Extending the Limits to Enzymatic Catalysis: Diffusion of Ribonuclease A in One Dimension†

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ABSTRACT: Bovine pancreatic ribonuclease A (RNase A) is a distributive endoribonuclease that catalyzes the cleavage of the P−$O^{5'}$ bond of RNA on the 3′ side of pyrimidine residues. Here, RNase A is shown to cleave the P−$O^{5'}$ bond of a pyrimidine ribonucleotide faster when the substrate is embedded within a longer tract of poly(adenylic acid) [poly(A)] or poly(deoxyadenylic acid) [poly(dA)]. These data indicate that a ribonuclease can diffuse in one dimension along a single-stranded nucleic acid. This facilitated diffusion is mediated by Coulombic interactions, as the extent is diminished by the addition of NaCl. RNase A is more effective at cleaving a pyrimidine ribonucleotide embedded within a poly(dA) tract than within a poly(deoxycytidylic acid) [poly(dC)] tract. T45G RNase A, which catalyzes the processive cleavage of poly(A) but the distributive cleavage of poly(cytidylic acid) [poly(C)], has the same preference. Apparently, processive catalysis by the T45G enzyme arises from the expanded substrate specificity of the variant superimposed upon an intrinsic ability to diffuse along poly(A). Homologous ribonucleases with cytotoxic activity may rely on facilitated diffusion along poly(A) tails for efficient degradation of the essential information encoded by cellular mRNA.

Diffusion poses a barrier on the free energy surface of all bimolecular reactions (*1*). Bovine pancreatic ribonuclease A [RNase A[1] (*2*−*4*), EC 3.1.27.5] catalyzes the cleavage of uridylyl(3′→5′)adenosine (UpA) at a rate that is not limited by the making or breaking of covalent bonds (*5*). In this respect, RNase A can be thought of as a "perfect" enzyme (*6*, *7*). Nonetheless, its catalytic efficacy would be extended further if RNase A were able to diffuse in the reduced dimension of polymeric RNA (*8*).

Facilitated diffusion enables a protein to limit the dimensions of a random diffusional search for a target. In an enzymatic mechanism in which facilitated diffusion is incorporated, the enzyme (E) binds to a nonspecific site (N) of a linear polymer to form a noncovalent complex (E·N). The E·N complex converts by one-dimensional diffusion along the polymer to a catalytically competent complex (E·S). Ultimately, turnover of the E·S complex forms product(s) (P). These steps are shown in eq 1.

$$\begin{aligned} E + N &\rightleftarrows E\cdot N \\ E\cdot N &\rightleftarrows E\cdot S \\ E\cdot S &\rightarrow\rightarrow E + P \end{aligned} \qquad (1)$$

von Hippel and co-workers first proposed the existence of facilitated diffusion to explain the unexpectedly large diffusional rate of the lac repressor along double-stranded DNA (*9*). Single-stranded nucleic acids can also support facilitated diffusion. Lohman provided evidence that the T4 gene 32 protein diffuses along RNA polymers (*10*, *11*). Others proposed a mechanism of facilitated diffusion for the filamentous phage gene 5 protein on single-stranded DNA and RNA (*12*). Restriction endonucleases (*13*) and DNA methyltransferases (*14*, *15*) provide examples of facilitated diffusion in enzymic systems. There does not exist, however, a precedent for facilitated diffusion by a ribonuclease.

RNase A catalyzes the cleavage of RNA after pyrimidine residues. This selectivity is mediated largely by a single conserved residue, Thr45, which is in the enzyme's B1 subsite (i.e., the active site) (*16*). There are two other known base-binding subsites. The B2 subsite has a preference for an adenine base, and the B3 subsite has a preference for a purine base.[2] Thus, the preferred substrate for RNase A is YAR, where Y refers to a pyrimidine nucleotide (C or U) and R refers to a purine nucleotide (A or G) (*17*).

While evaluating the importance of Thr45 to enzymic specificity, we discovered that variants of RNase A at Thr45

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[1] Abbreviations: DEPC, diethyl pyrocarbonate; DMF, dimethylformamide; EDTA, ethylenediaminetetraacetic acid; RNase A, bovine pancreatic ribonuclease A; PAGE, polyacrylamide gel electrophoresis; poly(A), poly(adenylic acid); poly(dA), poly(deoxyadenylic acid); poly(C), poly(cytidylic acid); poly(dC), poly(deoxycytidylic acid); MES, 2-(*N*-morpholino)ethanesulfonic acid; TBAF, tetrabutylammonium fluoride; Tris, tris(hydroxymethyl)aminomethane; UpA, uridylyl(3′→5′)-adenosine.

