNA (Breslow & Labelle, 1986) and the hydrolysis of ethylene phosphate (Covitz & Westheimer, 1963).] Poly(U) was also incubated at 70 °C in 2.5 M NaCl, as a control.

^{31}P NMR spectra were obtained at 161.972 MHz in Fourier transform mode on a Bruker AM400 spectrometer with the following parameters: pulse width, 13°; acquisition time, 0.28 s; relaxation delay, 3.2 s; line broadening, 5–10 Hz; number of scans, 64–100 when following a reaction, 200 when analyzing a static sample. Reactions were carried out in 10-mm NMR tubes with inserts containing D_2O. Chemical shifts were recorded relative to 0.3 M H_3PO_4.

Synthesis of [5,6 -^{3}H]UpA. [5,6-^{3}H]UpA was prepared by a combination of enzymatic and chemical methods, as shown in Figure 2. The template for transcription was generated by the PCR using standard conditions (Ausubel et al., 1989). Oligonucleotides ATACCGTCGACCTCGAG and CAGTGAGCGCGCGTAAT were used to generate a complementary strand of oligonucleotide CAGTGAGCGCGCGTAATACGACTCACTATAGGGATATATATATATATATATATATATATATATATACTCGAGGTCGACGGTAT [which contains a T7 RNA polymerase promoter, [(dA)p(dT)p]$_{16}$, and the recognition sequence for *Xho*I (underlined)] and to amplify the resulting duplex. The product from the PCR was digested with *Xho*I, and the resulting duplex was extracted with phenol:chloroform (1:1, v/v) to remove proteins and precipitated with aqueous EtOH (70%, v/v). This duplex (which contains [(dA)p(dT)p]$_{16}$·[(dA)p(dT)p]$_{16}$) served as a template for run-off transcription.

RNA containing ([5,6-^{3}H]UpAp)$_{16}$ was prepared by run-off transcription of the duplex template in the presence of [5,6-^{3}H]UTP. Transcription reactions were performed in solutions containing [5,6-^{3}H]UTP (50 μCi, 48 Ci/mmol; R_f 0.33, system B) and unlabeled ATP (0.5 mM), CTP (0.5 mM), GTP (0.5 mM), and UTP (12 μM). The resulting RNA (R_f 0.0, system A or B) was transphosphorylated with RNase U_2 (which cleaves RNA only after adenine residues) to yield [5,6-^{3}H]UpA>p (R_f 0.58, system A). [5,6-^{3}H]-UpA>p was hydrolyzed by treatment with 0.6 M $HClO_4$ for 45 min (Shapiro et al., 1986). Precipitate in this solution was removed by centrifugation, and the resulting supernatant was neutralized with saturated $NaHCO_3$ to yield a solution of [5,6-^{3}H]UpAp (R_f 0.25, system A). [5,6-^{3}H]UpAp was hydrolyzed with alkaline phosphatase (which cleaves only phosphomonoesters) to yield [5,6-^{3}H]UpA (R_f 0.56, system A).

[5,6-^{3}H]UpA was purified by extraction with phenol:chloroform (1:1, v/v, to remove proteins) and then by reverse-phase HPLC. The impure [5,6-^{3}H]UpA (0.27 μCi, 38 Ci/mmol) was mixed with a small amount of the unlabeled carriers UpA, U>p, and adenosine. The resulting mixture was injected at a flow rate of 0.8 mL/min onto an HPLC C-8 reverse-phase column that had been equilibrated with 0.1 M sodium phosphate buffer, pH 6.0. The loaded column was eluted first with 0.1 M sodium phosphate buffer, pH 6.0 (6.4 mL); then with a linear gradient (0.8 mL + 0.8 mL) of 0.1 M sodium phosphate buffer, pH 6.0, to aqueous methanol (10%, v/v); and finally with aqueous methanol (10%, v/v, 8.0 mL). Under these conditions, U>p eluted with the void volume, adenosine eluted at 12 mL, and [5,6-^{3}H]UpA eluted at 12.6 mL. The [5,6-^{3}H]UpA (0.27 μCi, 38 mCi/mmol) was collected, and solvent was removed under vacuum in a centrifuge. The purity of [5,6-^{3}H]UpA was assessed by TLC before and after its treatment with RNase A.

