

# Hypersensitive substrate for ribonucleases

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## ABSTRACT

**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 180-fold upon cleavage. Bovine pancreatic ribonuclease A (RNase A) cleaves this substrate with a  $k_{\text{cat}}/K_m$  of  $3.6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ . Human angiogenin, which is a homolog of RNase A that promotes neovascularization, cleaves this substrate with a  $k_{\text{cat}}/K_m$  of  $3.3 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ . 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.**

## 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 (1–3). 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) (4–6) or uridine 3'-(5-bromo-4-chloroindol-3-yl)-phosphate (7) results in a large change in optical absorption. These non-natural substrates suffer, however, from low values of  $k_{\text{cat}}/K_m$  during cleavage by bovine pancreatic ribonuclease A (RNase A, EC 3.1.27.5), which is the best characterized ribonuclease (8).

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 co-workers developed a sensitive

assay for RNase A based on fluorescence quenching (9,10). Their dinucleotide substrate undergoes a 60-fold increase in fluorescence after cleavage and has a  $k_{\text{cat}}/K_m$  of the order of  $10^7 \text{ M}^{-1} \text{ s}^{-1}$  (9). A pentanucleotide version has also been prepared and has an even higher  $k_{\text{cat}}/K_m$  for human RNase 4, a RNase A homolog (10). Recently, James and Woolley reported on a nonanucleotide fluorogenic substrate for RNase A (11). This substrate has a higher  $k_{\text{cat}}/K_m$  than the dinucleotide substrate of Hofsteenge and co-workers but suffers from a lower increase in fluorescence upon cleavage.

Here, we have searched for an optimized fluorogenic substrate for RNase A. We compared 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 determined empirically which substrate yielded the most sensitive assay. We then used that substrate to evaluate the ribonucleolytic activity of angiogenin, which is an RNase A homolog that promotes neovascularization (12). Finally, we used the substrate to develop a facile assay for ribonuclease inhibition.

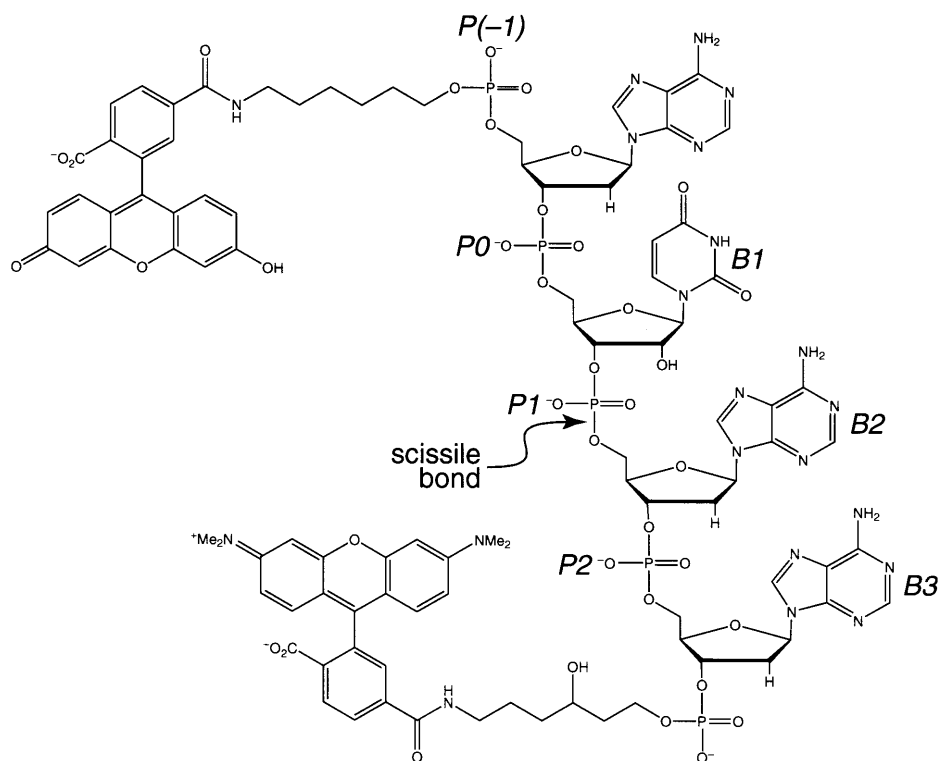
## MATERIALS AND METHODS

### Substrate design

Fluorescence quenching depends significantly on both the distance between the fluorophore and quencher and their relative orientation (13). 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 **1**), tetranucleotide (**2**; Fig. 1), hexanucleotide (**3**) and octanucleotide (**4**) composition with a fluorescein moiety at the 5'-end and a rhodamine moiety at the 3'-end (Table 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 (8). Each of our substrates preserves the rUdA unit of substrate **1**. The dArUdAdA nucleotides in substrate **2** are isologous to those observable in the crystalline RNase A–d(ATAAG) complex (14) and fill all of the known subsites of the enzyme (15,16). The interaction of RNase A and d(AUAA) has been analyzed in detail (17). Substrates **3** and **4** extend still further in both the 5' and 3' directions. Substrate **5** is identical to substrate **2**, but contains a cytosine rather than a uracil base. Substrate **6** is also identical to substrate **2**, but lacks the fluorescein and