[2] The existence of the B3 subsite has been inferred from kinetic data and chemical modification studies (*3*). In the crystalline RNase A·d(ATAAG) complex, the adenine base in the B3 subsite stacks with the adenine base in the B2 subsite (*28*). The B3 "subsite" could therefore result from $\pi-\pi$ stacking interactions that stabilize the enzyme·nucleic acid complex solely by preorganization or desolvation of the nucleic acid.

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Table 1: Labeled Substrates and Corresponding Detectable Products Formed by Transphosphorylation[a]

substrate (5′ → 3′)	detectable product (5′ → 3′) P_{1D}	P_{3D}
1 $^{32}P(dA)_5rU(rA)_5rU(dA)_{25}$	$^{32}P(dA)_5rU(rA)_5rU$	$^{32}P(dA)_5rU$
2 $^{32}P(dA)_{25}rU(rA)_5rU(dA)_5$	$^{32}P(dA)_{25}rU$	$^{32}P(dA)_{25}rU(rA)_5rU$

substrate (5′ → 3′)	detectable products (5′ → 3′) $P_{1D5'}$	$P_{3D5'}$	$P_{1D3'}$	$P_{3D3'}$
3 $Fl(dA)_5rU(dA)_5rU(dA)_{25}Fl$	$Fl(dA)_5rU(dA)_5rU$	$Fl(dA)_5rU$	$(dA)_{25}Fl$	$(dA)_5rU(dA)_{25}Fl$
4 $Fl(dA)_{25}rU(dA)_5rU(dA)_5Fl$	$Fl(dA)_{25}rU$	$Fl(dA)_{25}rU(dA)_5rU$	$(dA)_5rU(dA)_5Fl$	$(dA)_5Fl$
5 $Fl(rA)_5rU(rA)_5rU(rA)_{25}Fl$	$Fl(rA)_5rU(rA)_5rU$	$Fl(rA)_5rU$	$(rA)_{25}Fl$	$(rA)_5rU(rA)_{25}Fl$
6 $Fl(rA)_{25}rU(rA)_5rU(rA)_5Fl$	$Fl(rA)_{25}rU$	$Fl(rA)_{25}rU(rA)_5rU$	$(rA)_5rU(rA)_5Fl$	$(rA)_5Fl$

substrate (5′ → 3′)	detectable products (5′ → 3′) P_A	P_C
7 $(dA)_7rU(dA)_7(dC)_7rU(dC)_7Fl$	$(dA)_7(dC)_7rU(dC)_7Fl$	$(dC)_7Fl$

[a] Fl refers to a fluorescein label attached via a six-carbon spacer (*21*, *23*, *24*).

that cleave poly(A) do so processively (*18*). No other variants of RNase A are known to be capable of processive catalysis. Moreover, T45G RNase A and T45A RNase A are processive catalysts only with poly(A) as a substrate.

A processive catalytic mechanism is defined by the occurrence of multiple catalytic events without dissociation of an enzyme from its substrate. T45G RNase A can cleave poly(A) and then diffuse along this substrate to another phosphodiester bond to complete a cycle of processive catalysis (*18*). The discovery that T45G RNase A and T45A RNase A are processive catalysts suggested to us that wild-type RNase A itself may be equipped to diffuse along a single-stranded nucleic acid. In other words, we suspected that variants at Thr45 had gained only the ability to cleave the $P-O^{5'}$ bond on the 3′ side of adenosine residues. This expanded specificity along with an endogenous ability to diffuse in one dimension could be the genesis of processive catalysis.

If RNase A were capable of diffusing along RNA, then longer substrates would be cleaved faster than shorter substrates (*8*, *9*). Here, we test for facilitated diffusion of RNase A by comparing the rates of cleavage of substrates that differ in their effective lengths and nucleotide compositions. We use a set of all-RNA and DNA/RNA chimeric substrates to demonstrate that both wild-type RNase A and the T45G variant can indeed diffuse in one dimension along single-stranded RNA.