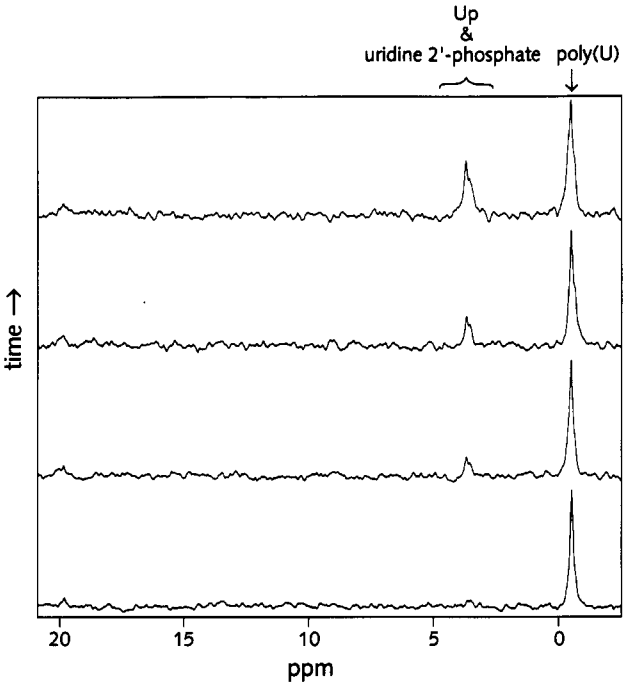


FIGURE 3: ^{31}P NMR spectra showing the time course of the hydrolysis of poly(U) by hydroxide ion. Spectra for the reaction catalyzed by imidazole buffer are similar.

Throughput Experiment. A sample (0.27 μCi, 38 mCi/mmol) of [5,6-^{3}H]UpA was dissolved in 0.1 M imidazole·HCl buffer, pH 7.0 (14 μL), containing U>p (5 mM). RNase A (1 ng in 1 μL of 0.1 M imidazole·HCl buffer, pH 7.0) was then added. At appropriate time intervals, portions (1.0 μL) were withdrawn, spotted onto PEI–cellulose plates, and dried. The plates were developed with system C, and elution profiles were constructed with the Bioscan scanner. The regions of the plates corresponding to UpA, U>p, and Up were excised, and the ^{3}H in each of these regions was quantitated by scintillation analysis.

RESULTS

^{31}P NMR Analysis. The phosphorous atoms in the three species (NpN, N>p, and Np) present during the ribonuclease-catalyzed hydrolysis of RNA had distinct chemical shifts: –0.5 ppm for NpN, 19.1 ppm for N>p, and 3.4 ppm for Np. Hence, ^{31}P NMR spectroscopy can be used to follow the time course for the hydrolysis of RNA (Cozzone & Jardetzky, 1977; delCardayré et al., 1994; delCardayré & Raines, 1994).

The time course for the hydrolysis of poly(U) as catalyzed by hydroxide ion showed that poly(U) was converted to Up without the accumulation of U>p (Figure 3). The reaction catalyzed by imidazole buffer (data not shown) gave a similar

