Probing Ribonuclease A
Catalysis
Using Non-Natural Nucleic Acids

By
Bradley Roger Kelemen

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ABSTRACT

Bovine pancreatic ribonuclease A (RNase A) catalyzes the cleavage of the P–O bond of RNA on the 3′ side of pyrimidine nucleotides. An active-site threonine residue at position 45 of RNase A enforces this specificity for pyrimidine nucleotides. Here, the consequences of an alteration to Thr45 to aspects of catalysis and ground state binding affinity is analyzed. This analysis is focused on the interaction of RNase A and its T45G variant with a variety of synthetic substrates and substrate analogs.

A hypersensitive fluorogenic substrate for RNase A was developed in an empirical manner. This class of substrates is based on quenching of a fluorescein moiety by a rhodamine or dabcyl moiety held in proximity by a ribouridine nucleotide embedded in a strand of deoxyadenosine nucleotides. When RNase A catalyzes the cleavage of the substrate at the ribonucleotide, the fluorescein becomes highly fluorescent. The optimal fluorogenic substrate is a tetranucleotide with 6-carboxytluorescein at the 5′ end and 6-carboxyrbodamine at the 3′ end. Cleavage of this substrate produces a 180-fold change in fluorescence, which is significantly greater than that for any previous substrate for a ribonuclease. The sensitivity of the optimal substrate enables a facile assay for the determination of values of competitive inhibition constants, $K_i$.

The contribution of ground state binding to the specificity of RNase A was evaluated using deoxynucleotide ligands and compared to catalytic specificity
measured with analogous fluorogenic substrates. Differences in ground state binding are responsible for only a small fraction of the substrate specificity of RNase A. The affinity for 2’-deoxy analogs of ribonuclease substrates is greater than that for 2’-deoxy-2’-fluoro substrate analogs, even though the 2’-deoxy-2’-fluoro analogs better mimic the stereoelectronics of RNA substrate.

Catalysis by RNase A is extremely efficient, and is not limited by a chemical step. RNA and DNA/RNA chimeric oligonucleotides were used to demonstrate that RNase A is capable of accelerating RNA cleavage by association with non-target RNA or DNA followed by one-dimensional diffusion. The ability to diffuse along a single-stranded nucleic acid is salt concentration dependent, indicating that Coulombic forces contribute to this diffusional process. Nucleotide type also strongly influences the capacity of RNase A to diffuse in one dimension.
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>3′-UMP</td>
<td>uridine 3′-phosphate</td>
</tr>
<tr>
<td>5′-ADP</td>
<td>adenosine 5′-diphosphate</td>
</tr>
<tr>
<td>CpA</td>
<td>cytidylyl(3′→5′)adenosine.</td>
</tr>
<tr>
<td>CPG</td>
<td>controlled pore glass</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>6-FAM</td>
<td>6-carboxyfluorescein</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulphonic acid</td>
</tr>
<tr>
<td>oligo(U)</td>
<td>oligo(uridylic acid)</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>poly(A)</td>
<td>poly(adenylic acid)</td>
</tr>
<tr>
<td>poly(C)</td>
<td>poly(cytidylic acid)</td>
</tr>
<tr>
<td>poly(dA)</td>
<td>poly(deoxyadenylic acid)</td>
</tr>
<tr>
<td>poly(dC)</td>
<td>poly(deoxycytidylic acid)</td>
</tr>
<tr>
<td>poly(U)</td>
<td>poly(uridylic acid)</td>
</tr>
<tr>
<td>RNase A</td>
<td>bovine pancreatic ribonuclease A</td>
</tr>
<tr>
<td>6-TAMRA</td>
<td>6-carboxytetramethylaminorhodamine.</td>
</tr>
<tr>
<td>T45G RNase A</td>
<td>Thr45 → Gly RNase A</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TBAF</td>
<td>tetrabutyl ammonium fluoride</td>
</tr>
<tr>
<td>TEA-3HF</td>
<td>triethylammonium-trihydrogen fluoride</td>
</tr>
<tr>
<td>TEAA</td>
<td>triethylammonium acetate</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>U^F</td>
<td>2'-deoxy-2'-fluoro uridine</td>
</tr>
<tr>
<td>UpA</td>
<td>uridylyl(3'→5')adenosine.</td>
</tr>
</tbody>
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Chapter 1

Introduction
Bovine pancreatic ribonuclease A (RNase A) catalyzes the depolymerization of ribonucleic acids (RNA) through the activation of the 2' hydroxyl of pyrimidine nucleotides (Thompson & Raines, 1994). This catalytic activity and specificity is present in all of the members of the RNase A superfamily of enzymes (Beintema, 1987). RNase A is produced in the bovine pancreas in large quantities as both RNase A and the glycosylated form, RNase B. Barnard speculated that RNase A functions as a digestive enzyme, but the true biological function is not known (Barnard, 1969). Homologues of RNase A have biological activities resulting from the toxicity of their ribonucleolytic activity within cells (Shapiro & Vallee, 1989; Kim et al., 1995).

RNase A catalyzes RNA breakage through the deprotonation of the 2' hydroxyl and the protonation of the 5' hydroxyl of RNA, as shown in Figure 1.1. Deprotonation and protonation is carried out in concert by His12 and His119 respectively (Sowa et al., 1997). The transition state of this transphosphorylation reaction is further stabilized by a hydrogen bond from Lys41, as shown in Figure 1.2 (Messmore et al., 1995).

The resulting cyclic phosphodiester product is also a substrate for RNase A, Figure 1.1. This product is not a true intermediate in that it is released and bound again by the enzyme (Thompson et al., 1994). The hydrolysis reaction is slow enough that RNA substrates are cleaved before the enzyme begins hydrolysis of the second-step substrates. The products of the hydrolysis reaction are competitive inhibitors of RNase A catalysis.
RNase A is not capable of DNA cleavage; however, it does bind to this substrate analogue. The crystalline complexes of RNase A with various DNA substrate analogues has offered structural information on the interactions with substrates made by RNase A (Pavlovsky et al., 1978; McPherson et al., 1986a; McPherson et al., 1986b; Birdsall & McPherson, 1992; Fontecilla-Camps et al., 1994; Zegers et al., 1994). Crystalline complex of a uridine 2',3'-cyclic vanadate transition state analogue has also provided valuable structural information about the RNase A–substrate interaction (Wlodawer et al., 1983).

Several residues of RNase A interact with polymeric substrates at sites distal from residues involved in bond breaking and bond forming (Moussaoui et al., 1995). These sites of interaction can be classified into two groups: phosphoryl-binding and base-binding subsites (Figure 1.3). The phosphoryl binding subsites consist of lysine and arginine residues and interact with RNA and DNA through Coulombic forces. Four such sites are currently defined through X-ray crystallography, kinetics, and equilibrium binding measurements and are labeled P(-1) through P2 (Fisher et al., 1998a). The P0 subsite is the active site and contains His12, His119, and Lys41.

Irie and coworkers have studied depolymerization of poly(adenylic acid) [poly(A)], poly(uridylic acid) [poly(U)] and oligo(uridylic acid) [oligo(U)] of varying lengths to establish how RNase A interacts with polymeric substrates (Imura et al., 1965; Irie et al., 1984). Nogués and coworkers have studied the degradation poly(C) by RNase A in an attempt to refine this understanding and propose that the subsites
described earlier are responsible for the endonucleolytic propensity of RNase A (Moussaoui et al., 1995).

Three defined base-binding subsites denoted B1, B2, and B3 produce a base specificity of YAR, where Y is a pyrimidine (C or U), A is adenosine, and R is a purine (A or G). The B1 subsite, composed of Thr45, Asp83, and Phe120, provides a slight preference of C over U (4-fold relative $k_{cat}/K_m$) while it excludes purine bases to a great extent (> 10^4-fold decrease in $k_{cat}/K_m$) (delCardayre & Raines, 1995b).

The B2 subsite, consisting of Asn71 and Glu111, favors adenosine such that RNase A cleaves cytidylyl(3'→5')cytidine more slowly ($k_{cat}/K_m \approx 10^4 \text{ M}^{-1}\text{s}^{-1}$) than cytidylyl(3'→5')adenosine [CpA; $k_{cat}/K_m \approx 10^6 \text{ M}^{-1}\text{s}^{-1}$] (Witzel & Barnard, 1962). However, the B2 subsite is less stringent if the enzyme is acting on polymeric substrates. For example, poly(cytidylic acid) [poly(C)] is a good substrate ($k_{cat}/K_m \approx 10^6 \text{ M}^{-1}\text{s}^{-1}$) despite placing a cytidine in the enzymic B2 subsite (Messmore et al., 1995).

The final base-binding site, B3, does not have any enzymic residues implicated by structural studies. The base occupying the B3 subsite stacks onto the base in the B2 subsite. This $\pi-\pi$ stacking interaction may be encouraged by the spacing of the phosphoryl-binding subsites and the lowest energy conformations of the substrate itself (McPherson et al., 1986a).

The residue Thr45 is the major contributor of the B1 subsite (Figure 1.4). This residue can also be considered a part of the active site, though it does not interact directly with the phosphoryl oxygen atoms of the scissile bond. The gamma oxygen
of Thr45 either accepts or donates a hydrogen bond to the pyrimidine nucleotide base (delCardayré & Raines, 1995b). This interaction provides the specificity of strand breakage at the 3' of pyrimidine nucleotides. The work described in this thesis explains the energetic contributions that provide specificity and the catalytic consequences of RNase A specificity.

Chapter 2 describes a group of fluorescence-based substrates. There exist among these substrates an optimal substrate for RNase A based on the kinetic parameter \( k_{cat}/K_m \) and the total change in fluorescence going from substrate to product. Nonetheless, this substrate does not possess the chemical stability to be used reliably in studies of the pH dependence of catalysis. For these studies, we present a similar substrate with a slightly lesser increase in fluorescence but a much greater chemical stability. With a variety of lengths of substrates, the significance of extended binding sites on the kinetic parameter \( k_{cat}/K_m \) is measured. Utilizing the sensitivity of the optimal substrate, we rapidly and efficiently determine values of the steady-state competitive inhibition constant, \( K_i \).

Chapter 3 describes ground-state contributions to the energetics of binding provided by substrate interactions with the residue Thr45. The crystalline structure of Thr45 \( \rightarrow \) Gly (T45G) RNase A is presented at 1.8 Å resolution to demonstrate the structural differences of this variant from wild-type RNase A. The binding of non-reactive substrate analogs to both wild-type and T45G RNase A were analyzed using fluorescence anisotropy. These data are compared to the kinetic parameters for the
cleavage of fluorescence based substrates similar in composition to the ligands analyzed in this chapter.

The ability of RNase A to use one-dimensional diffusion to locate target sites of action is described in Chapter 4. One-dimensional diffusion is the use of non-specific, non-target, binding to accelerate the location of specific, target sites. Due to this one-dimensional diffusion, the rate of RNase A catalysis is substrate-length dependent. This dependence is demonstrated using synthetic substrates. The salt concentration dependence of the facilitated diffusion indicates that this process is mediated by Coulombic forces. Still, there does exist a nucleotide base dependence to facilitated diffusion by RNase A.
Figure 1.1  Ribonuclease A catalyzes transphosphorylation and hydrolysis reactions in two distinct steps. The simplest mechanism consistent with available kinetic and structural data is presented with "A" representing histidine 119 and "B" representing histidine 12 (Thompson & Raines, 1994). These two reactions occur as distinct, uncoupled events with hydrolysis occurring more slowly (Thompson et al., 1994).
Transphosphorylation

Hydrolysis
Figure 1.2  The crystalline complex of ribonuclease A and the uridine 2',3'-cyclicvanadate complex (U>v) solved by X-ray and neutron diffraction hints at possible active-site interactions (Borah et al., 1985). These interactions include hydrogen bonds from Lys41, His12, His119, and the main-chain amide of Phe120.
The distal base and phosphoryl binding sites of ribonuclease A may be the source of endonucleolytic catalysis by the enzyme (Moussaoui et al., 1995). This figure presents all currently characterized interactions of ribonuclease A with substrate (Fisher et al., 1998a). The B1 subsite provides the primary specificity of the enzyme (delCardayré & Raines, 1995b), and the P0 effects catalysis (Thompson & Raines, 1994).
The specificity of ribonuclease A for purine nucleotides is enforced by a single residue, threonine 45. Threonine 45 forming a hydrogen bond with the base of uridine 2',3'-cyclicvanadate complex (U>v) is one example of how specificity is achieved in ribonuclease A (Borah et al., 1985).
Chapter 2

Hypersensitive Substrate for Ribonucleases

SUMMARY

A substrate for a hypersensitive assay of ribonucleolytic activity was developed in a systematic manner. This substrate is based on the fluorescence quenching of fluorescein held in proximity to rhodamine by a single ribonucleotide embedded within a series of deoxynucleotides. When the substrate is cleaved, the fluorescence of fluorescein is manifested. The optimal substrate is a tetranucleotide with a 5' 6-carboxyfluorescein label (6-FAM) and a 3' 6-carboxytetramethylrhodamine (6-TAMRA) label: 6-FAM-dArUdAdA-6-TAMRA. The fluorescence of this substrate increases by 180-fold upon cleavage. Bovine pancreatic ribonuclease A (RNase A) cleaves this substrate with a $k_{cat}/K_m$ of $3.6 \times 10^7$ M$^{-1}$s$^{-1}$. Human angiogenin, which is a homolog of RNase A that promotes neovascularization, cleaves this substrate with a $k_{cat}/K_m$ of $3.3 \times 10^2$ M$^{-1}$s$^{-1}$. This value is >10-fold larger than that for other known substrates of angiogenin. With these attributes, 6-FAM-dArUdAdA-6-TAMRA is the most sensitive known substrate for detecting ribonucleolytic activity. This high sensitivity enables a simple protocol for the rapid determination of the inhibition constant ($K_i$) for competitive inhibitors such as uridine 3'-phosphate and adenosine 5'-diphosphate. A substrate analogous to the optimal substrate with 6-TAMRA replaced by a dabcyl quencher provides for the analysis of kinetics of ribonucleases at different solution pH conditions.
INTRODUCTION

A sensitive assay is critical for the study of catalysis, and a continuous assay facilitates the evaluation of kinetic parameters. Continuous assays for ribonucleolytic activity often rely on a hyperchromicity shift or on coupling to catalysis by another enzyme, such as adenosine deaminase (Warshaw & Tinoco, 1966; Ipata & Felicioli, 1968; Hill & Tsuchiya, 1981). These assays are not particularly sensitive, as the substrates undergo only a modest change in optical absorption upon conversion to product. In contrast, cleavage of uridine 3'-(p-nitrophenylphosphate) (Davis et al., 1988; Thompson & Raines, 1994; delCardayré et al., 1995) or uridine 3'-(5-bromo-4-chloroindol-3-yl)-phosphate (Witmer et al., 1991) results in a large change in optical absorption. These nonnatural substrates suffer, however, from low values of $k_{cat}/K_m$ during cleavage by bovine pancreatic ribonuclease A (RNase A; EC 3.1.27.5), which is the best characterized ribonuclease (Raines, 1998).