EXPERIMENTAL PROCEDURES

Materials. All phosphoramidites and reagents for oligonucleotide synthesis were from Glen Research (Sterling, VA). Tetrabutylammonium fluoride (TBAF; 1.0 M in dimethylformamide) was from Aldrich Chemical Co. (Milwaukee, WI). RNase A (lyophilized), 2-(*N*-morpholino)ethanesulfonic acid (MES), and diethyl pyrocarbonate (DEPC) were from Sigma Chemical Co. (St. Louis, MO). Xylene cyanol was from MCB Manufacturing Chemists (Cincinnati, OH). Bromophenyl blue was from United States Biochemicals (Cleveland, OH). RNaseZAP was from Ambion (Austin, TX). Sephadex G-50 columns were from Pharmacia (Uppsala, Sweden). [γ-^{32}P]ATP was from duPont (Wilmington, DE). T4 polynucleotide kinase was from Promega (Madison, WI). Glycogen was from New England Biolabs (Beverly, MA). Siliconized microcentrifuge tubes were from Phenix (Hayworth, CA). Tris(hydroxymethyl)aminomethane (Tris), acrylamide, urea, boric acid, and ethylenediaminetetraacetic acid (EDTA) were from Fisher Chemical (Fairlawn, NJ). T45G RNase A was produced as described previously (*16*, *18*, *19*).

Substrate Design. RNase A can catalyze the cleavage of the $P-O^{5'}$ bond of an RNA nucleotide embedded in a DNA sequence (*20*, *21*). Substrates **1**−**4** and **7** are such DNA/RNA chimeras (Table 1). Substrates **5** and **6** are the all-RNA analogues of substrates **3** and **4**. Each of these seven substrates acts as two substrates in that each has two sites for RNase A cleavage, surrounded by distinct nucleotides. For substrates **1**−**6**, these differences are strictly in the number of adenosine nucleotides. For substrate **7**, the surrounding nucleotides are either adenosines or cytidines.

Substrates **1**−**6** were designed to enable a comparison of the ability of RNase A to cleave longer and shorter substrates. Each of these substrates can be used to test a particular aspect of facilitated diffusion. Comparing substrates **1** versus **2** (as well as **3** vs **4** and **5** vs **6**) can be used to reveal an effect of having the cleavage site closer to the 5′ versus the 3′ end. Effects of this kind have been observed in other interactions with single-stranded oligonucleotides (*22*).

Comparing substrates **1** versus **3** and **2** versus **4** can reveal an effect from the type of label. Such an effect is made apparent because substrates **1** and **2** are labeled with ^{32}P, and substrates **3** and **4** are otherwise identical but labeled instead with fluorescein. Moreover, cleavage of substrates **3**−**6**, which are doubly labeled, produces four detectable products ($P_{1D3'}$ and $P_{1D5'}$, or $P_{3D3'}$ and $P_{3D5'}$), enabling a more thorough product analysis than is possible with singly labeled substrates. Comparing substrates **3** versus **5** and **4** versus **6** can uncover a difference between diffusion along tracts of poly(dA) and tracts of poly(A). Finally, substrate **7** was designed to detect any preference of the enzyme for diffusion along adenine versus cytosine bases.

Substrate Synthesis. Nucleic acid substrates were synthesized on an ABI 492 DNA/RNA synthesizer from Applied Biosystems (Foster City, CA). Oligonucleotide substrates were synthesized according to standard protocols with extended coupling times (10 min) for fluorescein resin and fluorescein phosphoramidites. Oligonucleotides were removed from resin by treatment with ammonium hydroxide

(28% w/v NH_3 in H_2O) at room temperature overnight. Ammonium hydroxide was subsequently removed under vacuum. Deprotection of the 2′-hydroxyl was carried out by treatment with TBAF (1.0 M in dimethylformamide). Care was taken to eliminate contamination by ribonucleases. Containers used for reagents were cleaned with RNaseZAP, and water was treated with DEPC.

Oligonucleotide substrates were purified by polyacrylamide gel electrophoresis (PAGE). Glass plates and the gel apparatus were soaked in nitric acid (10% v/v) overnight before being used in purification. After electrophoresis, bands detected by illumination with UV light were excised from the gel and crushed to fine material. Oligonucleotides were eluted from the macerated polyacrylamide by incubation overnight in a solution of sodium acetate (0.30 M). The polyacrylamide was pelleted by centrifugation, and the supernatant was decanted. Ethanol precipitation of oligonucleotides from the supernatant was encouraged by the addition of glycogen (to 10 μg/mL).