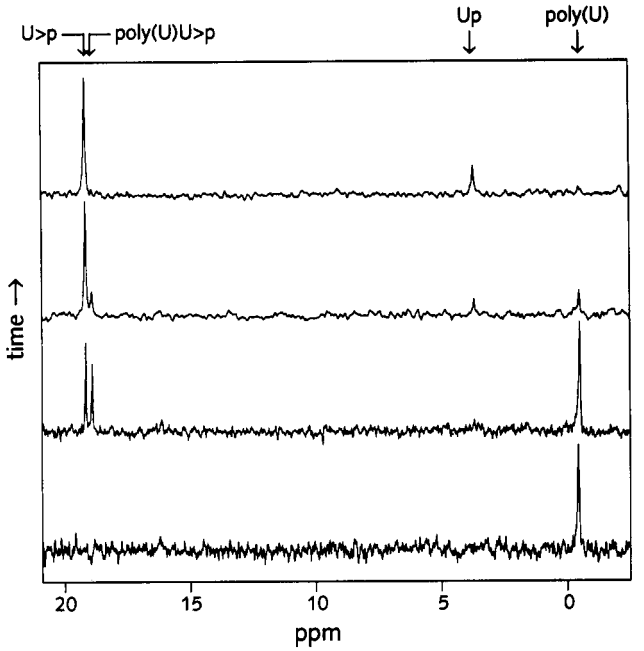


FIGURE 4: ^{31}P NMR spectra showing the time course of the hydrolysis of poly(U) by RNase A. Spectra for the reactions catalyzed by other ribonucleases are similar.

result. In contrast to these non-enzymatic reactions, during the RNase A-catalyzed hydrolysis of poly(U), a large fraction of the poly(U) was converted to U>p before any Up was detected (Figure 4). At short times, the U>p produced was heterogeneous, as evidenced by the multiple resonances between δ 19.1 and 19.5. The downfield resonance has a chemical shift identical to that of authentic U>p. The upfield resonance is likely to be from the cyclic phosphodiester in poly(U)U>p [that is, U>p at the 3′-end of poly(U)], which has a more shielded phosphorus atom. At long times, the poly(U)U>p was converted to U>p, which was eventually hydrolyzed to Up. The reactions catalyzed by dimeric RNase A, trimeric RNase A, dimeric bovine seminal ribonuclease, RNase T_1, barnase, and RNase I gave time courses (data not shown) similar to that shown in Figure 4. The RNase A-catalyzed hydrolysis of UpA showed complete conversion of UpA to U>p before any Up was detected (data not shown). No 2′,3′-cyclic phosphodiester intermediate was detected in RNA hydrolysis catalyzed by staphylcoccal nuclease, which is believed not to produce such an intermediate (Cotton et al., 1979). No poly(U) cleavage was detected after 2 days in 2.5 M NaCl at 70 °C, nor was any DNA cleavage detected after 1 h in 0.2 M NaOH at 25 °C.

Synthesis of [5,6-^{3}H]UpA. The synthesis of [5,6-^{3}H]-UpA was successful by the following criteria. Synthetic [5,6-^{3}H]UpA was a single peak by PEI–cellulose TLC (R_f 0.04, system C). Treatment of this material with RNase A produced initially a radiolabeled product that comigrated during TLC with U>p (R_f 0.37, system C). This material was eventually converted to a product that comigrated during TLC with Up (R_f 0.67, system C).

Throughput Experiment. RNase A catalyzes the transphosphorylation of [5,6-^{3}H]UpA to form labeled U>p and unlabeled A. The resulting enzyme-bound U>p can have two fates: release from the enzyme into solvent or hydrolysis by the enzyme to form Up. The throughput experiment was designed to measure the partitioning between these two possible fates. In this experiment, RNase A catalyzes the cleavage of labeled UpA in the presence of a large pool of unlabeled U>p. This large pool effectively traps any labeled U>p released by