The sensitivity of a substrate is a function of both the magnitude of the change in signal and the value of kinetic parameters. Hofsteenge and coworkers developed a sensitive assay for RNase A based on fluorescence quenching (Zelenko et al., 1994; Hofsteenge et al., 1998). Their dinucleotide substrate undergoes a 60-fold increase in fluorescence after cleavage and has a $k_{cat}/K_m$ on the order of $10^7 \text{ M}^{-1}\text{s}^{-1}$ (Zelenko et al., 1994). A pentanucleotide version has also been prepared, and has an even higher $k_{cat}/K_m$ for human RNase 4, an RNase A homolog (Hofsteenge et al., 1998). Recently, James and Woolley reported on a nonanucleotide fluorogenic substrate for
RNase A (James & Woolley, 1998). This substrate has a higher $k_{cat}/K_m$ than the dinucleotide substrate of Hofsteenge and coworkers, but suffers from a lower increase in fluorescence upon cleavage.

Here, we search for an optimized fluorogenic substrate for RNase A. We compare a series of substrates in which a labile pyrimidine residue is embedded within inert deoxyadenosine residues, with fluorescein as fluorophore and rhodamine as quencher. We determine empirically which substrate yields the most sensitive assay. We then use that substrate to evaluate the ribonucleolytic activity of angiogenin, which is an RNase A homolog that promotes neovascularization (Riordan, 1997). We use the substrate to develop a facile assay for ribonuclease inhibition. We also present a substrate similar to the optimal substrate with a greater chemical stability for use with determinations of enzymatic catalysis at various pH solution conditions.
MATERIALS AND METHODS

Substrate design. Fluorescence quenching depends significantly on both the distance between the fluorophore and quencher and their relative orientation (Selvin, 1995). To produce an optimal substrate, we varied the number of nucleotides between fluorophore and quencher within cleavable substrates. Specifically, we synthesized substrates of dinucleotide (substrate 2.1), tetranucleotide (2.2; Figure 2.1), hexanucleotide (2.3), and octanucleotide (2.4) composition with a fluorescein moiety at the 5' end and a rhodamine moiety at the 3' end (Table 2.1).

We designed the nucleic acid sequence of our substrate to be optimal for cleavage by an enzyme of the RNase A superfamily. These enzymes prefer to cleave after the pyrimidine residue in a YAR sequence, where Y refers to a pyrimidine and R refers to a purine (Raines, 1998). Each of our substrates preserves the rUdA unit of substrate 2.1. The dArUdAdA nucleotides in substrate 2.2 are isologous to those observable in the crystalline RNase A•d(ATAAG) complex (Fontecilla-Camps et al., 1994), and fill all of the known subsites of the enzyme (Nogués et al., 1995; Fisher et al., 1998a). The interaction of RNase A and d(AUAA) has been analyzed in detail (Fisher et al., 1998b). Substrates 2.3 and 2.4 extend still further in both the 5' and 3' directions. Substrate 2.5 is identical to substrate 2.2, but contains a cytosine rather than a uracil base. Substrate 2.6 is also identical to substrate 2.2, but lacks the fluorescein and rhodamine labels. This substrate, which is inexpensive to synthesize, is used to estimate the \( K_m \) of substrate 2.2.
Materials. Phosphoramidites were from Perkin Elmer (Foster City, CA). Amino-modifier-C7 CPG was from Glen Research (Sterling, VA). The 6-carboxytetramethylrhodamine succinimidylester (6-TAMRA-NHS-ester) labeling reagent was from Molecular Probes (Eugene, OR). RNase A (lyophilized), uridine 3'-phosphate (3'-UMP), adenosine 5'-diphosphate (5'-ADP), and 2-(N-morpholino)ethanesulphonic acid (MES) were from Sigma Chemical (St. Louis, MO). RNase A was purified further by gel filtration chromatography followed by cation exchange chromatography, as described elsewhere (delCardayré et al., 1995). Human angiogenin was produced from an Escherichia coli expression system (P.A. Leland and R.T. Raines, unpublished results), and purified by cation exchange chromatography. Purified angiogenin was judged to be free of contaminating ribonucleases by zymogram electrophoresis (Blank et al., 1982; Kim & Raines, 1993).

Substrate synthesis. Oligonucleotide substrates were synthesized with a 6-carboxyfluorescein (6-FAM) at the 5'-end and an amino-modifier-C7 on the 3'-end using standard phosphoramidite chemistry (Caruthers et al., 1987) on an Applied Biosystems Model 394 DNA/RNA synthesizer. Following synthesis, the controlled pore glass (CPG) solid support was transferred to a 1.5 mL microfuge tube. Oligonucleotides were cleaved from the CPG by incubation for 10 min at 65 °C in a solution of NH₄OH/methylamine (1:1). The supernatant was removed and the CPG
was washed with 1 mL of EtOH/MeCN/H₂O (3:1:1); supernatants were pooled and dried. The t-butyl-dimethylsilyl protecting group was removed from the RNA residue by treatment with fresh anhydrous triethylammonium-trihydrogen fluoride/N-methylpyrrolidinone (250 μL of a solution of 1.5 ml N-methylpyrrolidinone, 750 μL of triethylamine, and 1.0 ml of TEA-3HF) at 65 °C for 1.5 h. The oligonucleotide was precipitated by adding 25 μL of 3 M NaOAc and 1 ml of n-BuOH; the sample was cooled at −70 °C for 1 h, and then centrifuged at 10,000 × g for 30 minutes. The supernatant was decanted, and the pellet was washed with aqueous EtOH (70% v/v) and then dried (Wincott et al., 1995).

6-TAMRA succinimidyester (0.1 ml of a 10 mg/ml solution in dimethylsulfoxide) was added to the 3'-amino-modified oligonucleotide suspended in 1.0 ml sodium bicarbonate buffer, pH 8.5. The dye-labeling reaction was incubated for 12 h at 37 °C. Reactions were dried under vacuum. Labeled oligonucleotides were resuspended in water and passed through a G25 Nap-10 disposable desalting column to remove free dye. The oligonucleotides were then purified by high performance liquid chromatography (HPLC) using a linear gradient of acetonitrile in 0.1 M triethylammonium acetate (TEAA) buffer, pH 7.2. The entire sample was loaded on a Hamilton PRP-1 column (25 cm × 0.8 cm²) and eluted with a linear gradient of acetonitrile (5 − 50% v/v) over 40 min. Samples were monitored at 260 nm and 297 nm, and peaks corresponding to the dual-labeled oligonucleotide species were collected, pooled, and lyophilized. Substrate 2.6 was synthesized and purified in a manner analogous to substrate 2.2, except that a 5' amino modifier was used in place
of 6-FAM, and 6-TAMRA succinimidylester was not coupled to the 3’ amino group.
Substrate 2.7 was synthesized and purified in the same manner as substrate 2.2 except
that a dabcyl 3’ CPG was used in place of the 3’-amino-modifier C7 CPG.

**Analytical instruments.** Fluorescence measurements were carried out on a
QuantaMaster1 photon-counting fluorometer from Photon Technology International
(South Brunswick, NJ) equipped with sample stirring. Absorbance spectroscopy was
carried out on either a Cary 3 or a Cary 50 UV/Vis spectrophotometer from Varian
(Sugarland, TX).

Solution concentrations ([E]) of RNase A and angiogenin were determined by
assuming that $\varepsilon = 0.72 \text{ ml mg}^{-1} \text{ cm}^{-1}$ at 277.5 nm (Sela et al., 1957) and $\varepsilon = 0.85 \text{ ml mg}^{-1} \text{ cm}^{-1}$ at 278 nm (Raines et al., 1995), respectively. Solution concentrations of
substrates 2.1 – 2.6 were determined by assuming that $\varepsilon = 76,340 \text{ M}^{-1} \text{cm}^{-1}$, $\varepsilon =
102,400 \text{ M}^{-1} \text{cm}^{-1}$, $\varepsilon = 126,400 \text{ M}^{-1} \text{cm}^{-1}$, $\varepsilon = 150,400 \text{ M}^{-1} \text{cm}^{-1}$, $\varepsilon = 99,940 \text{ M}^{-1} \text{cm}^{-1}$, and
$\varepsilon = 49,500 \text{ M}^{-1} \text{cm}^{-1}$ at 260 nm, respectively (Wallace & Miyada, 1987). Solution
concentrations of 3’-UMP and 5’-ADP were determined by assuming that $\varepsilon =$
$10,000 \text{ M}^{-1} \text{cm}^{-1}$ at 260 nm and $\varepsilon = 15,400 \text{ M}^{-1} \text{cm}^{-1}$ at 259 nm, respectively (Beaven et
al., 1955).

**Assays of substrate cleavage.** Assays were carried out with stirring in 2.00 ml of
0.10 M MES-NaOH buffer, pH 6.0, containing NaCl (0.10 M), substrate (0.50 nM –
0.10 $\mu$M), and RNase A (1.0 pM – 0.20 nM) or angiogenin (0.130 $\mu$M – 0.50 $\mu$M).
An increase in fluorescence emission at 515 nm, upon excitation at 490 nm, indicates the progress of the reaction. An example of data collected for substrate 2.2 is presented in Figure 2.2. Kinetic parameters and fluorescent properties of substrates 2.1 – 2.5 were determined by using eq 2.1 and 2.2:

\[
I = I_f - (I_f - I_o)e^{\frac{k_{cat}[E]}{K_m}} \tag{2.1}
\]

\[
I = I_o + (I_f - I_o)\frac{k_{cat}}{K_m}[E]t \tag{2.2}
\]

The fluorescence intensity \((I_f)\), measured at a given time during the reaction, was recorded in units of photon counts per second (CPS). The intensity of product, \(I_o\), was determined by nonlinear least-squares regression analysis (eq 2.1) of data collected with the addition of sufficient enzyme to cleave all the substrate within a period of approximately 30 min. The intensity of substrate, \(I_o\), was determined from data collected prior to the addition of enzyme (typically 2 min). Values of \(k_{cat}/K_m\) were determined either by nonlinear least-squares regression analysis of all data using eq 2.1 or by linear least-squares regression analysis of initial velocity data using eq 2.2. In both analyses, we assume that the assays were done at substrate concentrations below the \(K_m\) (vide infra). Values of \(k_{cat}/K_m\) for RNase A derived with eq 2.1 and 2.2 were within error. The high ribonucleolytic activity of RNase A allows for complete cleavage of the substrate, and hence the generation of a complete data set. We therefore report values of \(k_{cat}/K_m\) for RNase A derived with eq 2.1 (Table
2.1). The low ribonucleolytic activity of angiogenin does not allow for complete cleavage within a reasonable time. We report values of $k_{\text{cat}}/K_m$ for angiogenin derived with 2.2 (Table 2.2), determining $l_f$ by adding RNase A to the reaction after approximately 10 min.

$K_m$ of substrate 2.6. The value of $K_m$ for the cleavage of substrate 2.6 by RNase A was determined by evaluating its ability to inhibit the turnover of substrate 2.2. This analysis is based on the relationship: $K_m = [E][S]/\Sigma [E\cdot S]$, where $\Sigma [E\cdot S]$ refers to the sum of all bound enzyme species (Fersht, 1999). Assays were carried out with stirring in 2.00 ml of 0.10 M MES-NaOH buffer, pH 6.0, containing NaCl (0.10 M), substrate 2.2 (0.60 μM), and RNase A (0.57 pM). After 5 min, an aliquot (0.4 μL of a 0.35 mM solution) of substrate 2.6 was added. Additional substrate was added at 5-min intervals until the substrate decreased the reaction rate by approximately 10-fold. The maximum substrate competition was achieved prior to the reaction progressing to more than 10% of completion. The addition of large volumes (>20 μL) of substrate caused a slight, but noticeable, loss of fluorescence (less than 1% of the total change in fluorescence). This phenomenon was caused by the dilution of the fluorophore, and we did not account for this slight change in fluorescence in our data analyses. The data of each 5-min interval were fitted by linear least-squares regression analysis to determine activity, $\Delta l/\Delta t$, in units of CPS/s. Values of $K_m$ were determined by nonlinear least-squares regression analysis of data fitted to eq 2.3:
In eq 2.3, \((\Delta I/\Delta t)_0\) is the activity prior to the addition of substrate 2.6. The use of eq 2.3 assumes that the cleavage of substrate 2.6 is competitive with the cleavage of substrate 2.2. This assumption is likely to be correct for RNase A and its homologs, which have only one known active site.

**\(K_i\) of 3'-UMP and 5'-ADP.** Assays for the determination of \(K_i\) were similar to that for the determination of \(K_m\). Here, aliquots of 3'-UMP (0.50 µL of a 4.9 mM solution) or 5'-ADP (0.50 µL of a 2.3 mM solution) were added to the reaction mixture. Values of \(K_i\) were determined by nonlinear least-squares regression analysis of data fitted to eq 2.4:

\[
\frac{\Delta I}{\Delta t} = \left(\frac{\Delta I}{\Delta t}\right)_0 \frac{K_i}{K_i + [I]} \tag{2.4}
\]

In eq 2.4, \((\Delta I/\Delta t)_0\) is the activity prior to the addition of inhibitor. The use of eq 2.4 requires that the inhibitor is competitive and that substrate 2.2 is at concentrations below \(K_m\) (*vide infra*).