Substrates **1** and **2** were labeled with ^{32}P on their 5′ end by treatment with [γ-^{32}P]ATP and T4 kinase, and desalted by gel filtration chromatography on Sephadex G-50 resin. Fluorescein with a six-carbon spacer was incorporated into substrates **3**−**7** as a phosphoramidite during synthesis (*23*, *24*). Residual salts and glycogen were removed from all substrates by Sephadex G-50 gel filtration chromatography.

Assays of Substrate Cleavage. Substrate and enzyme concentrations were determined with a Cary 3 UV/VIS spectrophotometer from Varian (Sugarland, TX), assuming that $\epsilon = 0.72$ mL mg^{-1} cm^{-1} at 277.5 nm for RNase A (*25*), $\epsilon = 5.4 \times 10^5$ M^{-1} cm^{-1} at 260 nm for substrates **1**−**6**, and $\epsilon = 3.3 \times 10^5$ M^{-1} cm^{-1} at 260 nm for substrate **7** (*26*). Reactions with substrates **1**−**7** were carried out in siliconized microcentrifuge tubes to limit loss of the enzyme. Reactions were performed in solutions (10 μL) of 50 mM MES/NaOH buffer (pH 6.0) containing enzyme (0.1−100 fM), substrate (0.4−1.0 μM), and NaCl (0, 0.10, or 1.00 M). Reactions were initiated by the addition of substrate. Aliquots (2 μL) of reaction mixtures were quenched after 1, 2, 5, and 10 min by combination with DMF (2 μL) containing xylene cyanol (0.2% w/v) and bromophenyl blue (0.2% w/v). Enzyme concentrations were adjusted so that <10% of the substrate was cleaved in 10 min. Under these conditions, reaction products were unlikely to become substrates for subsequent cleavage reactions. For reactions with substrates **3**−**7**, xylene cyanol was omitted because of its strong fluorescence. Products from quenched reactions were separated by electrophoresis through an 18% (w/v) polyacrylamide gel buffered by a solution of Tris base (0.045 M) and boric acid (0.045 M) containing urea (7 M) and EDTA (1 mM). Reaction products from substrates **1** and **2** were quantitated with a PhosphorImager from Molecular Dynamics (Sunnyvale, CA). Reaction products from substrates **3**−**7** were quantitated using a Vistra FluorImager SI from Molecular Dynamics. Phosphorimager and fluorimager data were analyzed using ImageQuant software from Molecular Dynamics. Each reaction was carried out at least four times.

RESULTS

Probes for Facilitated Diffusion. Oligonucleotide substrates were designed to test the hypothesis that RNase A uses facilitated diffusion. The design enabled the comparison of substrates containing one cleavable site within 11 and 31 nucleotides. Because both sites were embedded in one molecule, solution conditions, temperature, enzyme concentration, and substrate concentration were necessarily identical for the two cleavage reactions. Moreover, the rate of formation of one product was directly comparable to the rate of formation of the other product.

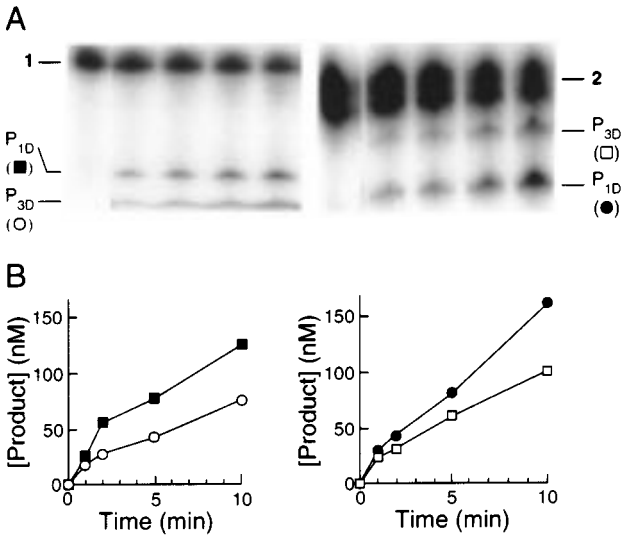

FIGURE 1: Time course (0, 1, 2, 5, and 10 min) for the cleavage of substrate **1** (left) and substrate **2** (right) by ribonuclease A. Reactions were performed in 50 mM MES/NaOH buffer (pH 6.0) containing NaCl (0.10 M). (A) Migration of the substrate and its P_{1D} and P_{3D} products (Table 1) during PAGE. (B) Quantitation of PAGE data by phosphorimager analysis. Data from replicate experiments did not differ substantially.