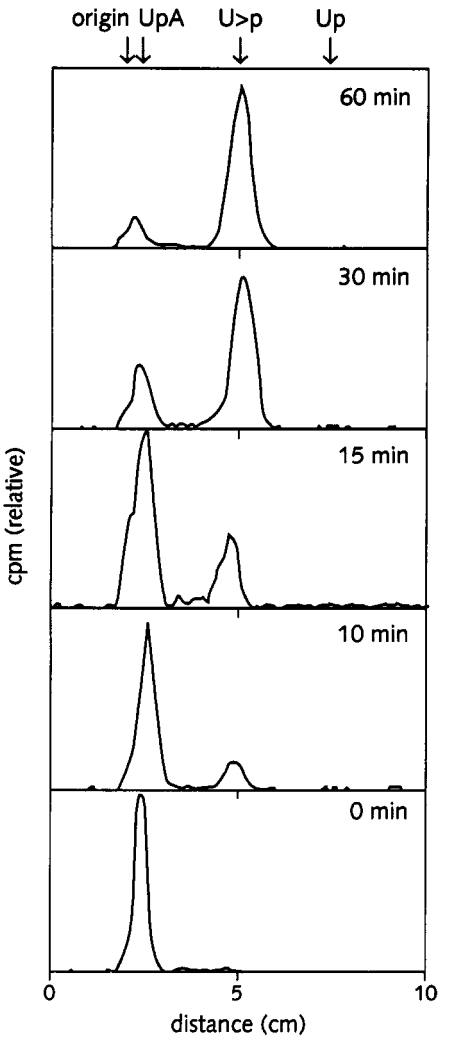
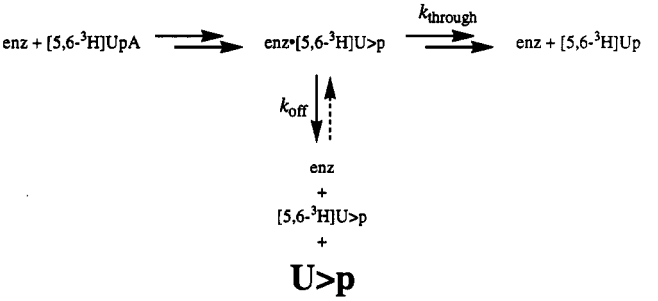


FIGURE 5: Elution profiles after PEI–cellulose TLC showing the time course of the hydrolysis of [5,6-^{3}H]UpA by RNase A.

Scheme 1



the enzyme by diluting its specific radioactivity. Rebinding and hydrolysis of labeled U>p therefore becomes insignificant. Under these conditions, labeled Up will only be formed if labeled U>p (from transphosphorylation of labeled UpA) suffers hydrolysis rather than release to solvent, as shown in Scheme 1.

Elution profiles for the throughput experiment are shown in Figure 5, and the resulting radiochemical analyses are shown in the inset of Figure 6. A plot of the time course of the labeled Up:labeled U>p ratio is also shown in Figure 6. The shape of this plot reports on the ability of unlabeled U>p to trap any labeled U>p that is released during catalysis. Complete trapping would yield a line of slope zero. Since the labeled Up:labeled U>p ratio increased only slowly during the time course of the experiment, the pool of unlabeled U>p

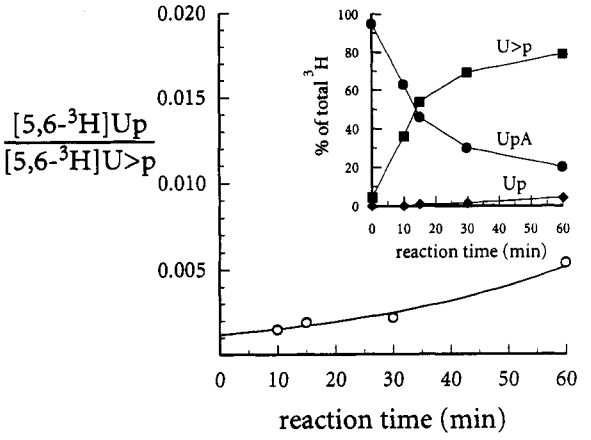


FIGURE 6: Throughput experiment for the hydrolysis of [5,6-^{3}H]-UpA by RNase A: time course of the ratio of radioactivity found in U>p to that found in Up. Inset: Percent of total ^{3}H found in UpA (●), U>p (■), and Up (◆) from radiochemical analysis of elution profiles shown in Figure 5.

was apparently effective at competing with labeled U>p for rebinding to the enzyme.

The *y*-intercept of the plot in Figure 6 describes the fate of the U>p produced during the initial turnover of UpA by RNase A. Any labeled Up formed during this turnover can only derive from the throughput of labeled UpA, since no labeled U>p is present. In theory, the intercept in Figure 6 can range from zero (for no throughput) to infinity (for complete throughput). Although the intercept is unaffected by the degree of trapping, it can be estimated more accurately if the trap is effective so that the labeled Up:labeled U>p ratio is constant. An exponential fit of the data in Figure 6 gives an intercept of approximately 1×10^{-3}, which indicates that labeled U>p was released by RNase A 1×10^3 times more often than it was hydrolyzed. A control reaction containing no RNase A was unchanged over the time course of the experiment.