**Kinetic determinations at various solution pH.** The chemical stability of 6-TAMRA at acidic pH is not sufficient for reliable measurements of enzymatic kinetic
parameters. For this reason, we synthesized substrate 2.7. Substrate 2.7 is analogous to substrate 2.2 with 6-TAMRA replaced with the stable quencher dabcyl. Assays were carried out with stirring in 2.00 ml of 0.10 M buffer containing NaCl (0.10 M), substrate, and RNase A. Buffers were sodium citrate (pH 3.22), sodium succinate (pH 3.84 and 4.97), MES-NaOH (pH5.99), MOPS-NaOH (pH 7.04), Tris-HCl (pH 7.97), CHES-NaOH (pH 9.14), and CAPS-NaOH (10.0). Because of fluorescence loss of fluorescein in acidic conditions (pKa 7.5), substrate was used at a higher concentration (40 – 80 nM) at pH 3.22 than at pH 10.0 (5 – 10 nM). More RNase A is required at pH 3.22 and pH 10.0 (10 – 200 nM) than at pH 5.99 (0.1 – 0.5 nM). Increase of fluorescence intensity at 515 nm when excited at 490 nm indicates the progress of the reaction. Kinetic data was fit to eq 2.1 or 2.2 depending on the level of RNase A activity. The dependence on the values of $k_{cat}/K_m$ with respect to solution pH was fitted by non-linear least squares regression analysis to eq 2.5.

$$\log\left(\frac{k_{cat}}{K_m}\right) = \log\left[\frac{\frac{k_{cat}}{K_m}}{10^{(pH-pK_{a1})} + 1 + 10^{(pK_{a2}-pH)}}\right]$$

(2.5)

In eq. 2.5, $(k_{cat}/K_m)_0$ is the pH-independent rate constant and $pK_{a1}$ and $pK_{a2}$ are the two acid dissociation constants significant to catalysis. In RNase A, $pK_{a1}$ and $pK_{a2}$ are the acid dissociation constants for His12 and His119 respectively.
RESULTS

Efficacy of substrates. Spectroscopic and kinetic parameters for substrates 2.1 – 2.5 were determined in a continuous assay system. As shown for substrate 2.2 in Figure 2.2, the 6-TAMRA label quenches almost completely the fluorescence emission at 515 nm of the 6-FAM label. The emission maximum of intact substrate 2.2 at 577 nm is likely the result of fluorescence resonance energy transfer (FRET) from the 6-FAM label to the 6-TAMRA label. Cleavage of substrate 2.2 produces a large increase in fluorescence emission at 515 nm, with $I/I_0 = 180$ (Figure 2.2; Table 2.1). Replicate synthetic preparations of substrate 2.2 gave spectroscopic and kinetic parameters that did not differ significantly. We also synthesized 6-FAM–d(ATAA)–6-TAMRA, which is a deoxynucleotide version of substrate 2.2. We could not detect cleavage of this deoxynucleotide by RNase A (data not shown), suggesting that the cleavage of substrate 2.2 is caused by transphosphorylation to the 2' hydroxyl group of its single ribonucleotide.

Values of $k_{cat}/K_m$ for RNase A acting on substrates 2.1 – 2.5 are listed in Table 2.1. All the substrates have similar values of $k_{cat}/K_m$ (i.e., within 3-fold). Substrate 2.5 has the largest value of $k_{cat}/K_m$ at $6.6 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$. This value is insensitive to the changes in substrate concentration and enzyme concentration used in our assays.

Values of $k_{cat}/K_m$ for angiogenin acting on substrates 2.2 and 2.5 are listed in Table 2.2. The two substrates have similar values of $k_{cat}/K_m$ (i.e., within twofold), with substrate 2.5 having the larger value of $5.4 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$. 
To quantify the utility of fluorogenic substrates, we define sensitivity \( S \) as the increase in fluorescence intensity brought about by the action of the enzyme on a low concentration of substrate. We express sensitivity as the product of the kinetic parameter \( k_{\text{cat}}/K_m \) and the spectroscopic parameter \( I/I_0 \), as in eq 2.5:

\[
S = (k_{\text{cat}}/K_m)(I/I_0)
\]  

(2.5)

According to this analysis, substrate 2.2 is the optimal substrate for RNase A, with a sensitivity of \( S_{\text{RNase A}} = 6.5 \times 10^9 \text{M}^{-1}\text{s}^{-1} \). Likewise, substrate 2.2 is a better substrate for angiogenin than is substrate 2.5, with a sensitivity of \( S_{\text{angiogenin}} = 5.9 \times 10^4 \text{M}^{-1}\text{s}^{-1} \).

**Determination of \( K_m \) value.** Substrate 2.6 is an unlabeled analog of substrate 2.2. The value of \( K_m \) for substrate 2.6, as determined in a continuous assay by monitoring inhibition of the cleavage of substrate 2.2, is 22 \( \mu \text{M} \). This value is >25-fold larger than the substrate concentration in any assay described herein.

**Determination of \( K_i \) values.** Inhibition of wild-type RNase A by 3'-'UMP and 5'-ADP was assessed in a continuous assay. The effect of 3'-UMP and 5'-ADP concentration on relative activity \([\Delta I/\Delta t]/(\Delta I/\Delta t)_0\] is shown in Figure 2.3. The effect of 5'-ADP concentration on absolute activity \([\Delta I/\Delta t]\) is shown in the inset of Figure 2.3. The \( K_i \) values for inhibition by 3'-UMP and 5'-ADP were \((60 \pm 3) \mu \text{M}\) and \((8.4 \pm 1.0) \mu \text{M}\), respectively.
Determination of enzymic $pK_a$ values. The bell-shaped dependence of the values of $k_{\text{cat}}/K_m$ on the solution pH is shown in Figure 2.4. The value of the pH-independent $k_{\text{cat}}/K_m$ is $6.3 \times 10^8$ M$^{-1}$s$^{-1}$. The values of the acid dissociation constants $pK_{a1}$ and $pK_{a2}$ are 5.7 and 6.7 respectively.
DISCUSSION

*Substrate sensitivity with ribonuclease A.* 6-FAM-(dA)rU(dA)₂-6-TAMRA (substrate 2.2; Figure 2.1) is the smallest substrate for RNase A that both fills the known subsites of the enzyme and can be synthesized on a solid phase with commercial reagents. The cleavage of substrate 2.2 is accompanied by a 180-fold increase in fluorescence (Figure 2.2; Table 2.1). This increase is the largest reported for a ribonuclease substrate.

The kinetic parameters of the substrates follow a predictable trend. The value of $k_{cat}/K_m$ increases with increasing nucleotide length. Cationic residues of RNase A make contact with the phosphoryl groups of a bound substrate at several subsites remote from the active site (Fisher et al., 1998b). These Coulombic interactions allow RNase A to use a mechanism of facilitated diffusion in which binding to adenosine nucleotides accelerates contact with specific sites of cleavage (Kelemen & Raines, 1999). Thus as substrate length increases, the value of $k_{cat}/K_m$ increases. The value of $k_{cat}/K_m$ for the cleavage of the dinucleotide substrate of Hofsteenge and coworkers is $2.06 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$. This value is similar to that for the dinucleotide substrate 2.1 (Table 2.1). The value of $k_{cat}/K_m$, $6.9 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, for the nonanucleotide substrate of James and Woolley is similar to that for octanucleotide substrate 2.4.

RNase A has a slight preference for the substrate cytidylyl(3'→5')adenosine (CpA) in comparison to the substrate uridylyl(3'→5')adenosine (UpA) (Witzel & Barnard, 1962). Accordingly, we synthesized substrate 2.5, which is identical to
substrate 2.2 but with cytidine in place of uridine. Substrate 2.5 has a greater value of $k_{cat}/K_m$ than does substrate 2.2. However, substrate 2.5 has a twofold lower value of $I/I_0$. The structural reason for this discrepancy is not readily apparent. One possibility is that protonation of cytidine at N3 [$pK_a$ 4.3 (Manzini et al., 1990)] may alter the conformation of the oligonucleotide or provide an interaction with fluorescein in the substrate that disrupts quenching.

The sensitivity, defined as the product of $k_{cat}/K_m$ and $I/I_0$, of substrate 2.2 cleaved by RNase A is larger at $S_{RNase A} = 6.5 \times 10^9$ M$^{-1}$s$^{-1}$ than that of the other substrates listed in Table 2.1. The kinetic parameters for all the substrates are, however, within an order of magnitude of one another. The sensitivity is greatest for substrate 2.2 because of its large change in fluorescence intensity.

We also synthesized substrates analogous to substrates 2.1 – 2.4 with 2',4,5,6,7,7'-hexachlorofluorescein rather than fluorescein as the fluorophore. The emission spectrum of 2',4,5,6,7,7'-hexachlorofluorescein has a greater overlap with the excitation spectrum of 6-TAMRA. All four members of this class of substrates had kinetic parameters similar to their fluorescein counterparts, but smaller increases in fluorescence after cleavage (data not shown).

**Substrate sensitivity with angiogenin.** Angiogenin is a homolog of RNase A that likewise catalyzes RNA cleavage (Riordan, 1997). The ribonucleolytic activity of angiogenin is essential for its angiogenic activity (Shapiro & Vallee, 1989). Indeed, variants of angiogenin with greater ribonucleolytic activity are more effective at
promoting neovascularization (Harper & Vallee, 1988). Angiogenin is, however, a much less effective catalyst of RNA cleavage than is RNase A in typical assays. Accordingly, assays of catalysis by angiogenin are most often done in a discontinuous manner. Like RNase A, angiogenin cleaves CpA faster than it does UpA (Shapiro, 1998). This nucleotide specificity is apparent, though less pronounced, in the cleavage of substrates 2.2 and 2.5 (Table 2.2). Most remarkable, however, is our finding that angiogenin cleaves substrate 2.2 102-fold faster than it does UpA. Additional interactions with substrates 2.2 and 2.5 apparently have a profound effect on catalysis by angiogenin. Thus, substrates 2.2 and 2.5 are exceptional substrates with which to analyze catalysis by angiogenin.

Value of \( K_m \). The value of \( K_m \) for substrate 2.6 determined herein is 22 µM. This value is similar to the value of \( K_d = 88 \) µM for the RNase A•d(AUAA) complex, which was studied under similar conditions (Fisher et al., 1998b). Likewise, \( K_m = 33 \) µM for the nonanucleotide substrate of James and Woolley (James & Woolley, 1998). Using the values of \( k_{cat}/K_m \) for substrate 2.2 and \( K_m \) for substrate 2.6, we can estimate that the value of \( k_{cat} \) for substrate 2.2 is \( 6 \times 10^2 \) s\(^{-1}\). This value of \( k_{cat} \) is similar to that for the cleavage of the dinucleotide UpA [350 s\(^{-1}\) (Fisher et al., 1998c)].

Values of \( K_i \). Substrate 2.2 enables an efficient method to evaluate inhibition of ribonucleolytic activity. Because the entire assay is performed by the cumulative
addition of inhibitor to one 2-ml solution, determining the potency of an inhibitor requires little over an hour of time and requires minimal materials. By using this assay, we determined that the values of $K_i$ for the inhibition of RNase A by 3'-UMP and 5'-ADP are 60 μM and 8.4 μM, respectively.

In the RNase A•3'-UMP complex, the phosphoryl group of 3'-UMP interacts directly with the active-site residues (Fisher et al., 1998c). The value of $K_i = 60 \mu M$ for 3'-UMP is in gratifying agreement with the value of $K_d = 54 \mu M$ for the RNase A•3'-UMP complex determined under identical conditions by isothermal titration calorimetry (Fisher et al., 1998c). In the RNase A•5'-diphosphoadenosine 3'-phosphate complex, the pyrophosphoryl group of 5'-diphosphoadenosine moiety interacts directly with the active-site residues (Leonidas et al., 1997). The value of $K_i = 8.4 \mu M$ for 5'-ADP is somewhat larger than a value of $K_d = 1.2 \mu M$ determined in a solution of lower ionic strength (Russo et al., 1997), as expected for an interaction that relies on Coulombic forces (Fisher et al., 1998b).

This assay to evaluate competitive inhibition has other applications. For example, some active-site variants of RNase A have a ribonucleolytic activity much lower than that of the wild-type enzyme (Thompson & Raines, 1994). If a preparation of one of these variants is contaminated by another ribonuclease, then the value of $K_i$ would be that for inhibition of the contaminant. If the predominant catalytic agent were the site-directed variant, then the $K_i$ would match the $K_d$ determined by other methods, such as isothermal titration calorimetry.
**pH dependence of RNase A catalysis.** The values of the acid dissociation constants $pK_{a1}$ and $pK_{a2}$ of 5.7 and 6.7 respectively are similar to the values reported for the hydrolysis of cytidine 2',3' cyclic phosphate of 5.7 and 6.0 (Schultz et al., 1998) and the values reported for cleavage of UpA of 5.7 and 5.9 (Thompson, 1995). The value of the pH independent $k_{cat}/K_m$ of $6.3 \times 10^8$ M$^{-1}$s$^{-1}$ is similar to the reported value for the pH independent $k_{cat}/K_m$ of UpA of $1.6 \times 10^7$ M$^{-1}$s$^{-1}$ (Thompson, 1995).

**Conclusions.** We report the systematic optimization of a fluorogenic substrate for RNase A. We find that the tetranucleotide 6-FAM–dArUdA–6-TAMRA (Figure 2.1) provides the most sensitive continuous assay for ribonucleolytic activity reported to date. This substrate, which can be prepared from commercial reagents by solid-phase synthesis, is likely to have many uses. For example, we have demonstrated its use in a rapid assay to screen for inhibitors of RNase A. Other possible applications include detecting ribonucleolytic activity in “ribonuclease-free” samples and evaluating new enzymic or small-molecule catalysts of RNA cleavage.

**Acknowledgment.** I am grateful to Brian Elliott for assistance with the synthesis and purification of oligonucleotides.
Table 2.1. Parameters for the cleavage of fluorogenic substrates by ribonuclease A.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}/K_m$ (10^7 M⁻¹ s⁻¹)ᵃ</th>
<th>$I_f/I_o$ᵇ</th>
<th>Sensitivity (10^8 M⁻¹ s⁻¹)ᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 6-FAM–rUdA–6-TAMRA</td>
<td>2.5 ± 0.3</td>
<td>15 ± 2</td>
<td>3.8 ± 0.7</td>
</tr>
<tr>
<td>2.2 6-FAM–(dA)rU(dA)₂–6-TAMRA</td>
<td>3.6 ± 0.4</td>
<td>180 ± 10</td>
<td>65 ± 8</td>
</tr>
<tr>
<td>2.3 6-FAM–(dA)₂rU(dA)₃–6-TAMRA</td>
<td>4.7 ± 0.6</td>
<td>26 ± 3</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>2.4 6-FAM–(dA)₃rU(dA)₄–6-TAMRA</td>
<td>4.8 ± 0.5</td>
<td>62 ± 2</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>2.5 6-FAM–(dA)rCp(dA)₂–6-TAMRA</td>
<td>6.6 ± 0.4</td>
<td>83 ± 1</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>6-FAM–(dA)₄rU(dA)₄–6-TAMRAᵈ</td>
<td>6.9 ± 0.7</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td>DUPAAAAᵉ</td>
<td>2.06 ± 0.08</td>
<td>60</td>
<td>12</td>
</tr>
</tbody>
</table>

ᵃ Data were obtained in 0.10 M MES-NaOH buffer, pH 6.0, containing NaCl (0.10 M). For comparison, the value of $k_{cat}/K_m$ for the cleavage of uridylyl(3'→5')adenosine by ribonuclease A is 1.7 × 10⁶ M⁻¹ s⁻¹ (Fisher et al., 1998c).

ᵇ $I_f/I_o$ is ratio of the fluorescence intensity of product ($I_f$) and substrate ($I_o$).

ᶜ Sensitivity ($S$) for catalysis by ribonuclease A is defined by eq 2.5.

ᵈ From ref (James & Woolley, 1998).