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**Figure 1.** Chemical structure of substrate **2**, 6-FAM-dArUdAdA-6-TAMRA, where 6-FAM refers to 6-carboxyfluorescein and 6-TAMRA refers to 6-carboxy-tetramethylrhodamine. The italicized text refers to RNase A subsites known to interact with nucleic acid bases (B1, B2 and B3) and phosphoryl groups [P(-1), P0, P1 and P2] (16).

**Table 1.** Parameters for the cleavage of fluorogenic substrates by RNase A

Substrate	$k_{\text{cat}}/K_m$ ( $10^7 \text{ M}^{-1} \text{ s}^{-1}$ ) <sup>a</sup>	$I_f/I_o$ <sup>b</sup>	Sensitivity ( $10^8 \text{ M}^{-1} \text{ s}^{-1}$ ) <sup>c</sup>
<b>1</b> 6-FAM-rUdA-6-TAMRA	$2.5 \pm 0.3$	$15 \pm 2$	$3.8 \pm 0.7$
<b>2</b> 6-FAM-(dA)rU(dA) <sub>2</sub> -6-TAMRA	$3.6 \pm 0.4$	$180 \pm 10$	$65 \pm 8$
<b>3</b> 6-FAM-(dA) <sub>2</sub> rU(dA) <sub>3</sub> -6-TAMRA	$4.7 \pm 0.6$	$26 \pm 3$	$12 \pm 2$
<b>4</b> 6-FAM-(dA) <sub>3</sub> rU(dA) <sub>4</sub> -6-TAMRA	$4.8 \pm 0.5$	$62 \pm 2$	$30 \pm 3$
<b>5</b> 6-FAM-(dA)rCp(dA) <sub>2</sub> -6-TAMRA	$6.6 \pm 0.4$	$83 \pm 1$	$55 \pm 3$
6-FAM-(dA) <sub>4</sub> rU(dA) <sub>4</sub> -6-TAMRA <sup>d</sup>	$6.9 \pm 0.7$	25	17
DUPAAA <sup>e</sup>	$2.06 \pm 0.08$	60	12

<sup>a</sup>Data were obtained in 0.10 M MES/NaOH buffer, pH 6.0, containing 0.10 M NaCl. For comparison, the value of  $k_{\text{cat}}/K_m$  for the cleavage of uridylyl(3'→5')adenosine by ribonuclease A is  $1.7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  (33).

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

<sup>c</sup>Sensitivity ( $S$ ) for catalysis by ribonuclease A is defined by equation 5.

<sup>d</sup>From James and Woolley (11).

<sup>e</sup>From Zelenko *et al.* (9).

rhodamine labels. This substrate, which is inexpensive to synthesize, is used to estimate the  $K_m$  of substrate **2**.

## Materials

Phosphoramidites were from Perkin Elmer (Foster City, CA). Amino-modifier-C7 controlled pore glass (CPG) was from Glen Research (Sterling, VA). The 6-carboxytetramethyl-

rhodamine 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 Co. (St Louis, MO). RNase A was purified further by gel filtration chromatography followed by cation exchange chromatography, as described

elsewhere (6). 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 (18,19).

### Substrate synthesis

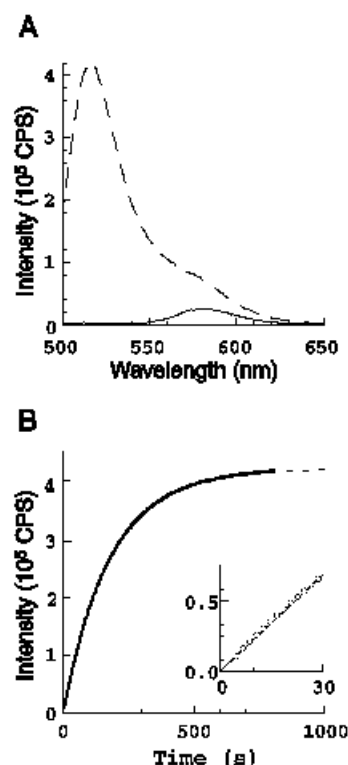
Oligonucleotide substrates were synthesized with a 6-carboxy-fluorescein (6-FAM) at the 5'-end and an amino-modifier-C7 on the 3'-end using standard phosphoramidite chemistry (20) on an Applied Biosystems Model 394 DNA/RNA synthesizer. Following synthesis, the CPG solid support was transferred to a 1.5 ml microfuge tube. Oligonucleotides were cleaved from the CPG by incubation for 10 min at 65°C in a solution of NH<sub>4</sub>OH/methylamine (1:1). The supernatant was removed and the CPG was washed with 1 ml of EtOH/MeCN/H<sub>2</sub>O (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 min. The supernatant was decanted and the pellet was washed with aqueous EtOH (70% v/v) and then dried (21).