DISCUSSION

Ribonucleases catalyze the in vivo maturation and catabolism of various types of RNA. We are interested in understanding the molecular basis for the rate enhancements mediated by these enzymes, particularly RNase A. This understanding requires detailed knowledge of the mechanism and energetics of phosphodiester cleavage.

Many important advances in phosphodiester chemistry resulted from seminal studies by Westheimer (Westheimer, 1968). Of particular interest to us was his discovery that the non-enzymatic hydrolysis of ethylene phosphate is faster than that of dimethyl phosphate. Each of these hydrolysis reactions is intermolecular. In contrast, RNA is hydrolyzed in two steps, the first of which is intramolecular (Figure 1). The proximity of the 2′-OH groups of RNA to its phosphorus atoms is likely to accelerate the cleavage of the P–O$^{5'}$ bonds.

We have developed a facile ^{31}P NMR assay to monitor simultaneously both steps in Figure 1 and thereby assess which of these two steps limits the rate of hydrolysis of the P–O$^{5'}$ bond of RNA. In imidazole buffer or at high pH, transphosphorylation limits this rate. Under these conditions, the intermolecular reaction of water and a cyclic phosphodiester occurs faster than does the intramolecular transphosphorylation of RNA, and the cyclic phosphodiester does not accumulate (Figure 3). The data from the imidazole-catalyzed reaction are particularly salient because the imidazole groups of two histidine residues act as the general acid–base catalysts in the active site of RNase A (Thompson & Raines, 1994).

The ^{31}P NMR assay also enabled us to determine the relative rates for the intermolecular and intramolecular reaction as they occur in the active sites of a wide variety of ribonucleases. In each of the ribonuclease-catalyzed reactions studied here, most of the cyclic phosphodiester generated by the transphosphorylation of RNA is released, rather than remaining bound to the enzyme to suffer hydrolysis. Further, the extensive accumulation of this intermediate indicates that the value of k_{cat}/K_m for the transphosphorylation of RNA is greater than that for the hydrolysis of the 2′,3′-cyclic phosphodiester intermediate (Raines & Hansen, 1988). For RNase A, this finding is consistent with steady-state kinetic parameters. Under the conditions used here, the value of k_{cat}/K_m (=2.3 $\times 10^6$ M^{-1} s^{-1}; delCardayré & Raines, 1994) for transphosphorylation of UpA is 10^3-fold higher than that (=1.7 $\times 10^3$ M^{-1} s^{-1}; D. J. Quirk and R. T. Raines, unpublished results) for hydrolysis of U>p. As predicted from these values, little Up is detected by ^{31}P NMR spectroscopy (data not shown) or TLC (Figure 5 and 6) until all of the UpA has been converted to U>p. In contrast, Up is detected before poly(U) is cleaved completely by RNase A (Figure 4). This result is also consistent with steady-state parameters, since the value of k_{cat}/K_m (=3.88 $\times 10^4$ M^{-1} s^{-1}; Hammes & Rossario, 1969) for the transphosphorylation of UpU is only 10-fold higher than that for the hydrolysis of U>p.

Studies relying on product isolation have demonstrated that ribonucleases can release 2′,3′-cyclic phosphodiesters (Cuchillo et al., 1993). For example, Markham and Smith (1952) were able to isolate cyclic phosphodiesters from yeast RNA exposed to RNase A. Also, Cozzone and Jardetzky (1977) used ^{31}P NMR spectroscopy to monitor the slow transphosphorylation of poly(A) by RNase A to form products having cyclic phosphodiesters. Recently, Fersht and co-workers showed that catalysis by barnase yields product mixtures containing only cyclic phosphodiesters (Day et al., 1992). The results of our ^{31}P NMR studies suggest that *all* ribonucleases may release cyclic phosphodiesters as products, and that these products rebind to the enzyme and are hydrolyzed to phosphomonoesters in a separate, slower step.