ᵉ From ref (Zelenko et al., 1994).
**Table 2.2. Parameters for the cleavage of fluorogenic substrates by human angiogenin.**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}/K_m$  ((10^2 M^{-1}s^{-1})^a)</th>
<th>$I_f/I_o^b$  (10^4 M^{-1}s^{-1})^c)</th>
<th>Sensitivity  ((10^4 M^{-1}s^{-1})^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2 6-FAM-(dA)rU(dA)r6-TAMRA</td>
<td>3.3 ± 0.4</td>
<td>180 ± 10</td>
<td>5.9 ± 0.8</td>
</tr>
<tr>
<td>2.5 6-FAM-(dA)rC(dA)r6-TAMRA</td>
<td>5.4 ± 0.4</td>
<td>83 ± 1</td>
<td>4.5 ± 0.3</td>
</tr>
</tbody>
</table>

\(^a\) Data were obtained in 0.10 M MES-NaOH buffer pH 6.0, containing NaCl (0.10 M). For comparison, the value of $k_{cat}/K_m$ for the cleavage of uridylyl(3'→5')adenosine by human angiogenin is 1.2 $M^{-1}s^{-1}$ (Shapiro, 1998).

\(^b\) $I_f/I_o$ is ratio of the fluorescence intensity of product ($I_f$) and substrate ($I_o$), as in Table 2.1.

\(^c\) Sensitivity ($S$) for catalysis by human angiogenin is defined by eq 2.5.
Figure 2.1 Chemical structure of substrate 2.2, 6-FAM–dArGdA–6-TAMRA, where 6-FAM refers to 6-carboxyfluorescein and 6-TAMRA refers to 6-carboxytetramethylrhodamine. The italicized text refers to Ribonuclease A subsites known to interact with nucleic acid bases (B1, B2, and B3) and phosphoryl groups [P(–1), P(0), P(1), and P(2)] (Fisher et al., 1998a).
Figure 2.2  A. Fluorescence emission intensity of substrate 2.2 (solid line) and its cleavage products (dashed line) as a function of wavelength upon excitation at 490 nm.

B. Fluorescence emission intensity (515 nm; excitation: 490 nm) of substrate 2.2 (84 nM) at 2-s intervals after addition of RNase A (0.11 nM). Reaction was performed in 0.10 M MES-NaOH buffer, pH 6.0, containing NaCl (0.10 M). Inset: data collected during the first 30 s of the reaction.
Figure 2.3  The dependence of the relative ribonucleolytic activity of ribonuclease A \([\Delta I/\Delta t]/(\Delta I/\Delta t)_0\) on the concentration of 3'-UMP (open symbols) or 5'-ADP (closed symbols). Triangle, circle, and square symbols represent data collected in independent reactions. Inset shows dependence of absolute Ribonuclease A activity \([(\Delta I/\Delta t)]\) on the concentration of 5'-ADP. Reactions were carried out in 0.10 M MES-NaOH buffer, pH 6.0, containing NaCl (0.10 M). Data were analyzed using eq 2.4.
Figure 2.4  The dependence of the values of $k_{cat}/K_m$ of ribonuclease A on the solution pH. Each pH is represented in triplicate and all data is used in the non-linear least-squares fitting to eq 2.5. Experimental conditions are described in the text.
Chapter 3

Interplay Between the Specificity and Activity of Ribonuclease A

SUMMARY

Bovine pancreatic ribonuclease A (RNase A) catalyzes the specific degradation of RNA after pyrimidine nucleotides. When bound in the active site, the base of a pyrimidine nucleotide forms a hydrogen bond with the side chain of Thr4S. Here, the nucleotide specificity of wild-type RNase A and its T45G variant are quantitated and compared. The crystalline structure of T45G RNase A, determined at 1.8 Å resolution by X-ray diffraction analysis, shows little change from that of the wild-type enzyme. The crystalline structure of T45G RNase A indicates only minor variance and an increase in flexibility with respect to the structure of wild-type RNase A near Gly45, and does not reveal new potential interactions with a nucleic acid base. To assess the catalytic specificity of RNase A, we measured the cleavage rates of fluorogenic substrates of the form: 6-carboxyfluorescein-dArXdAdA-6-carboxytetramethylrhodamine (6-FAM-dArXdAdA-6-TAMRA), where X is cytidine, uridine, adenosine, and guanine. Wild-type RNase A cleaves 6-FAM-dArCdAdA-6-TAMRA 10^6-fold faster than it cleaves 6-FAM-dArAdAdA-6-TAMRA. In contrast, the value of the equilibrium dissociation constant, $K_d$, for the complex of RNase A with the non-hydrolyzable nucleotide 6-carboxyfluorescein-d(CAA) [6-FAM-d(CAA)] is merely 50-fold tighter than that for the complex with 6-FAM-d(AAA). T45G RNase A cleaves 6-FAM-dArAdAdA-6-TAMRA with a value of $k_{cat}/K_m$ more than 10^2-fold greater than that of the wild-type enzyme. T45G RNase A binds 6-FAM-d(AAA) with a value of $K_d$ that is
approximately the same as that for the wild-type enzyme. Thus, the enhanced ability of the T45G enzyme to cleave after adenosine nucleotides appears to arise simply from an enlarging of the active site to accommodate adenosine. The importance of the conformation of the ribose ring to nucleotide specificity of RNase A was investigated using 2'-deoxy-2'-fluoro residues. The significance of the ribose ring conformation is evident from a decrease in binding affinity of wild-type RNase A for a ligand containing 2'-deoxy-2'-fluorouridine (U$^F$), 6-carboxyfluorescein-d(AU$^F$AA), rather than 2'-deoxyuridine. T45G RNase A does not discriminate between dU$^F$ and dU, indicating that the conformation of the ribose ring is not significant without a hydrogen bond from Thr45 to the uridine base. From these data, we propose that the specificity of RNase A is generated by both favorable interactions with pyrimidine nucleotides and unfavorable steric interactions with purine nucleotides.
INTRODUCTION

Bovine pancreatic ribonuclease A [RNase A (D'Alessio & Riordan, 1997; Raines, 1998; Raines, 1999); EC 3.1.27.5] catalyzes a transphosphorylation reaction resulting in RNA strand breakage on the 3' side of pyrimidine nucleotides. The specificity of RNase A for pyrimidine nucleotides is enforced by an interaction of the pyrimidine nucleotide with an active-site threonine residue, Thr45 (delCardayre & Raines, 1995b). This threonine residue, present in all members of the RNase A superfamily (Beintema et al., 1988), is a component of the primary specificity subsite (B1) where it forms hydrogen bonds with a pyrimidine base. The catalytic specificity of RNase A for poly(cytidylic acid) [poly(C)] over poly(adenylic acid) [poly(A)] is profound (i.e., $10^4$ relative $k_{cat}/K_m$)(delCardayre & Raines, 1995b).

The active site of RNase A is composed of two histidine residues, His12 and His119, and one lysine residue, Lys41. These residues are completely conserved in the RNase A superfamily and are also referred to as the primary phosphoryl group binding subsite (P1). Acid/base catalysis by RNase A is accomplished by the two histidines, with His12 deprotonating the 2' hydroxyl group of a pyrimidine nucleotide and His119 protonating the 5' alkoxide ion of the subsequent nucleotide (Thompson & Raines, 1994). RNase A also stabilizes the transition state through a hydrogen bond donated by Lys41 (Messmore et al., 1995). This transphosphorylation reaction most likely proceeds in a concerted manner (Sowa et al., 1997).
The residues His12, His119 and Lys41 interact with phosphoryl groups of RNA and DNA through Coulombic forces and hydrogen bonds (Fisher et al., 1998c). The residue Thr45 interacts with pyrimidine bases through a hydrogen bond. From data quantitating enzymic interactions with substrate and substrate analogues, we herein propose that the catalytic specificity of RNase A arises from the interaction of His12, His119, and Lys41 with Thr45 through the bound substrate.
MATERIALS AND METHODS

Protein Production and Purification. T45G RNase A was produced and purified by the methods described previously (delCardayre & Raines, 1994; delCardayré & Raines, 1995b). Briefly, T45G RNase A was produced in inclusion bodies in *E. coli*. Cells were lysed by passage through a French pressure cell. Inclusion bodies were isolated by centrifugation, then reduced and denatured. T45G RNase A was oxidatively refolded, and purified by gel filtration chromatography followed by cation exchange chromatography. The protein solution was then dialyzed exhaustively against water and lyophilized. Wild-type RNase A was produced by a similar method and was a generous gift of Dr. Barbara M. Fisher.

Analytical Instrumentation. Time-based fluorescence readings were collected with excitation at 490 nm and emission at 515 nm using a QuantaMaster QM1 fluorometer from Photon Technologies International (South Brunswick, NJ) equipped with stirring and temperature control. Fluorescence anisotropy was measured at room temperature (23 ± 2 °C) on a Beacon fluorescence polarization system from PanVera (Madison, WI). Wild-type and T45G RNase A concentration was determined assuming ε = 0.72 ml mg⁻¹ cm⁻¹ measured at 277.5 nm in a Cary Model 50 spectrophotometer from Varian (Sugar Land, TX) equipped with a temperature controller.

Crystalline Structure of T45G Ribonuclease A. Protein Crystallization. Crystals of T45G RNase A were prepared by vapor diffusion using the hanging drop method.
Drops (6 μL) of 0.050 M sodium acetate buffer, pH 5.6, containing T45G RNase A (60 mg/mL), ammonium sulfate (15% w/v) and sodium chloride (25% w/v) were suspended over 1.0 mL wells of 0.10 M sodium acetate buffer, pH 5.6, containing ammonium sulfate (30% w/v) and sodium chloride (50% w/v). Single tetragonal crystals grew out of amorphous precipitate at 20 °C, appeared within 3 days, and grew to a final size of 0.7 × 0.7 × 0.7 mm.

**Data Collection.** The crystals of T45G RNase A were of space group P3_21, with \(a = 64.32\) Å, \(c = 64.84\) Å, \(\alpha = \beta = 90^\circ\), and \(\gamma = 120^\circ\). X-ray data were collected with a Siemens HI-STAR detector mounted on a Rigaku rotating anode operating at 50 kV, 90 mA, and a 300 μm focal spot. The X-ray beam was collimated by double-focusing mirrors. The crystal-to-detector distance was 12.0 cm. Data were obtained in 512 × 512 pixel format, processed with the program XDS (Kabsch, 1988a; Kabsch, 1988b), and scaled using the program Xscalibre (G. Wesenberg and I. Rayment, unpublished results). Frames of data were collected from a single crystal using \(\phi\)-scans. Reflections with \(|I|/|\sigma| < 0.33\) were rejected. The crystal was cooled in a 4 °C air stream, resulting in negligible crystal decay for the entire data collection. Full crystallographic details are listed in Table 3.1.

**Refinement.** Prior to least-squares refinement \(2|F_o| - |F_c|, |F_o| - |F_c|,\) and \(\sigma A\) (Collaborative Computational Project, Number 4 1994) difference maps were calculated using the data from 30–3.5 Å. The model was examined and was continuous in the density for the entire chain. However, there was some disorder apparent in the C-terminus, from His119 to Ala122. The starting model (pdb entry
7RSA minus residues 119 – 122) was subjected to 10 cycles of least-squares refinement using TNT (Tronrud et al., 1987) and the data from 30 – 3.0 Å, giving an initial R-factor of 0.252. A negative difference fourier map (|F_o| – |F_c|) showed clearly the absence of the Thr45 side chain. The Thr45 side chain was removed and the model refined to 2.0 Å. Manual adjustments to the model were performed in TurboFrodo (Cambillau et al., 1997). The model was further refined to 1.8 Å and the difference map (|F_o| – |F_c|) showed two clear conformations for residues 119 – 122. These residues were fitted and refined in two separate conformations with 50% occupancy for each (determined iteratively). After several cycles of manual adjustments and least-squares refinement, water molecules were added to the model. The peak-searching algorithm in TNT was used to place ordered water molecules. Water molecules were retained if they had at least 1σ of 2|F_o| – |F_c| density, 3σ of |F_o| – |F_c| density, and were within hydrogen bonding distance of the protein or other water molecules. Atomic coordinates for T45G RNase A have been deposited in the Brookhaven Protein Data Bank with accession code 3RST.

**Fluorogenic Substrates.** Substrates for the analysis of the catalytic specificity of RNase A were a generous gift from Integrated DNA Technologies (Coralville, IA) and were synthesized from commercial reagents as we described previously (Kelemen et al., 1999). The sequence of these substrates is 6-carboxyfluorescein–dArXdAdA–6-carboxytetramethylrhodamine (6-FAM–dArXdAdA–6-TAMRA), where X is uridine, cytidine, adenosine, or guanine. The concentrations of 6-FAM–dArCdAdA–6-TAMRA, 6-FAM–dArUdAdA–6-
TAMRA, 6-FAM~dArAdAdA~6-TAMRA, or 6-FAM~dArGdAdA~6-TAMRA were determined by assuming \( \varepsilon = 99,900 \text{ M}^{-1}\text{cm}^{-1} \), \( 102,000 \text{ M}^{-1}\text{cm}^{-1} \), \( 108,000 \text{ M}^{-1}\text{cm}^{-1} \), and \( 104,000 \text{ M}^{-1}\text{cm}^{-1} \) respectively measured at 260 nm.

Assays were carried out in a 2.00 mL volume of 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M), substrate (5.0 nM – 20 nM), and wild-type RNase A (1.0 pM – 1.0 \( \mu \text{M} \)) or T45G RNase A (5.0 nM – 0.30 \( \mu \text{M} \)). Data collected in this manner were fitted to eq 3.1 or 3.2, depending on the extent of product formation during the course of data collection, using Deltagraph 4.0 from DeltaPoint (Monterey, CA).

\[
I = I_t - (I_t - I_o) \text{e}^{-kt}
\]  
(3.1)

\[
I = I_o + (I_t - I_o) \text{O}^{obs}t
\]  
(3.2)

The intensity of fluorescence \( (I) \) was recorded in units of photon counts per second (CPS). Product fluorescence intensity \( (I_t) \) was determined for each experiment by nonlinear least-squares regression analysis using eq 3.2 after the addition of sufficient enzyme to complete the reaction within 30 min. T45G RNase A was used to determine product intensity for 6-FAM~dArAdAdA~6-TAMRA and ribonuclease T1 from Sigma Chemical (St. Louis, MO) was used to determine product intensity for 6-FAM~dArGdAdA~6-TAMRA. Substrate intensity was determined by averaging data collected prior to enzyme addition (for 2 – 5 min). The pseudo-first order rate constants, \( k \) and \( k_{obs} \), were determined at substrate concentrations below \( K_m \) (Kelemen
et al., 1999). Values of $k_{cat}/K_m$ were determined by dividing the pseudo-first order rate constant by the enzyme concentration. Values of $k_{cat}/K_m$ determined in this manner were independent of enzyme and substrate concentration within the range of substrate and enzyme concentrations used.