We determined the concentration of each product at different times using a discontinuous assay. For all assays, the results from replicate reactions did not differ substantially. We refer to the product formed from cleavage of the "longer" substrate as P_{1D}, and that from the cleavage of the "shorter" substrate as P_{3D} (Table 1). The results of the assay used to identify P_{1D} and P_{3D} for substrates **1** and **2** are shown in Figure 1. Product P_{1D} formed faster than P_{3D} from both substrates **1** and **2**. At a total Na^+ concentration of 0.125 M, P_{1D} and P_{3D} had formed at a ratio of approximately 1.6:1 at all time points. Onconase, which is a cytotoxic homologue of RNase A (*2*), likewise generated P_{1D} faster than P_{3D} (data not shown).

We express the relative rates of product formation as the ratio of product concentrations at each time point ($[P_{1D}]/[P_{3D}]$). The $[P_{1D}]/[P_{3D}]$ ratios for substrates **1** and **2** at various concentrations of Na^+ are shown in Figure 2. The data in this figure demonstrate a trend in the product ratio that is consistent with facilitated diffusion mediated by Coulombic interactions. At a low Na^+ concentration (25 mM), the indication of facilitated diffusion is strong ($[P_{1D}]/[P_{3D}] \approx 2$). At a high Na^+ concentration (1.025 M), facilitated diffusion is not apparent ($[P_{1D}]/[P_{3D}] \approx 1$). Also, the overall rate of substrate cleavage is diminished greatly, as would be expected from a shift to a three-dimensional diffusion mechanism.

Doubly Labeled Substrates. Substrates **1** and **2** lack a label at their 3′ ends and thus cannot reveal whether RNase A moves from one cleavage site to another without release of

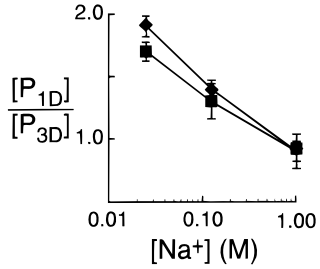

FIGURE 2: Salt concentration dependence of the $[P_{1D}]/[P_{3D}]$ product ratio from the cleavage of substrate **1** (■) and substrate **2** (◆) by ribonuclease A. Reactions were performed in 50 mM MES/NaOH buffer (pH 6.0) containing NaCl (0, 0.10, or 1.00 M). Each value (±standard error) is the average of data obtained after reaction for 1, 2, 5, and 10 min.

Table 2: Concentration Ratios ($[P_{1D}]/[P_{3D}]$) of 5′- and 3′-Fluorescein-Labeled Products Generated by Wild-Type Ribonuclease A and T45G Ribonuclease A[a]

	wild-type		T45G	
substrate	5′	3′	5′	3′
3	1.5 ± 0.1	2.5 ± 0.2	2.0 ± 0.1	2.0 ± 0.1
4	1.9 ± 0.2	1.7 ± 0.2	2.1 ± 0.2	1.9 ± 0.2
5	1.6 ± 0.2	1.5 ± 0.3	1.9 ± 0.2	2.1 ± 0.3
6	2.1 ± 0.4	1.8 ± 0.2	2.6 ± 0.4	1.7 ± 0.1

[a] Reactions were carried out in 0.050 M MES/NaOH buffer (pH 6.0) containing NaCl (0.10 M). Each value (±standard error) is the average of data obtained after reaction for 1, 2, 5, and 10 min.