We devised the throughput experiment to extend the understanding provided by the ^{31}P NMR assay. The throughput experiment provides a quantitative measure of the rate at which the cyclic phosphodiester intermediate is released (k_{off}) compared to that at which it is hydrolyzed ($k_{through}$). This experiment is powerful because $k_{through}/k_{off}$ is obtained for a ribonuclease molecule that has just transphosphorylated an RNA molecule (Figure 1, top). This ratio cannot be deduced from the steady-state kinetic parameters alone. The value of $(k_{through}/k_{off})_{t=0} = 1 \times 10^{-3}$ obtained from Figure 6 indicates that after RNase A catalyzes the transphosphorylation of UpA, the resulting U>p dissociates from the enzyme 1×10^3 times for each time that it remains bound to the enzyme and is hydrolyzed. According to Scheme 1, $k_{through} = k_{cat}^{U>p}$ (=4.4 s^{-1}; D. J. Quirk and R. T. Raines, unpublished results). Since $k_{through}/k_{off} = 1 \times 10^{-3}$, the value of k_{off} is 4×10^3 s^{-1}, which is close to that of k_{cat}^{UpA} (=1.4 $\times 10^3$ s^{-1}; delCardayré & Raines, 1994).[2] Thus, the release of U>p may partially limit the rate of turnover of UpA by RNase A.

The results of the throughput experiment have implications for the mechanism of the reaction catalyzed by RNase A. The active sites of ribonucleases typically contain two residues with side chains that can function as general acid–base catalysts. In RNase A, these residues are His12 and His119

[2] Under different conditions, Erman and Hammes (1966) found k_{off} = 1×10^4 or 2×10^4 s^{-1}.

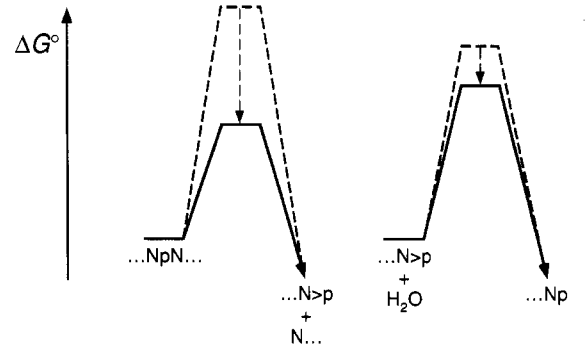


FIGURE 7: Free energy profiles for the reactions in Figure 1. The profiles portray the relative free energy barriers for the ribonuclease-catalyzed (—) or non-enzymatic (- - -) transphosphorylation (left) and hydrolysis (right) reactions. Dashed arrows depict the catalysis effected by ribonucleases.

(Thompson & Raines, 1994). The imidazole group of His12 acts as a general base in the transphosphorylation reaction and a general acid in the hydrolysis reaction. The imidazole group of His119 has a complementary role, acting as a general acid in the transphosphorylation reaction and a general base in the hydrolysis reaction.[3] After catalysis of transphosphorylation, each histidine residue in the active site of RNase A is protonated appropriately for catalysis of hydrolysis of the bound cyclic phosphodiester intermediate. After hydrolysis of this substrate, each histidine residue is returned to its initial protonation state, completing the catalytic cycle (Figure 1). Apparently, RNase A interrupts this cycle by releasing rather than hydrolyzing the cyclic intermediate. Thus, RNase A (and perhaps other ribonucleases) has an iso mechanism (Medwedew, 1937) in which protonation states of the unliganded enzyme are interconverted by a pathway that does not involve substrate molecules.[4]

We conclude that the step in Figure 1 that limits the rate of the ribonuclease-catalyzed hydrolysis of RNA is different from that that limits the rate of the non-enzymatic hydrolysis of RNA. This difference is illustrated in the free energy profiles shown in Figure 7. To draw these profiles, we have assumed that the value of k_{cat}/K_m for the hydrolysis reaction is limited by the chemical interconversion of the enzyme-bound substrates (Figure 1, bottom) rather than by the diffusion together or apart of the enzyme and its substrate. This assumption is correct for RNase A (Eftink & Biltonen, 1983a,b,c).