Fluorescence Anisotropy. We determined the affinities of wild-type RNase A and the T45G variant for a variety of fluorescein-labeled oligonucleotide ligands using methods outlined previously (Fisher et al., 1998b). These oligonucleotides were from Promega (Madison, WI) and include 6-FAM–d(UAA), 6-FAM–d(CAA), 6-FAM–d(AAA), 6-FAM–d(ØAA), 6-FAM–d(AUAA) and Fl-(dAU₅AA), where Ø refers to an abasic analog of DNA, U₅ refers to 2′-deoxy-2′-fluorouridine, and 6-FAM– refers to 6-carboxyfluorescein attached to the 5′ end of the oligonucleotides by a six-carbon spacer through a terminal 5′ phosphoryl group (Figure 3.1). All ligands were produced from commercially available reagents and were purified by extraction from a polyacrylamide gel after electrophoresis. Concentration of ligands 6-FAM–d(AUAA) and 6-FAM–d(AU₅AA) were determined by assuming ε = 66,300 M⁻¹cm⁻¹ measured at 260 nm. Concentration of ligands 6-FAM–d(UAA), 6-FAM–d(CAA), 6-FAM–d(AAA), and 6-FAM–d(ØAA) by assuming ε = 51,100 M⁻¹cm⁻¹, 49,700 M⁻¹cm⁻¹, 57,900 M⁻¹cm⁻¹, and 42,700 M⁻¹cm⁻¹ respectively measured at 260 nm.

Wild-type RNase A or the variant T45G was dissolved to a concentration of between 1 mM and 2 mM in 2.00 mL of 0.020 M MES-NaOH buffer (pH 6.0) containing NaCl (0.050 M) for the tetranucleotide ligands or in 2.00 mL of 0.10 M
MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M) for the trinucleotide ligands. The protein sample was filtered and partitioned between a sample tube (0.90 mL) and a blank tube (0.90 mL). Oligonucleotide ligand (0.10 mL of a 100 nM solution in the appropriate buffer) was added to the sample tube, and buffer (0.10 mL) was added to the blank tube. The anisotropy of each sample was measured three to five times, with the blank reading taken before each sample. The anisotropy values were averaged. The sample was diluted by removing a 0.25 mL aliquot, which was replaced with an equal volume of ligand (10 nM) in buffer. The blank was diluted similarly, with an equal volume of buffer added. The anisotropy of each sample was again measured three to five times, with the blank read before each sample and the anisotropy values averaged. This process was repeated up to twenty times to complete each binding isotherm. At the end of the experiment, the exact protein concentrations of both the blank and the sample for the first five protein concentrations were determined. The remaining protein concentrations were calculated from the dilutions at each step.

The equilibrium dissociation constants, $K_d$, were determined by weighted, non-linear least squares fitting of the data to eq 3.3 using the program Deltagraph 4.0 from DeltaPoint (Monterrey, CA).

$$A = \frac{\Delta A[RNase \ A]}{K_d + [RNase \ A]} + A_{min} \quad (3.3)$$
In eq 3.3, $A$ is the average of the measured anisotropy values, $\Delta A (= A_{\text{max}} - A_{\text{min}})$ is the difference in anisotropy values of bound and free ligand, $A_{\text{min}}$ is the anisotropy of the unbound ligand, [RNase A] is the total protein concentration, and $K_d$ is the equilibrium dissociation constant.
RESULTS

*Crystalline Structure of T45G Ribonuclease A.* The structure of T45G RNase A was refined to an $R$-factor of 0.176 using data from 30 – 1.8 Å, Table 3.1. The RMS deviation from ideal bond length is 0.011 Å, and RMS deviation from ideal bond angle is 2.4°. Average $B$-factors are 25.1 Å$^2$ for main-chain atoms and 38.7 Å$^2$ for side-chain atoms. The electron density is continuous for the main chain and most side chains. The presence of Gly45 (or absence of Thr45) is unambiguous and clearly defined in both $2|F_o| - |F_c|$ density and annealed omit $|F_o| - |F_c|$ density.

The overall structure of T45G RNase A shows little variation from that of wild-type RNase A (main-chain RMS 0.61 Å from pdb entry 7rsa) with the largest deviation occurring at the main chain of residues Lys66 and Asn67 (Figure 3.2). The side-chain residues in proximity of Gly45 are less resolved than those residues around the corresponding Thr45 of wild-type RNase A (Figure 3.3). The side chain of Ile81 is directly adjacent to Gly45 and occupies two conformations ($\chi_{1A} = 64°$ and $\chi_{1B} = 173°$). The side chain of Asp83 also occupies two conformations ($\chi_{1A} = -72°$ and $\chi_{1B} = -165°$). The residue Asp83 of wild-type RNase A (pdb: 7rsa) also exists in two conformations; however these two conformations are not the same as those observed in the T45G RNase A structure ($\chi_{1A} = -110°$ and $\chi_{1B} = -179°$).

The side chain of Phe120, part of the B1 subsite, has density for all but the $C_{el}$, $C_{e2}$ and $C_\zeta$ atoms and occupies a different conformation ($\chi_1 = -115°$ and $\chi_2 = 160°$) than that observed in the wild-type RNase A crystalline structure ($\chi_1 = -169°$ and $\chi_2 =$...
−85°). The main-chain carbonyl of Phe120 exists in two positions (ϕ = −90° and ψ = 149°; ϕ = −100° and ψ = 100°). This dichotomy has not been observed for Phe120 of wild-type RNase A (ϕ = −98° and ψ = 116°).

As in some crystalline structures of wild-type RNase A, the active-site residue His119 in the T45G variant also occupies two positions, referred to as A and B (χ1A = 175° and χ1B = −59°). The electron density defining the A position is not, however, substantial enough to establish exact rotation about the χ2 angle of this residue. For this reason, we caution against over interpretation of distances to his119 in the A position. In the A position of the T45G RNase A crystalline structure, His119 is within hydrogen bonding distance from one of two conformations of the main-chain carbonyl of Phe120 (distance of 2.5 Å from Nε2 of His119 to NH of Phe120). In the wild-type structure, the side chain of His119 forms a hydrogen bond with Asp121 (distance of 2.91 Å from Nε2 of His119 to Oδ1 of Asp121). This hydrogen bond is not present in the structure of T45G RNase A (distance of 4.35 Å from Nε2 of His119 to Oδ1 of Asp121).

The main-chain carbonyl of residue 45 forms a hydrogen bond with Nε1 of His12 as is observed in the wild-type structure. Likewise, the side chain of Asp121 forms a hydrogen bond with the main chain of Lys66, as in the wild-type structure, and a conserved water molecule is also present in a hydrogen-bonding network despite the movement of residues Lys66 and Asn67 away from the active site (displaced by 1.5 Å RMS from wild-type RNase A).
An acetate molecule is present in the active site and interacts with His12 (distance of 2.65 Å from Nε2 to O2 of acetate), main chain of Phe120 (distance 3.22 Å from NH to O1 of acetate), and His119 (distance of 2.59 Å from Nδ1 to O1 of acetate). There also exist three chloride ions in the structure in positions similar to those observed by Almo and coworkers (Federov et al., 1996).

**Kinetic Specificity of Ribonuclease A.** The values of the kinetic parameter $k_{cat}/K_m$ for the cleavage of four tetranucleotide substrates by wild-type RNase A and the T45G variant were determined in a continuous manner by following the increase of fluorescence after enzyme addition (Figure 3.4A). The values $k_{cat}/K_m$ for the cleavage of 6-FAM–dArUdAdA–6-TAMRA and 6-FAM–dArCdAdA–6-TAMRA by wild-type RNase A were determined previously to be $(3.6 \pm 0.4) \times 10^7$ and $(6.6 \pm 0.4) \times 10^7$ M$^{-1}$s$^{-1}$ (Kelemen et al., 1999). The value of $k_{cat}/K_m$ for the cleavage of 6-FAM–dArAdAdA–6-TAMRA by wild-type RNase A is $(1.8 \pm 0.2) \times 10^1$ M$^{-1}$s$^{-1}$. The cleavage of 6-FAM–dArGdAdA–6-TAMRA by wild-type RNase A is extremely slow; we estimate the value of $k_{cat}/K_m$ to be $< 0.1$ M$^{-1}$s$^{-1}$. The values of $k_{cat}/K_m$ for the cleavage of 6-FAM–dArUdAdA–6-TAMRA, 6-FAM–dArCdAdA–6-TAMRA, and 6-FAM–dArAdAdA–6-TAMRA by T45G RNase A are $(7.5 \pm 0.8) \times 10^5$, $(3.3 \pm 0.3) \times 10^6$, and $(2.3 \pm 0.2) \times 10^3$, respectively. The cleavage rate of 6-FAM–dArGdAdA–6-TAMRA by T45G RNase A is low; we estimate the value of $k_{cat}/K_m$ to be $< 0.5$ M$^{-1}$s$^{-1}$.

**Ground-State Base Specificity of Ribonuclease A.** The value of the dissociation constant, $K_d$, for the complex of wild-type RNase A and the T45G variant with four
trinucleotides, 6-FAM~d(CAA), 6-FAM~d(UAA), 6-FAM~d(AAA), and 6-
FAM~d(ØAA), was determined by fluorescence anisotropy (Figure 3.5A). Wild-type
RNase A has a tighter affinity for nucleotides containing pyrimidine, 6-
FAM~d(CAA) at $70 \pm 2 \mu M$ and 6-FAM~d(UAA) at $130 \pm 10 \mu M$, than for the
nucleotides that contain either adenosine or an abasic analog of DNA, 6-
FAM~d(AAA) at $3.2 \pm 0.1 \text{mM}$ and 6-FAM~d(ØAA) at $2.5 \pm 200 \text{mM}$ (Table 3.3).
The affinity of T45G RNase A for 6-FAM~d(CAA), 6-FAM~d(UAA), 6-
FAM~d(AAA), and 6-FAM~d(ØAA) is approximately the same at $0.97 \pm 0.05$, $1.5 \pm
0.1$, $2.3 \pm 0.1$, and $1.3 \pm 0.1 \text{mM}$, Table 3.3.
The affinity of RNase A for the fluorescein labeled tetranucleotides 6-
FAM~d(AUAA) and 6-FAM~d(AU\text{F}AA) was determined by fluorescence
anisotropy. The affinity of wild-type RNase A and the T45G variant for these two
ligands is listed in Table 3.2. The value of the dissociation constant, $K_d$, for wild-type
RNase A bound to 6-FAM~d(AUAA) and 6-FAM~d(AU\text{F}AA) is $12 \pm 3 \mu M$ and $640
\pm 220 \mu M$. The value of the dissociation constant, $K_d$, for T45G RNase A bound to 6-
FAM~d(AUAA) and 6-FAM~d(AU\text{F}AA) is $120 \pm 20 \mu M$ and $87 \pm 47 \mu M$. 
DISCUSSION

*Crystalline Structure of T45G Ribonuclease A.* In the structure of T45G RNase A, the absence of van der Waals contacts with Gly45 has resulted in some disorder and rotation of the side chain of Phe120. The lack of side-chain rigidity has allowed movement in the main chain of Phe120. The multiple positions of the main-chain atoms of Phe120 could have dramatic consequences for catalysis. The main-chain nitrogen of Phe120 donates a hydrogen bond to the transition state (Hondal et al., 1999). The hydrogen bond observed between His119 and the main-chain carbonyl of Phe120 could be a result of the flexibility of the C-terminal main-chain atoms induced by the loss of interactions near Gly45. Regardless, the interaction of the active-site residue His119 with Asp121 has been lost in this structure. This interaction is, however, more important to the stability of RNase A than to its catalytic activity (Quirk et al., 1998). Indeed, the $T_m$ of T45G RNase is 10 °C lower than the wild-type enzyme (delCardayré & Raines, 1995b).

The absence of van der Waals contact of Gly45 C$_\alpha$ with Ile81 allows for rotation of Ile81, causing this residue to occupy two positions. Like Ile81, Asp83 is in two positions because of space vacated by replacing Thr45 with Gly. Asp83 hydrogen bonds with Thr45 when the wild-type enzyme binds to uridine nucleotides (delCardayré & Raines, 1995b).

The structure of the active site of T45G RNase A does not give any indication of an improved complementarity with adenine bases. The ground state binding data also
indicates that no new binding interactions with adenosine were created in T45G RNase A. Therefore, the dramatic increase of catalytic competency of T45G RNase A for cleavage on the 3' side of adenosine nucleotides over that of wild-type RNase A arises from an opening and flexibility of the B1 subsite, allowing key active-site residues to access the scissile phosphodiester bond. From the crystalline structure, we conclude that Thr45 provides specificity not only through complementarity with pyrimidine bases, but also through exclusion of purine bases.

Kinetic Specificity of Ribonuclease A. We determined the kinetic specificity of RNase A using four substrates. These fluorogenic substrates are based on the quenching of fluorescence of 6-carboxytluorescein (6-FAM) held in proximity to the quencher 6-carboxytetramethylRhodamine (6-TAMRA) by a single ribonucleotide embedded within deoxyadenosine nucleotides (Kelemen et al., 1999). When RNase A cleaves the sole ribosyl unit, fluorescein is released from its proximity to TAMRA and manifests its intrinsic fluorescence.

Wild-type RNase A catalyzes the degradation of the fluorogenic substrate 6-FAM~dArCdAdA~6-TAMRA the most proficiently of the fluorogenic substrates used here. Wild-type RNase A cleaves the substrate 6-FAM~dArUdAdA~6-TAMRA roughly half as fast as 6-FAM~dArCdAdA~6-TAMRA with a value of 

\[
\frac{(k_{cat}/K_m)_{RT}}{(k_{cat}/K_m)_{RC}} = 0.55 \quad (\text{Figure 3.4B})
\]

This difference in catalytic proficiency is similar to that with the substrates cytidylyl(3' → 5')adenosine (CpA) and uridylyl(3' → 5')adenosine (UpA) with a value of 

\[
\frac{(k_{cat}/K_m)_{UpA}}{(k_{cat}/K_m)_{CpA}} = 0.46 \quad \text{from Witzel and Barnard (Witzel & Barnard, 1962)}
\]

\[
\frac{(k_{cat}/K_m)_{UpA}}{(k_{cat}/K_m)_{CpA}}
\]
0.21 from Follmann and coworkers (Follmann et al., 1967). The difference in catalytic capacity of RNase A for cytidine 2',3' cyclic phosphate (C>p) and uridine 2',3' cyclic phosphate (U>p) with a value of \( \frac{k_{\text{cat}}/K_m}{k_{\text{cat}}/K_m} \) of 0.23 (delCardayré & Raines, 1995b) is also similar to that of the fluorogenic substrates.

T45G RNase A cleaves 6-FAM–dArUdAdA–6-TAMRA roughly one-fourth as fast as 6-FAM–dArCdAdA–6-TAMRA with a value of \( \frac{k_{\text{cat}}/K_m}{k_{\text{cat}}/K_m} \) of 0.23 (Figure 3.4B). This substrate comparison is significantly different from the value of \( \frac{k_{\text{cat}}/K_m}{k_{\text{cat}}/K_m} \) of 0.0096 from delCardayré and Raines (delCardayré & Raines, 1995b). The difference in catalytic capacity for C>p and U>p may be accentuated by the inability of these substrates to occupy the enzymic B2 subsite.