products. Substrates **3**−**6** are labeled on both their 5′ end and their 3′ end with fluorescein. For each of these substrates, there exist four detectable products: $P_{1D3'}$, $P_{1D5'}$, $P_{3D3'}$, and $P_{3D5'}$ (Table 1). Hence, there exist $[P_{1D}]/[P_{3D}]$ ratios for the 3′ and 5′ labels, which we represent as $[P_{1D}]/[P_{3D}]_{3'}$ and $[P_{1D}]/[P_{3D}]_{5'}$, respectively. The product ratios for the reaction carried out at 0.10 M NaCl are listed in Table 2. Assays of fluorescein-labeled substrates are not as sensitive as assays of ^{32}P-labeled substrates. The quality of the fluorimager data suffers significantly from light scattering by the polyacrylamide gel. Still, as listed in Table 2, all values of $[P_{1D}]/[P_{3D}]$ (≈2) are similar to each other and similar to $[P_{1D}]/[P_{3D}]$ ratios for substrates **1** and **2**. The value of the $[P_{1D}]/[P_{3D}]$ ratio is in reasonable agreement with the difference in the number of adenosine nucleotides (30 vs 10) that flank the cleavable sites. The similarity between the data from substrates **1** and **2** and those from substrates **3**−**6** indicates that the type of label is not affecting the results (Figure 3). The picture provided by doubly labeled substrates **3**−**6**, though more complete than that provided by substrates **1** and **2**, is essentially identical. The similarity between the data from substrates **3** and **4** and those from substrates **5** and **6** indicates that wild-type RNase A and the T45G variant treat adenosine and deoxyadenosine nucleotides equivalently in this assay.

Facilitated Diffusion along Poly(dA) versus Poly(dC). The cleavage of substrate **7** by RNase A results in two products, P_A and P_C (Table 1). Product P_A results from the cleavage of substrate **7** within the region of deoxyadenosine nucleotides, whereas P_C is the result of cleavage at the uridine within the deoxycytidine region. Product P_C can also be formed by the cleavage of P_A. The cleavage of substrate **7** by wild-type RNase A and T45G RNase A results almost exclusively in P_A (Figure 4). Excess RNase A generated P_C

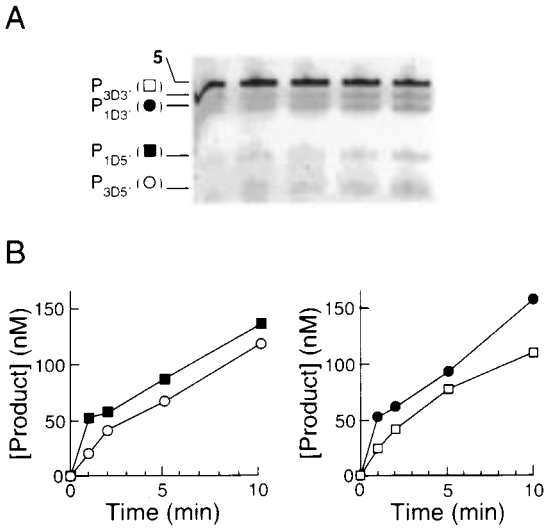

FIGURE 3: Time course (0, 1, 2, 5, and 10 min) for the cleavage of substrate **5** by ribonuclease A. Reactions were performed in 50 mM MES/NaOH buffer (pH 6.0) containing NaCl (0.10 M). (A) Migration of substrate **5** and its $P_{1D5'}$, $P_{3D5'}$, $P_{1D3'}$, and $P_{3D3'}$ products (Table 1) during PAGE. (B) Quantitation of PAGE data by fluorimager analysis. Data from replicate experiments did not differ substantially.

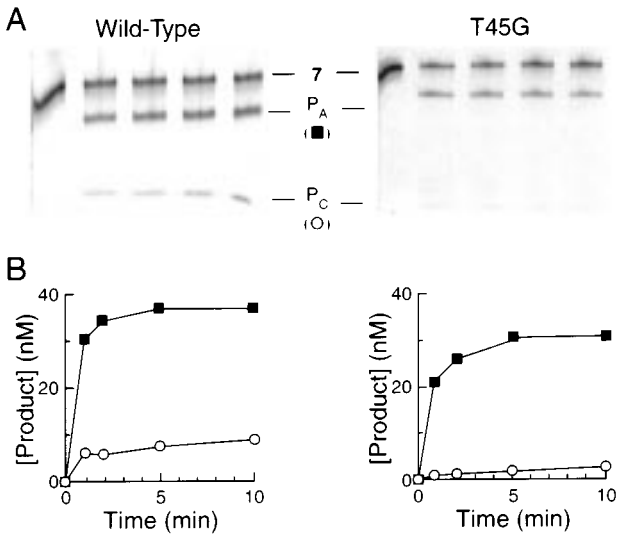

FIGURE 4: Time course (0, 1, 2, 5, and 10 min) for the cleavage of substrate **7** by wild-type ribonuclease A (left) and T45G ribonuclease A (right). Reactions were performed in 50 mM MES/NaOH buffer (pH 6.0) containing NaCl (0.10 M). (A) Migration of substrate **7** and its P_A and P_C products (Table 1) during PAGE. (B) Quantitation of PAGE data by fluorimager analysis. Data from replicate experiments did not differ substantially.

to significant levels, but P_A was still produced much more rapidly (data not shown).