The profiles in Figure 7 are surprising for several reasons. First, binding energy can be used to eliminate translational and rotational degrees of freedom and thereby to effect catalysis (Hansen & Raines, 1990). By this criterion, the intermolecular reaction of water and the cyclic phosphodiester is a better candidate for an enzymatic rate acceleration than is the intramolecular transphosphorylation of RNA. Second, after catalyzing a transphosphorylation reaction, the cyclic intermediate is bound in an active site in which His12 is protonated and His119 is unprotonated (Figure 1), leaving RNase A poised to catalyze a hydrolysis reaction. Finally, the catalytic effectiveness of enzymes can evolve (Burbaum et al., 1989). For example, the rate enhancement for the RNase A-catalyzed hydrolysis of C>p is 10^{10}-fold (Eftink & Biltonen, 1983b). Yet, none of the ribonucleases studied here (which are of widely disparate phylogenetic origin[5]) have evolved to catalyze the hydrolysis of the 2′,3′-cyclic phosphodiester intermediate as effectively as they catalyze the transphosphorylation of RNA.

A structural feature of RNase A and other ribonucleases is consistent with the idea that the primary role of these enzymes is to catalyze RNA transphosphorylation. This feature is the enzymic subsites that bind to portions of RNA flanking the scissile P–O5′ bond (Parés et al., 1991; delCardayré & Raines, 1994). These subsites affect the ability of the enzyme to catalyze the transphosphorylation of different phosphodiesters. For example, the values of k_{cat} for the transphosphorylation of CpA, CpU, and CpOMe by RNase A are 3000, 27, and 0.5 s^{-1}, respectively (Witzel & Barnard, 1962). These differences exist even though the leaving groups in these substrates have conjugate acids with similar values of pK_a.[6] These data suggest that catalysis by RNase A is enhanced by subsite interactions that orient a bound substrate favorably for transphosphorylation. The absence of similar interactions between water and enzymic residues may be the reason that the hydrolysis step is relatively slow (Cuchillo et al., 1993).

To cleave RNA, ribonucleases do not require water as a substrate. If the primary role of ribonucleases is indeed to cleave RNA and thereby simply commit it to catabolism [rather than to hydrolyze it, as has been suggested (Barnard, 1969)], then these enzymes may not have been subjected to selective pressure to bind substrate water effectively. In contrast, enzymes that catalyze the cleavage of proteins, DNA, or carbohydrates require water as a substrate. These enzymes may therefore be more likely than ribonucleases to bind water optimally for catalysis.

[3] In unliganded RNase A, the pK_a of His12 is 5.8, and the pK_a of His119 is 6.2 (Markley, 1975). This difference in pK_a is maintained in various RNase A·ligand complexes (Blackburn & Moore, 1982). Thus at all values of pH, more enzyme is in the proper protonation state for catalysis of transphosphorylation (B: and A–H^+ in Figure 1) than for catalysis of hydrolysis (B–H^+ and A:). Steady-state kinetic parameters therefore underestimate the efficiency of RNase A as a catalyst of hydrolysis, but not by orders of magnitude.

[4] The interconversion of unliganded enzyme forms can limit the rate of an enzyme-catalyzed reaction (Raines & Knowles, 1987; Rebholz & Northrop, 1994). This situation is evidenced by noncompetitive inhibition by the reaction product (Northrop & Rebholz, 1994). Detailed analyses of progress curves for the RNase A-catalyzed transphosphorylation of UpA or hydrolysis of U>p showed that product inhibition was competitive (K. L. Rebholz, J. E. Thompson, R. T. Raines, and D. B. Northrop, unpublished results). Apparently, the interconversion of unliganded enzyme forms does not limit the ability of RNase A to catalyze either reaction in Figure 1.

[5] *Aspergillus oryzae* (RNase T_1), *Bacillus amyloliquefaciens* (barnase), *Bos taurus* (bovine seminal ribonuclease and RNase A), and *Escherichia coli* (RNase I).

[6] $CH_3OCH_2CH_2OH$ (which has a hydroxyl group analogous to that of A or U) has pK_a = 14.8, and MeOH has pK_a = 15.5 (Ballinger & Long, 1960).

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