The most dramatic specificity demonstrated by wild-type RNase A is against adenosine and guanine nucleotides. The substrate 6-FAM–dArAdAdA–6-TAMRA has only one labile residue, its adenosine ribonucleotide. Wild-type RNase A cleaves after this ribonucleotide six orders of magnitude slower than after the analogous cytidine or uridine ribonucleotides, with \( \frac{k_{\text{cat}}/K_m}{k_{\text{cat}}/K_m} \) of 3.6 \( \times \) 10\(^{-6}\) and \( \frac{k_{\text{cat}}/K_m}{k_{\text{cat}}/K_m} \) of 2.0 \( \times \) 10\(^{-6}\) (Figure 3.4B).

The catalytic specificity of T45G RNase A against adenosine relative to cytidine or uridine is only three orders of magnitude with \( \frac{k_{\text{cat}}/K_m}{k_{\text{cat}}/K_m} \) of 7.0 \( \times \) 10\(^{-4}\) and \( \frac{k_{\text{cat}}/K_m}{k_{\text{cat}}/K_m} \) of 3.1 \( \times \) 10\(^{-3}\) (Figure 3.4B). T45G RNase A is one thousand times less specific for pyrimidine nucleotides over purine nucleotides than is the wild-type enzyme. The rate of cleavage of 6-FAM–dArGdAdA–6-TAMRA by both wild-type RNase A and the T45G variant is extremely low (< 0.5 M\(^{-1}\)s\(^{-1}\)). We cannot
quantitate the level of catalytic discrimination of these enzymes against guanine nucleotides, but we can estimate it to be >10^7.

The ability of T45G RNase A to readily cleave the phosphodiester bond on the 3' side of adenosine residues was first observed with poly(A) as the substrate (delCardayré & Raines, 1994). T45G RNase A cleaves poly(A) 100-fold faster than 6-FAM-dArAdAdA-6-TAMRA \((k_{cat}/K_m)_{\text{poly}(A)}/(k_{cat}/K_m)_{6-\text{FAM-dArAdAdA-6-TAMRA}} = 1.1 \times 10^2\). This comparison is not rigorously correct because the cleavage of poly(A) is processive (delCardayré & Raines, 1994) processive catalysis does not satisfy Michaelis-Menten kinetic approximations (Chou et al., 1994). However, the increase in apparent second-order rate constant for poly(A) could be a result of the ability of the enzyme to limit the diffusional search for cleavable phosphodiester bonds through processive catalysis. The same kinetic comparison for the wild-type enzyme shows a 16-fold faster cleavage of poly(A) over 6-FAM-dArAdAdA-6-TAMRA \((k_{cat}/K_m)_{\text{poly}(A)}/(k_{cat}/K_m)_{6-\text{FAM-dArAdAdA-6-TAMRA}} = 1.6 \times 10^1\). The lack of a significant benefit of a polymeric substrate for the wild-type enzyme probably results from the distributive kinetic mechanism of wild-type RNase A.

The ability of wild-type RNase A to cleave the phosphodiester bond on the 3' end of adenosine nucleotides at all may arise from the conformation adopted by the adenine base. The lowest energy conformation of the glycosidic bond results in an anti conformation of the adenosine base. However, the adenine base can rotate about the glycosidic bond, putting the adenosine base in a syn conformation (Yathindra & Sundaralingham, 1973). In the syn conformation, the smaller aspect of the adenosine
base can fit into the B1 subsite of wild-type RNase A (Ko et al., 1996). This energetic rotation to the higher energy conformation could explain the low rate of cleavage by the wild-type enzyme. The ability of T45G RNase A to cleave after adenosine nucleotides arises from the ability of the enzyme to accommodate the large adenosine base in an anti conformation within its enlarged B1 subsite. The increased catalytic capacity does not arise from an increased ground-state interaction (vide infra).

**Ground State Base Specificity of Ribonuclease A.** T45G RNase A has a significantly greater catalytic capacity for cleavage after adenosine residues than does the wild-type enzyme. To determine if this change in catalytic capacity arises from a greater ground state affinity for adenine bases at the B1 subsite, we measured the binding of four trinucleotide DNA ligands to wild-type and T45G RNase A. The binding of 6-FAM-d(CAA) and 6-FAM-d(UAA) to wild-type RNase A is only about 20-fold tighter than the binding of 6-FAM-d(AAA) and 6-FAM-d(ØAA) (Figure 3.4B). This ground state specificity is all but eliminated by replacing Thr45 with a glycine residue. Interestingly, the loss in specificity was not due to an increase in binding to adenosine nucleotides, but to the loss in binding affinity for cytidine and uridine nucleotides (Figure 3.5B). Thus the T45G variant has less catalytic specificity and less ground state specificity than does wild-type RNase A. With the same ground state adenosine binding as wild-type RNase A, T45G RNase A has a much greater capacity to cleave on the 3' side of adenosine than does wild-type RNase A.

**Interaction of Fluorine Substituted Nucleotides with Ribonuclease A.** RNase A interacts with DNA oligonucleotides in a manner assumed to be similar to the manner
in which RNase A interacts with RNA oligonucleotides. The structure of the crystalline complex of RNase A•d(ATAAG) shows interactions with this extended substrate analog (Fontecilla-Camps et al., 1994). However, the catalytic residue His12 forms a hydrogen bond with the 3' phosphoryl of thymidine and is more than 4 Å away from the 2' carbon of the thymidine in this structure.

If the actual Michaelis complex is like this RNase A•d(ATAAG) complex, some rearrangement would be necessary for His12 to activate the 2' hydroxyl group. The difference in the pucker of a ribose ring from that of a deoxyribose ring may move the 2' carbon closer to His12, allowing for a hydrogen bond to form between His12 and the 2' hydroxyl group of RNA. The structure of the crystalline complex of RNase S•U^FpA shows the 2' fluorine, rather than the phosphoryl group in contact with His12 (Pavlovsky et al., 1978). The 2'-fluoro substitution on DNA makes the sugar pucker more like that of RNA (Guschlbauer & Jankowski, 1980; Ikehara, 1984; Kawasaki et al., 1993). If the contact between His12 and the 2' fluorine is a hydrogen bond, as has been asserted (Pavlovsky et al., 1978), then this hydrogen bond is weak (Dunitz & Taylor, 1997). These structural examples could explain the difference we observe for the binding affinities of 6-FAM•d(AUAA) and 6-FAM•d(AU^FAA) with wild-type RNase A. In the RNase A•6-FAM•d(AUAA) complex a hydrogen bond can form between the 3'-phosphoryl group of the deoxyuridine residue and His12. In the RNase A•6-FAM•d(AU^FAA) complex only the weaker interaction between the 2' fluorine and His12 may be available. Hence, wild-type RNase A binds more tightly to 6-FAM•d(AUAA) than 6-FAM•d(AU^FAA). With T45G RNase A, the uridine base
is not held rigidly, and the phosphoryl group of a ligand is able interact equally well with His12 regardless of the conformation of the sugar.

**Conclusion.** RNase A provides prodigious catalytic selectivity ($10^6$ relative $k_{cat}/K_{m}$) without significant ground state specificity (50-fold tighter binding). From the crystalline structure of T45G RNase A and the capacity of this enzyme to cleave after adenosine, we contend that the specificity of wild-type RNase A relies on the exclusion of adenosine nucleotides from the active site. The ground-state binding of deoxyuridine and 2' fluorine-substituted deoxyuridine ligands to wild-type RNase A and the T45G variant indicate that the specificity of RNase A is generated by a synergy of the interaction of the base-binding subsite with a nucleotide base and the interaction of the active site with a phosphoryl group through the conformation of the bound substrate.
Table 3.1. X-ray Diffraction Analysis Statistics for T45G Ribonuclease A.

<table>
<thead>
<tr>
<th>Space group</th>
<th>P3₁21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell dimensions (Å)</td>
<td></td>
</tr>
</tbody>
</table>
a = 64.32 (1)  
b = 64.32 (1)  
c = 64.84 (2)  
α = β = 90°  
γ = 120° |
| Protein molecules/unit cell | 6 |

**Data Collection Statistics**

- Resolution (Å): 1.8
- No. of measured reflections (I/σ > 0.33): 62164
- No. of unique reflections: 18761
- Average redundancy: 3.3
- Average I/σ: 23.2
- Completeness of data (30 - 1.8): 96%
- Completeness of high-resolution shell (1.9 - 1.8): 87%
- Rsym (I/σ > 0.33)$^a$: 0.036
- Rsym (1.9 - 1.8 Å)$^a$: 0.19

**Final Refinement Statistics**

- RNase A atoms: 968
- Solvent atoms: 120
- R-factor (30.0 - 1.8 Å)$^b$: 0.176
- Rms deviations from ideal geometry:
  - Bond distances (Å): 0.011
  - Bond angles (deg): 2.4
- Average B-factors (Å²):
  - Protein (main chain): 25.1
  - Protein side chain and solvent: 35.7
  - Acetate: 38.5
  - Chloride: 11

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$^a$ Rsym = $\Sigma_{hkli} |I| - \langle I \rangle |\Sigma_{hkli}|$, where $I$ = the observed intensity and $\langle I \rangle$ = the averaged intensity obtained from multiple observations of symmetry related reflections.

$^b$ R-factor = $\Sigma_{hkli}|F_o - F_c|/\Sigma_{hkli}|F_o|$, where $F_o$ and $F_c$ are the observed and calculated structure factors, respectively.
Table 3.2. Kinetic Parameters of $k_{cat}/K_m$ for the Cleavage of Fluorescence Based Substrates by Wild-Type Ribonuclease A and the T45G Variant.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$(k_{cat}/K_m)_{Wild-Type}$ $(10^6 \text{ M}^{-1}\text{s}^{-1})^a$</th>
<th>$(k_{cat}/K_m)_{T45G}$ $(10^6 \text{ M}^{-1}\text{s}^{-1})^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-FAM–dArCdAdA–6-TAMRA</td>
<td>$66 \pm 4^b$</td>
<td>$3.3 \pm 0.3$</td>
</tr>
<tr>
<td>6-FAM–dArUdAdA–6-TAMRA</td>
<td>$25 \pm 3^b$</td>
<td>$0.75 \pm 0.08$</td>
</tr>
<tr>
<td>6-FAM–dArAdAdA–6-TAMRA</td>
<td>$0.000018 \pm 0.000002$</td>
<td>$0.0023 \pm 0.0002$</td>
</tr>
<tr>
<td>6-FAM–dArGdAdA–6-TAMRA</td>
<td>$&lt; 0.0000001$</td>
<td>$&lt; 0.0000005$</td>
</tr>
</tbody>
</table>

$^a$ Values of $k_{cat}/K_m$ were determined at 25 °C in 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M). Values are average ± standard deviation of triplicate determinations.

$^b$ From ref (Kelemen et al., 1999).
Table 3.3. Equilibrium Dissociation Constants, $K_d$, for the Binding of Wild-Type Ribonuclease A and the T45G Variant to 5' Fluorescein-Labeled DNA as Ligands Determined by Fluorescence Anisotropy.

<table>
<thead>
<tr>
<th>RNase A</th>
<th>$K_d$ Wild-Type (mM)</th>
<th>$K_d$ T45G (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-FAM-d(AUAA)</td>
<td>0.012 ± 0.003</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>6-FAM-d(AU&lt;sup&gt;F&lt;/sup&gt;A)</td>
<td>0.62 ± 0.22</td>
<td>0.087 ± 0.047</td>
</tr>
<tr>
<td>6-FAM-d(CAA)</td>
<td>0.070 ± 0.002</td>
<td>0.97 ± 0.05</td>
</tr>
<tr>
<td>6-FAM-d(UAA)</td>
<td>0.13 ± 0.01</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>6-FAM-d(AAA)</td>
<td>3.2 ± 0.1</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>6-FAM-d(ØAA)</td>
<td>2.5 ± 0.2</td>
<td>1.3 ± 0.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Equilibrium dissociation constants were determined at 23 ± 2 °C in 0.020 M MES-NaOH buffer (pH 6.0) containing NaCl (0.050 M).

<sup>b</sup> dU<sup>F</sup> refers to 2'-deoxy-2'-fluorouridine.

<sup>c</sup> Equilibrium dissociation constants were determined at 23 ± 2 °C in 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M).

<sup>d</sup> dØ refers to an abasic analog of DNA (see text).
Figure 3.1. Deoxynucleotides labeled with 6-carboxyfluorescein used to assess binding to the B1 subsite. The italicized text refers to Ribonuclease A subsites known to interact with nucleic acid bases (B1, B2, and B3) and phosphoryl groups [P(0), P(1), and P(2)] (Fisher et al., 1998a).
Figure 3.2. Stereoview of the least-squares superposition of the crystalline structures of wild-type ribonuclease A from protein data bank accession code 7rsa (red) and T45G ribonuclease A (blue).
The electron density \((2|F_a| - |F_c|)\) contoured at 1.0 \(\sigma\) of residues surrounding Gly45. Ile81 and Asp83 show static disorder and have been modeled as two side-chain conformations. Phe120 has weak density for \(C_{\alpha1}\), \(C_{\alpha1}\), and \(C_\zeta\) atoms, indicating some mobility. A water molecule (W1) occupies the position of the side chain of Phe120 from the wild-type enzyme, and a chloride ion is also present.
Figure 3.4.  

A. Time dependence of product formation from the ribonuclease-catalyzed cleavage of 6-carboxyflourescein-dAdAdA-6-carboxytetramethylaminorhodamine. Values were calculated from the fluorescence emission at 515 nm with excitation at 490 nm upon addition of wild-type ribonuclease A (0.23 μM; ○) and the T45G variant (0.16 μM; ●) using the initial and final fluorescence intensity data of substrate (23 nM). Data for wild-type and variant enzymes were fitted to a straight line with slopes of 0.13 pM/s and 9.5 pM/s, respectively. Reactions were performed at 25 °C in 0.10 M Mes-NaOH buffer (pH 6.0) containing NaCl (0.10 M). Inset: Data from wild-type enzyme plotted with an expanded ordinate.

B. Values of the kinetic parameter $k_{cat}/K_m$ for the cleavage of 6-carboxyflourescein-dArXdAdA-6-carboxytetramethylaminorhodamine, where X is uridine, cytidine, or adenine by wild-type ribonuclease A and its T45G variant.
Figure 3.5.  

A. Measurement of ground-state binding affinities of wild-type ribonuclease A and the T45G variant for 6-carboxyfluorescein –d(UAA) made using fluorescence anisotropy. Binding isotherms were performed in 0.10 M Mes-NaOH buffer (pH 6.0) containing NaCl (0.10 M).