DISCUSSION

Probes for Facilitated Diffusion. We designed labeled oligonucleotide substrates to eliminate the hypothesis that RNase A does not use facilitated diffusion. This antipodal hypothesis depends on RNase A diffusing to a specific site of cleavage directly in a one-step mechanism. If the hypothesis of strictly three-dimensional diffusion were true, then the length of substrate beyond that directly contacting the enzyme would have no effect on the rate of cleavage of the substrate. RNase A interacts simultaneously with no more

than 10 phosphoryl groups (*27*, *28*) and has smaller K_m values for longer substrates (*29*). Still, values of k_{cat}/K_m plateau for substrates that are three or more nucleotides long (*29*). We designed our substrates with at least five nucleotides flanking each cleavable site.

RNase A prefers to cleave "longer" oligonucleotide substrates (Figure 1). The identity and location of the label (5′-^{32}P vs 5′-fluorescein and 3′-fluorescein) do not alter this preference (Figures 1 and 3). This preference is diminished by Na^+ (Figure 2), as would be expected from an effect mediated by Coulombic interactions (*23*, *24*, *27*). Indeed, RNase A is known to interact with the anionic phosphoryl groups of nucleic acids via cationic histidine, lysine, and arginine residues (*23*, *24*). These data are consistent with facilitated diffusion of RNase A along poly(dA) that is mediated (at least in part) by Coulombic interactions.

All-RNA Substrates. DNA/RNA chimera are nonnatural substrates for RNase A. We were concerned that the linear diffusion that we observed along the deoxyadenosine residues of substrates **1**−**4** was not relevant to a biological process. Accordingly, we synthesized and tested substrates **5** and **6**, which are composed entirely of ribose nucleotides. These all-RNA substrates were comparable to substrates **1**−**4** at fostering facilitated diffusion (Figures 1 and 3). Thus, facilitated diffusion can occur along a poly(A) tract as well as along a poly(dA) tract.

Processivity along DNA/RNA Chimera. We considered the possibility that RNase A may be processing from one cleavage site to the other on substrates **1**−**6**. Such processivity would not be readily detectable with substrates **1** and **2**. Substrates **3**−**6** provide a sensitive test for processivity. These substrates, which are labeled with fluorescein at both ends, enable us to detect cleavage at both uridine nucleotides. If RNase A diffuses from one site of cleavage to the next, then $[P_{1D}]/[P_{3D}]_{5'}$ and $[P_{1D}]/[P_{3D}]_{3'}$ would not be the same. Specifically, if RNase A releases the 3′ product after cleavage, effectively diffusing in the 5′ direction, then substrates **4** and **6** would have a $[P_{1D}]/[P_{3D}]_{5'}$ that was larger than $[P_{1D}]/[P_{3D}]_{3'}$. If RNase A diffuses in the 3′ direction after cleavage, then substrates **3** and **5** would have a $[P_{1D}]/[P_{3D}]_{3'}$ that was larger than $[P_{1D}]/[P_{3D}]_{5'}$. Neither of these two possibilities was observed (Table 2). We conclude that processivity is insignificant during the cleavage of substrates **3**−**6**.

Facilitated Diffusion along Poly(dA) versus Poly(dC). We designed substrate **7** to probe the relative ability of RNase A to diffuse along tracts of deoxyadenosine and deoxycytidine nucleotides. We chose to make this comparison because poly(C), a polypyrimidine like the 3′ half of substrate **7**, is an excellent substrate for RNase A (*18*). Moreover, in the crystalline structure of an RNase A·d(ATAAG) complex, a pyrimidine nucleotide rather than a purine nucleotide is bound in the active site (*30*). Time courses for the cleavage of substrate **7** indicate that both wild-type RNase A and the T45G variant diffuse much more efficiently along a poly(dA) tract than along a poly(dC) tract (Figure 4). What is the basis for this preference? Both poly(A) and poly(C) have helical structures in aqueous solution at neutral pH (*31*−*34*). Poly(C) can form a left-handed helix stabilized by a hydrogen bond between adjacent bases (*33*). In contrast, poly(A) can form a right-handed helix stabilized by base stacking (*32*). In a crystalline RNase A·$(dA)_4$ complex, the $(dA)_4$ ligand forms a helical structure similar to that of poly(A), with the phosphoryl groups in continuous contact with the surface of RNase A (*28*, *35*). Thus, Coulombic interactions with preorganized phosphoryl groups are likely to lead to efficient diffusion along poly(dA). Favorable interactions between the enzymic B2 and B3[2] subsites and adenine bases may also contribute to the preference for diffusing along a poly(dA) tract rather than along a poly(dC) tract.