B. Ground-state base specificity of ribonuclease A and the T45G variant determined using fluorescence anisotropy of 6-carboxyfluorescein –d(XAA) nucleotide ligands, where X is cytidine, uridine, adenosine, and an abasic analog of DNA (Θ).
Chapter 4

Extending the Limits to Enzymatic Catalysis:

Diffusion of Ribonuclease A in One Dimension

SUMMARY

Bovine pancreatic ribonuclease A (RNase A) is a distributive endoribonuclease that catalyzes the cleavage of the P–O₅' bond of RNA on the 3' side of pyrimidine residues. Here, RNase A is shown to cleave the P–O₅' 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 it 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.
INTRODUCTION

Diffusion poses a barrier on the free energy surface of all bimolecular reactions (Blacklow et al., 1988). Bovine pancreatic ribonuclease A [RNase A (D'Alessio & Riordan, 1997; Raines, 1998; Raines, 1999); 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 (Thompson et al., 1995). In this respect, RNase A can be thought of as a “perfect” enzyme (Albery & Knowles, 1976; Burbaum et al., 1989). Nonetheless, its catalytic efficacy would be extended further if RNase A were able to diffuse in the reduced dimension of polymeric RNA (von Hippel & Berg, 1989).

Facilitated diffusion enables a protein to limit the dimensions of a random diffusional search for a target. In an enzymatic mechanism that incorporates facilitated diffusion, 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 4.1.

\[
\begin{align*}
E+N & \rightleftharpoons E\cdot N \\
E\cdot N & \rightleftharpoons E\cdot S \\
E\cdot S & \rightarrow \rightarrow E+P
\end{align*}
\] (4.1)
von Hippel and coworkers first proposed the existence of facilitated diffusion to explain the unexpectedly large diffusional rate of lac repressor along double-stranded DNA (Berg et al., 1981). Single-stranded nucleic acids can also support facilitated diffusion. Lohman provided evidence that T4 gene 32 protein diffuses along RNA polymers (Lohman, 1984a; Lohman, 1984b). Others proposed a mechanism of facilitated diffusion for the filamentous phage gene 5 protein on single-stranded DNA and RNA (Pörschke & Rauh, 1983). Restriction endonucleases (Jeltsch & Pingoud, 1998) and DNA methyltransferases (Surby & Reich, 1996a; Surby & Reich, 1996b) 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) (delCardayre & Raines, 1995b). 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.¹ 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) (Nogués et al., 1995).

¹ 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 (Fontecilla-Camps et al., 1994). The B3 "subsite" could therefore result from π – π stacking interactions that stabilize the enzyme-nucleic acid complex solely by preorganization or desolvation of the nucleic acid.
While evaluating the importance of Thr45 to enzymic specificity, we discovered that variants of RNase A at Thr45 that cleave poly(A) do so processively (delCardayré & Raines, 1994). 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 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 (delCardayré & Raines, 1994). 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–O5' bond on the 3' side of adenosine residues. This expanded specificity plus 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 would shorter substrates (Berg et al., 1981; von Hippel & Berg, 1989). Here, we test for facilitated diffusion of RNase A by comparing the rate 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.
MATERIALS AND METHODS

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 (Milwaukee, WI). RNase A (lyophilized), 2-(N-morpholino)ethanesulphonic acid (MES), and diethylpyrocarbonate (DEPC) were from Sigma Chemical (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). \([\gamma^{32}P]\)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 (delCardayre & Raines, 1994; delCardayre & Raines, 1995a; delCardayre & Raines, 1995b). Briefly, T45G RNase A was produced in inclusion bodies in *E. coli*. Cells were lysed by passage through a French pressure cell. Inclusion bodies were isolated by centrifugation, then reduced and denatured. T45G RNase A was oxidatively refolded, and purified by gel filtration chromatography followed by cation exchange chromatography.
**Substrate Design.** RNase A can catalyze the cleavage of the P–O³' bond of an RNA nucleotide embedded in a DNA sequence (Jenkins et al., 1996; Kelemen & Raines, 1997). Substrates 4.1 – 4.4 and 4.7 are such DNA/RNA chimeras (Table 4.1). Substrates 4.5 and 4.6 are the all-RNA analogs of substrates 4.3 and 4.4. 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 4.1 – 4.6, these differences are strictly in the number of adenosine nucleotides. For substrate 4.7, the surrounding nucleotides are either adenosine or cytosine.

Substrates 4.1 – 4.6 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 4.1 vs 4.2 (as well as 4.3 vs 4.4 and 4.5 vs 4.6) can be used to reveal an effect of having the cleavage site closer to the 5' vs 3' end. Effects of this kind have been observed in other interactions with single-stranded oligonucleotides (Zhang et al., 1999).

Comparing substrates 4.1 vs 4.3 and 4.2 vs 4.4 can reveal an effect from the type of label. Such an effect is made apparent because substrates 4.1 and 4.2 are labeled with ³²P, and substrates 4.3 and 4.4 are otherwise identical but labeled instead with fluorescein. Moreover, cleavage of substrates 4.3 – 4.6, which are labeled doubly, produces four detectable products (P₁D₃' and P₁D₅', or P₃D₃' and P₃D₅'), enabling a more thorough product analysis than is possible with substrates labeled singly. Comparing substrates 4.3 vs 4.5 and 4.4 vs 4.6 can uncover a difference between diffusion along
tracts of poly(dA) and tracts of poly(A). Finally, substrate 4.7 was designed to detect on any preference of the enzyme for diffusion along adenine vs 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 NH₃ in H₂O) 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 use 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 4.1 and 4.2 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 4.3 - 4.7 as a phosphoramidite during synthesis (Fisher et al., 1998a; Fisher et al., 1998b). 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 $\varepsilon = 0.72$ (mg/mL)$^{-1}$cm$^{-1}$ at 277.5 nm for RNase A (Sela et al., 1957), $\varepsilon = 5.4 \times 10^5$ M$^{-1}$cm$^{-1}$ at 260 nm for substrates 4.1 - 4.6 and $\varepsilon = 3.3 \times 10^5$ M$^{-1}$cm$^{-1}$ at 260 nm for substrate 4.7 (Wallace & Miyada, 1987). Reactions with substrates 4.1 - 4.7 were carried out in siliconized microcentrifuge tubes to limit loss of 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 4.3 - 4.7, 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 4.1 and 4.2 were quantitated with a PhosphorImager from Molecular Dynamics (Sunnyvale, CA). Reaction products from substrates 4.3 – 4.7 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 run 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.

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 4.1). The results of the assay used to determine $P_{1D}$ and $P_{3D}$ for substrates 4.1 and 4.2 are shown in Figure 4.1. Product $P_{1D}$ formed faster than does $P_{3D}$ from both substrates 4.1 and 4.2. 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 homolog of RNase A (D'Alessio & Riordan, 1997), 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 4.1 and 4.2 at various concentrations of Na$^+$ are shown in Figure 4.2. The data in this
figure demonstrate a trend in the product ratio that is consistent with facilitated diffusion mediated by Coulombic interactions. At low Na\(^+\) concentration (25 mM), the indication of facilitated diffusion is strong ([P\(_{1D}\)/P\(_{3D}\)] ≈ 2). At high Na\(^+\) concentration (1.025 M) facilitated diffusion is not apparent ([P\(_{1D}\)/P\(_{3D}\)] ≈ 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 4.1 and 4.2 lack a label at their 3' ends and thus cannot reveal whether RNase A moves from one cleavage site to another without release of products. Substrates 4.3 – 4.6 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 4.1). Hence, there exist [P\(_{1D}\)/P\(_{3D}\)] ratios for the 3' and 5' labels, which we refer to 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 4.2. Assays of fluorescein-labeled substrates are not as sensitive as are assays of \(^{32}\)P-labeled substrates. The quality of fluorimager data suffers significantly from light scattering by the polyacrylamide gel. Still, as listed in Table 4.2, all values of [P\(_{1D}\)/P\(_{3D}\)] (≈ 2) are similar to each other and similar to [P\(_{1D}\)/P\(_{3D}\)] ratios for substrates 4.1 and 4.2. 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 of the data from substrates 4.1 and 4.2 with those from substrates 4.3 – 4.6 indicate that the type of label is not affecting the results (Figure 4.3). The picture provided by the doubly-labeled substrates 4.3 –
4.6, though more complete than that provided by substrates 4.1 and 4.2, is essentially identical. The similarity of the data from substrates 4.3 and 4.4 with those from substrates 4.5 and 4.6 indicate that wild-type RNase A and the T45G variant treat adenosine and deoxyadenosine nucleotides equivalently in this assay.

Facilitated Diffusion along Poly(dA) vs Poly(dC). The cleavage of substrate 4.7 by RNase A results in two products, P_A and P_C (Table 4.1). Product P_A results from the cleavage of substrate 4.7 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 4.7 by wild-type RNase A and T45G RNase A results almost exclusively in P_A (Figure 4.4). Excess RNase A generated P_C 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 ten phosphoryl groups (Jensen & von Hippel, 1976; McPherson et al., 1986a) and has smaller $K_m$ values for longer substrates (Irie et al., 1984). Still, values of $k_{cat}/K_m$ plateau for substrates of three nucleotides in length or greater (Irie et al., 1984). We designed our substrates with at least five nucleotides flanking each cleavable site.

RNase A prefers to cleave "longer" oligonucleotide substrates (Figure 4.1). The identity and location of the label ($5'^{-32}$P vs $5'$-fluorescein and $3'$-fluorescein) does not alter this preference (Figures 4.1 and 4.3). This preference is diminished by $Na^+$ (Figure 4.2), as would be expected from an effect mediated by Coulombic interactions (Jensen & von Hippel, 1976; Fisher et al., 1998a; Fisher et al., 1998c). Indeed, RNase A is known to interact with the anionic phosphoryl groups of nucleic acids via cationic histidine, lysine, and arginine residues (Fisher et al., 1998a; Fisher et al., 1998b). 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 4.1 – 4.4 was not relevant to a biological process. Accordingly, we synthesized and tested substrates 4.5 and 4.6, which are composed entirely of ribose nucleotides. These all-RNA substrates were comparable to substrates 4.1 – 4.4 at fostering facilitated diffusion (Figures 4.1 and 4.3). Thus, facilitated diffusion can occur along a poly(A) tract as well as 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 4.1 – 4.6. Such processivity would not be readily detectable with substrates 4.1 and 4.2. Substrates 4.3 – 4.6, 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 

\[
\frac{[P_{1D}]}{[P_{3D}]_3^{'}} \text{ and } \frac{[P_{1D}]}{[P_{3D}]_3^{'}} \text{ would not be the same. Specifically, if RNase A releases the 3'} \text{ product after cleavage, effectively diffusing in the 5'} \text{ direction, then substrates 4.4 and 4.6 would have a larger } \frac{[P_{1D}]}{[P_{3D}]_3^{'}} \text{ than } \frac{[P_{1D}]}{[P_{3D}]_3^{'}}. \text{ If RNase A diffuses in the 3'} \text{ direction after cleavage, then substrates 4.3 and 4.5 would have a larger } \frac{[P_{1D}]}{[P_{3D}]_3^{'}} \text{ than } \frac{[P_{1D}]}{[P_{3D}]_3^{'}}. \text{ Neither of these two possibilities were observed (Table 4.2). We conclude that processivity is insignificant during the cleavage of substrates 4.3 – 4.6.}

Facilitated Diffusion along Poly(dA) vs Poly(dC). We designed substrate 4.7 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 polypyrrimidine like the 3' half of substrate 4.7, is an excellent substrate for RNase A (delCardayré & Raines, 1994). 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 (Fontecilla-Camps et al., 1994). Time courses for the cleavage of substrate 4.7 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 4.4). What is the basis for this preference? Both poly(A) and poly(C) have helical structures in aqueous solution at neutral pH (Arnott et al., 1976; Olsthoorn et al., 1980; Broido & Kearns, 1982; Saenger, 1991). Poly(C) can form a left-handed helix stabilized by a hydrogen bond between adjacent bases (Broido & Kearns, 1982). In contrast, poly(A) can form a right-handed helix stabilized by base stacking (Olsthoorn et al., 1980). 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 (McPherson et al., 1986a; McPherson et al., 1986b). 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 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) (delCardayré & Raines, 1994),
does not appear to catalyze the processive cleavage of the poly(A) tracts of substrates 4.5 and 4.6 (Figure 4.3). 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 less 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 4.5 and 4.6 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 (delCardayré & Raines, 1994). 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 4.5 and 4.6. This same affinity may be responsible for the product inhibition that occurs during the turnover of substrate 4.7 (Figure 4.4).

An Alternative Explanation? Our data with substrates 4.1 – 4.6 are consistent with a hypothesis alternative to that of facilitated diffusion—RNase A could be attracted to the midpoint of oligonucleotides. Specifically, P₁D could be formed preferentially because RNase A is drawn by Coulombic forces to the more central uridine nucleotide in substrates 4.1 – 4.6. This hypothesis is eliminated by the results from substrate 4.7. 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 it does P_C (Figure 4.4). The catalytic behavior of RNase A on substrate 4.7 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 (von Hippel & Berg, 1989). These mechanisms are “sliding,” “interdomain transfer,” and “intradomain dissociation and association.” These mechanisms elaborate the second step in eq 4.1. 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 (Nogués et al., 1995), as would be required by an interdomain transfer mechanism. Moreover, cleavage of substrates 4.1–4.7 by an intradomain dissociation and association (i.e., hopping) mechanism would likely yield [P_D]/[P_3D] ratios close to unity. These ratios were typically near 2 (Figures 4.1 and 4.3; Table 4.2). 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 homologs (D'Alessio & Riordan, 1997) and variants (Di Donato et al., 1994; Leland et al., 1998) of RNase A are cytotoxic to mammalian cells. Poly(A) tails of up to 300 nucleotides are a feature of the mRNA's
in all eukaryotic organisms (Brawerman, 1973). In vivo, cytotoxic ribonucleases may use the poly(A) tract of mammalian mRNA's 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.