T45G RNase A is also capable of facilitated diffusion. Yet, T45G RNase A, which catalyzes the processive cleavage of poly(A) (*18*), does not appear to catalyze the processive cleavage of the poly(A) tracts of substrates **5** and **6** (Figure 3). This surprising result is likely to be due to the manner in which RNase A finds its substrate and releases its products. The B1 subsite of T45G RNase A has a greater affinity for a uracil base than for an adenine base, though this preference is weaker than that of the wild-type enzyme (B. R. Kelemen and R. T. Raines, unpublished results). We suspect that the T45G variant diffuses randomly along substrates **5** and **6** until a uracil base occupies its B1 subsite. After cleavage, the uracil base remains in the B1 subsite, just as it would in the wild-type enzyme. Yet, processive catalysis of poly(A) cleavage requires release of the 5′ product, as it proceeds in the 5′ → 3′ direction (*18*). The slow release of a 5′ uracil product may allow time for the release of the 3′ adenine product. In other words, the affinity of the B1 subsite for uracil may slow release of the 5′ product, obviating processive catalysis for both T45G RNase A and the wild-type enzyme on substrates **5** and **6**. This same affinity may be responsible for the product inhibition that occurs during the turnover of substrate **7** (Figure 4).

An Alternative Explanation? Our data for substrates **1**−**6** are consistent with a hypothesis alternative to that of facilitated diffusion: RNase A could be attracted to the midpoint of oligonucleotides. Specifically, P_{1D} could be formed preferentially because RNase A is drawn by Coulombic forces to the more central uridine nucleotide in substrates **1**−**6**. This hypothesis is eliminated by the results for substrate **7**. This substrate has an equal number of DNA nucleotides surrounding two RNA nucleotides. One RNA nucleotide is embedded within a tract of deoxycytidine residues; the other is embedded within a tract of deoxyadenosine residues. If RNase A were not to use facilitated diffusion to locate specific sites of cleavage, then P_A and P_C would be produced at identical rates. Yet, RNase A produces P_A much more rapidly than P_C (Figure 4). The catalytic behavior of RNase A on substrate **7** is thus consistent with the hypothesis that RNase A uses facilitated diffusion.

Mechanism of Facilitated Diffusion. Diffusion in reduced dimensions can be accomplished by several mechanisms, as described by von Hippel (*8*). These mechanisms are "sliding", "interdomain transfer", and "intradomain dissociation and association". These mechanisms describe in detail the second step in eq 1. In theory, each could describe the facilitated diffusion of RNase A along poly(A). Yet, RNase A is not known to have two distal sites with which to bind RNA independently (*17*), as would be required by an interdomain transfer mechanism. Moreover, cleavage of substrates **1**−**7** by an intradomain dissociation and association (i.e., hopping) mechanism would likely yield $[P_{1D}]/[P_{3D}]$ ratios that were close to unity. These ratios were typically

near 2 (Figures 1 and 3 and Table 2). We therefore favor a sliding mechanism mediated by contact between the phosphoryl groups of poly(A) and the cationic residues of RNase A.

Biological Implications. The ability of RNase A to diffuse along poly(A) tracts may have a sinister ramification. Several homologues (*2*) and variants (*36*, *37*) of RNase A are cytotoxic to mammalian cells. Poly(A) tails of up to 300 nucleotides are a feature of the mRNAs in all eukaryotic organisms (*38*). In vivo, cytotoxic ribonucleases may use the poly(A) tract of mammalian mRNAs as a conduit that leads the enzyme to pyrimidine nucleotides in the indispensable coding region. Such direct routing would be more efficacious than a tortuous three-dimensional search for a substrate.

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