Acknowledgment. We are grateful to Prof. M.T. Record, Jr. for thoughtful advice throughout the course of this work.
### Table 4.1. Labeled Substrates and Corresponding Detectable Products Formed by Transphosphorylation

<table>
<thead>
<tr>
<th>Substrate (5'→3')</th>
<th>Detectable Products (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Substrate</strong></td>
<td><strong>P&lt;sub&gt;1D&lt;/sub&gt;</strong></td>
</tr>
<tr>
<td>4.1 32p(dA)&lt;sub&gt;5&lt;/sub&gt;rU(rA)&lt;sub&gt;3&lt;/sub&gt;rU(dA)&lt;sub&gt;25&lt;/sub&gt;</td>
<td>32p(dA)&lt;sub&gt;5&lt;/sub&gt;rU(rA)&lt;sub&gt;3&lt;/sub&gt;rU</td>
</tr>
<tr>
<td>4.2 32p(dA)&lt;sub&gt;25&lt;/sub&gt;rU(rA)&lt;sub&gt;3&lt;/sub&gt;rU(dA)&lt;sub&gt;3&lt;/sub&gt;</td>
<td>32p(dA)&lt;sub&gt;25&lt;/sub&gt;rU</td>
</tr>
<tr>
<td>4.3 FI(dA)&lt;sub&gt;2&lt;/sub&gt;rU(dA)&lt;sub&gt;3&lt;/sub&gt;rU(dA)&lt;sub&gt;25&lt;/sub&gt;Fl</td>
<td>FI(dA)&lt;sub&gt;2&lt;/sub&gt;rU(dA)&lt;sub&gt;3&lt;/sub&gt;rU</td>
</tr>
<tr>
<td>4.4 FI(dA)&lt;sub&gt;25&lt;/sub&gt;rU(dA)&lt;sub&gt;3&lt;/sub&gt;rU(dA)&lt;sub&gt;3&lt;/sub&gt;Fl</td>
<td>FI(dA)&lt;sub&gt;25&lt;/sub&gt;rU(dA)&lt;sub&gt;3&lt;/sub&gt;rU</td>
</tr>
<tr>
<td>4.5 FI(rA)&lt;sub&gt;2&lt;/sub&gt;rU(rA)&lt;sub&gt;3&lt;/sub&gt;rU(rA)&lt;sub&gt;25&lt;/sub&gt;Fl</td>
<td>FI(rA)&lt;sub&gt;2&lt;/sub&gt;rU(rA)&lt;sub&gt;3&lt;/sub&gt;rU</td>
</tr>
<tr>
<td>4.6 FI(rA)&lt;sub&gt;25&lt;/sub&gt;rU(rA)&lt;sub&gt;3&lt;/sub&gt;rU(rA)&lt;sub&gt;3&lt;/sub&gt;Fl</td>
<td>FI(rA)&lt;sub&gt;25&lt;/sub&gt;rU(rA)&lt;sub&gt;3&lt;/sub&gt;rU</td>
</tr>
<tr>
<td>4.7 (dA)&lt;sub&gt;5&lt;/sub&gt;rU(dA)&lt;sub&gt;3&lt;/sub&gt;(dC)&lt;sub&gt;3&lt;/sub&gt;rU(dC)&lt;sub&gt;25&lt;/sub&gt;Fl</td>
<td>(dA)&lt;sub&gt;5&lt;/sub&gt;(dC)&lt;sub&gt;3&lt;/sub&gt;rU(dC)&lt;sub&gt;25&lt;/sub&gt;Fl</td>
</tr>
</tbody>
</table>

<sup>a</sup> "Fl" refers to a 6-carboxyfluorescein label attached via a six-carbon spacer (21, 23, 24).
Table 4.2. Concentration Ratios ([P₁D]/[P₃D]) of 5'- and 3'-Fluorescein Labeled Products Generated by Wild-Type Ribonuclease A and T45G Ribonuclease A.\(^a\)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Wild-Type</th>
<th>T45G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5'</td>
<td>3'</td>
</tr>
<tr>
<td>4.3</td>
<td>1.5 ± 0.1</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>4.4</td>
<td>1.9 ± 0.2</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>4.5</td>
<td>1.6 ± 0.2</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>4.6</td>
<td>2.1 ± 0.4</td>
<td>1.8 ± 0.2</td>
</tr>
</tbody>
</table>

\(^a\) Reactions were carried out in 0.050 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M). Each value (± SE) is the average of data obtained after reaction for 1, 2, 5, and 10 min.
Figure 4.1: Time course (0, 1, 2, 5, and 10 min) for the cleavage of substrate 4.1 (left) and substrate 4.2 (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 4.1) during PAGE.

B. Quantitation of PAGE data by phosphorimager analysis. Data from replicate experiments did not differ substantially.
Figure 4.2: Salt concentration dependence of the P_{1D}/P_{3D} product ratio from the cleavage of substrate 4.1 (■) and substrate 4.2 (●) 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 (± SE) is the average of data obtained after reaction for 1, 2, 5, and 10 min.
Figure 4.3: Time course (0, 1, 2, 5, and 10 min) for the cleavage of substrate 4.5 by ribonuclease A. Reactions were performed in 50 mM MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M).

A. Migration of substrate 4.5 and its P₁D₅', P₃D₅', P₁D₃', and P₃D₃' products (Table 4.1) during PAGE.

B. Quantitation of PAGE data by fluorimagner analysis. Data from replicate experiments did not differ substantially.
Time course (0, 1, 2, 5, and 10 min) for the cleavage of substrate 4.7 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 4.7 and its $P_A$ and $P_C$ products (Table 4.1) during PAGE.

B. Quantitation of PAGE data by fluorimeter analysis. Data from replicate experiments did not differ substantially.
APPENDIX

INTRODUCTION

A sensitive assay for the activity of ribonucleases is essential for the study of catalytic activity. Measuring a change in fluorescence as substrate is converted to product enables a more sensitive signal than detection of other optical parameters such as optical absorption. However, natural substrates of ribonucleases do not have significant fluorescence. Several commercially available dyes exist which fluoresce only when bound to RNA or DNA (Jones et al., 1998). A sensitive fluorescence based substrate for ribonucleases could be generated from a polymeric substrate bound to a conditionally fluorescent dye (Yazynin & Deyev, 1996; Lee & Choi, 1997). This appendix describes an approach of using a polymeric substrates and the dye SYBR Green II for such a substrate.

MATERIALS AND METHODS

Poly (adenylic acid) [poly (A)] was from Midland Certified Reagent (Midland, TX). The dye SYBR Green II was from Molecular Probes (Eugene, OR). Ribosomal RNA from E. coli (16s and 23s) was from Boehringer Mannheim (Indianapolis, IN). Reagents for runoff transcription were from Promega (Madison, WI). Bovine pancreatic ribonuclease A (RNase A) and the T45G variant were produced as
described previously (delCardayré et al., 1995). 2-(N-morpholino)ethanesulphonic acid (MES) and all other reagents were from Sigma Chemical (St. Louis, MO). The substrate (UpAp)₃₀ was made by runoff transcription of the template [(dA)p(dT)p]₃₀ produced from a plasmid containing this sequence bounded by XhoI restriction endonuclease cleavage sites by methods described in Thompson et al. (Thompson et al., 1994).

The concentration of wild type RNase A and the T45G variant were determined assuming $\varepsilon_{277.5} = 0.72$ mL mg⁻¹ cm⁻¹ using a Cary3 spectrophotometer from Varian (Sugar Land, TX). The concentration of poly (A) in phosphodiester units was determined assuming $\varepsilon_{257} = 10,000$ M⁻¹ cm⁻¹. The concentration of (UpAp)₃₀ in UpAp units was determined assuming $\varepsilon_{260} = 16,300$ M⁻¹ cm⁻¹.

Fluorescence measurements were made using a QuantaMaster1 photon-counting fluorometer from Photon Technologies International (South Brunswick, NJ) equipped with stirring and temperature control. Assays were carried out in 2.0 mL volume of 0.10 M MES-NaOH pH6.0 containing NaCl (0.10 M) with stirring. Cleavage of poly(A) (100 μM) substrate by T45G RNase A (11 nM) was detected by the loss of fluorescence upon the release of SYBR Green II (1:4,000 dilution from stock) from product. Cleavage of (UpAp)₃₂ (0.20 – 2.0 μM) substrate by RNase A (10 - 20 nM) was detected by the loss of fluorescence upon the release of SYBR Green II (1:4,000 dilution from stock) from product. Cleavage of ribosomal RNA (20 ng/mL) substrate by RNase A (470 nM) was detected by the loss of fluorescence upon the release of SYBR Green II (1:4,000 dilution from stock) from product.
Cleavage of (UpAp)_{32} (0.20 – 2.0 μM) substrate by RNase A (10 - 20 nM) was also detected by the increase in absorbance detected at 257 nm. The change in absorbance per time was converted to change in concentration of product per time using the molar change in extinction coefficient determined empirically to be \( \Delta \varepsilon_{257} = 1,380 \text{ M}^{-1} \text{cm}^{-1} \). These reactions were carried out in 120 μL volume of 0.10 M MES-NaOH pH 6.0 containing NaCl (0.10 M). Reactions were initiated with the addition of enzyme followed by ten seconds of thorough mixing.

RESULTS

Fluorescence of the dye SYBR Green II is roughly 10-fold greater in the presence of polymeric RNA than in the presence of RNA that has been degraded by a ribonuclease, Figure A.1 and Figure A.2. Although the fluorescent properties of the combination of SYBR Green II and the substrate poly(A) are good, this substrate is only slowly degraded by wild-type RNase A. Poly(A) is more rapidly cleaved by T45G RNase A (delCardayré & Raines, 1994). For this reason, we used T45G RNase A with this substrate. The substrate poly(A) is limited in utility for mammalian ribonucleases which all cleave on the 3' side of pyrimidine nucleotides. Ribosomal RNA contains sufficient quantity of pyrimidine nucleotides to be used as a substrate for mammalian ribonucleases and provides a large change in fluorescence upon cleavage (Figure A.2). However, this substrate is a heteropolymer and is not suitable for kinetic analysis. For example, the data presented in Figure A.2 cannot be fitted to
an equation of a single exponential decay. For this reason, we prefer homopolymeric substrates for this assay.

The commercially available poly(cytidylic acid) [poly(C)] does not significantly change the fluorescent properties of SYBR Green II (data not shown). Therefore, we produced the substrate (UpAp)$_{30}$. This RNA does change the fluorescent properties of SYBR Green II and is readily cleaved by RNase A. The rates of cleavage determined by this assay are linearly dependent on the enzyme concentration (data not shown) and linearly dependent on the RNA concentration up to 1 µM (Figure A.3). The data presented in Figure A.3 was fitted by linear least-squares regression analysis forced through the origin to determine a value of $k_{cat}/K_m$ of $(1.2 \pm 0.2) \times 10^7$ M$^{-1}$s$^{-1}$.

The possibility that SYBR Green II interferes with the interaction of RNase A and RNA is a concern with substrates of this nature. We have also determined kinetic parameters of (UpAp)$_{30}$ cleavage by RNase A without SYBR Green II present by following the loss of hypochromicity of (UpAp)$_{30}$ at 257 nm due to depolymerization. The enzyme-concentration normalized rates of cleavage depend on the concentration of (UpAp)$_{30}$ present in the assay (Figure A.4). The data presented in this figure were fitted by non-linear least squares regression analysis to eq A.1.

$$\frac{v}{[\text{RNase A}]} = \frac{\frac{k_{cat}[(\text{UpAp})_{30}]}{k_{cat} + [(\text{UpAp})_{30}]/(k_{cat}/K_m)}}{[(\text{UpAp})_{30}]}$$  (A.1)
From this analysis, we determine values of $k_{\text{cat}} = (1.8 \pm 0.6) \times 10^3$ s$^{-1}$ and $k_{\text{cat}}/K_m = (6.6 \pm 2.8) \times 10^7$ M$^{-1}$s$^{-1}$.

**DISCUSSION**

Depolymerization of RNA by wild-type and T45G RNase A produces significant loss of fluorescence of the dye SYBR Green II (Figure A.1 and Figure A.2). The type of RNA used in this assay is significant to the efficiency of fluorescence of SYBR Green II, to the type of kinetic analysis possible and to the catalytic capacity of the enzyme.

The values of the catalytic parameters $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ determined for RNase A acting on (UpAp)$_{30}$ (1,700 s$^{-1}$ and $6 \times 10^7$ M$^{-1}$s$^{-1}$ respectively) are similar to but slightly greater than those determined for poly(C) (510 s$^{-1}$ and $5.7 \times 10^6$ M$^{-1}$s$^{-1}$ respectively). RNase A is specific for cleavage 3' of pyrimidine nucleotides but also has some preference for 5' of adenosine nucleotides (Katoh et al., 1986). The substrate (UpAp)$_{30}$ is for this reason a better substrate for RNase A than poly(C). In fact, the value of the $k_{\text{cat}}/K_m$ of (UpAp)$_{30}$ ($6 \times 10^7$ M$^{-1}$s$^{-1}$) is very similar to the value determined for the fluorescence based substrate 6-carboxyfluorescein–dArUdAdA–6-carboxytetramethylrhodamine ($3.6 \times 10^7$ M$^{-1}$s$^{-1}$). The dye does diminish the catalytic action of RNase A on (UpAp)$_{30}$ by at most ten-fold. This interference of the dye can be thought of as a competition of the enzyme and the dye for binding to RNA.
CONCLUSION

The conditionally fluorescent dye SYBR Green II and polymeric RNA provide a feasible substrate for the sensitive detection of ribonucleolytic activity. This combination substrate, however, is not optimal for rigorous determinations of kinetic parameters due to a competition of enzyme with the dye for binding of substrate.
Figure A.1  Fluorescence emission spectra (excited at 490 nm) of SYBR Green II (1:4000 dilution of stock) bound to poly(A) (98 μM) diminishes uniformly 10, 20 and 30 min upon addition of T45G ribonuclease A (110 nM). Fluorescence measurements were taken of a 2.0 mL sample of RNA•SYBR Green II complex in 0.10 M MES-NaOH pH6.0 containing NaCl (0.10 M).
Figure A.2  Fluorescence emission (excited at 490 nm and detected at 515 nm) of SYBR Green II (1:4000 dilution of stock) bound to ribosomal RNA (20 ng/mL) diminishes rapidly from $5.7 \times 10^5$ counts per second (CPS) to $3.3 \times 10^4$ CPS after the addition of wild-type ribonuclease A (470 nM). Fluorescence measurements were made with sample in 2.0 mL volume of 0.10 M MES-NaOH pH6.0 containing NaCl (0.10 M).
Figure A.3  Dependence of enzyme-concentration normalized rate of (UpAp)$_{30}$ cleavage by wild-type ribonuclease on the concentration of (UpAp)$_{30}$ with SYBR Green II present in the reaction solution. Reactions were carried out in 2.0 mL volume of 0.10 M MES-NaOH pH6.0 containing NaCl (0.10 M) and SYBR Green II (1:4000 dilution of stock) with stirring. Data was fit to a straight line forced through the origin by linear least-squares regression analysis resulting in a value of $k_{cat}/K_m = (1.2 \pm 0.2) \times 10^7 \text{M}^{-1}\text{s}^{-1}$. 
Figure A.4  Dependence of enzyme-concentration normalized rate of \((\text{UpAp})_30\) cleavage by wild-type ribonuclease on the concentration of \((\text{UpAp})_30\) without SYBR Green II present in the reaction solution. Reactions were carried out in 2.0 mL volume of 0.10 M MES-NaOH pH6.0 containing NaCl (0.10 M) with stirring. Data was fit to eq A.1 by non-linear least-squares regression analysis resulting in a values of \(k_{\text{cat}} = (1.8 \pm 0.6) \times 10^3 \text{ s}^{-1}\), \(k_{\text{cat}}/K_m = (6.6 \pm 2.8) \times 10^7 \text{ M}^{-1}\text{s}^{-1}\).