6-TAMRA succinimidylester (0.1 ml of a 10 mg/ml solution in dimethyl sulfoxide) 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 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 cm<sup>2</sup>) and eluted with a linear gradient of acetonitrile (5–50% v/v) over 40 min. Samples were monitored at 260 and 297 nm and peaks corresponding to the dual labeled oligonucleotide species were collected, pooled and lyophilized. Substrate **6** was synthesized and purified in a manner analogous to substrate **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.

### 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  $\epsilon = 0.72 \text{ ml mg}^{-1} \text{ cm}^{-1}$  at 277.5 nm (22) and  $\epsilon = 0.85 \text{ ml mg}^{-1} \text{ cm}^{-1}$  at 278 nm (23), respectively. Solution concentrations of substrates **1–6** were determined by assuming that  $\epsilon = 76\,340 \text{ M}^{-1} \text{ cm}^{-1}$ ,  $\epsilon = 102\,400 \text{ M}^{-1} \text{ cm}^{-1}$ ,  $\epsilon = 126\,400 \text{ M}^{-1} \text{ cm}^{-1}$ ,  $\epsilon = 150\,400 \text{ M}^{-1} \text{ cm}^{-1}$ ,  $\epsilon = 99\,940 \text{ M}^{-1} \text{ cm}^{-1}$  and  $\epsilon = 49\,500 \text{ M}^{-1} \text{ cm}^{-1}$  at 260 nm, respectively (24). Solution concentrations of 3'-UMP and 5'-ADP



**Figure 2.** (A) Fluorescence emission intensity of substrate **2** (solid line) and its cleavage products (dashed line) as a function of wavelength upon excitation at 490 nm. (B) Fluorescence emission intensity (emission 515 nm, excitation 490 nm) of substrate **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 0.10 M NaCl. (Inset) Data collected during the first 30 s of the reaction.

were determined by assuming that  $\epsilon = 10\,000 \text{ M}^{-1} \text{ cm}^{-1}$  at 260 nm and  $\epsilon = 15\,400 \text{ M}^{-1} \text{ cm}^{-1}$  at 259 nm, respectively (25).

### 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 0.10 M NaCl, 0.50–100 nM substrate and 1–200 pM RNase A or 0.130–0.50 µM angiogenin. 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** is presented in Figure 2. Kinetic parameters and fluorescent properties of substrates **1–5** were determined using equations **1** and **2**:

$$I = I_f - (I_f - I_o)e^{-(k_{cat}/K_m)[E]t} \quad \mathbf{1}$$

$$I = I_o + (I_f - I_o) (k_{cat}/K_m)[E]t \quad \mathbf{2}$$

The fluorescence intensity (*I*), measured at a given time during the reaction, was recorded in units of photon counts per second (c.p.s.). The intensity of product (*I<sub>f</sub>*) was determined by non-linear least squares regression analysis (equation **1**) of data collected with the addition of sufficient enzyme to cleave all the substrate within a period of ~30 min. The intensity of substrate (*I<sub>o</sub>*) was determined from data collected prior to the addition of enzyme (typically 2 min). Values of  $k_{cat}/K_m$  were determined either by non-linear least squares regression analysis

**Table 2.** Parameters for the cleavage of fluorogenic substrates by human angiogenin

Substrate	$k_{\text{cat}}/K_m$ ( $10^2 \text{ M}^{-1} \text{ s}^{-1}$ ) <sup>a</sup>	$I_f/I_o$ <sup>b</sup>	Sensitivity ( $10^4 \text{ M}^{-1} \text{ s}^{-1}$ ) <sup>c</sup>
2 6-FAM-(dA)rU(dA) <sub>2</sub> -6-TAMRA	$3.3 \pm 0.4$	$180 \pm 10$	$5.9 \pm 0.8$
5 6-FAM-(dA)rC(dA) <sub>2</sub> -6-TAMRA	$5.4 \pm 0.4$	$83 \pm 1$	$4.5 \pm 0.3$

<sup>a</sup>Data were obtained in 0.10 M MES/NaOH buffer pH 6.0, containing 0.10 M NaCl. For comparison, the value of  $k_{\text{cat}}/K_m$  for the cleavage of uridylyl(3'→5')adenosine by human angiogenin is  $1.2 \text{ M}^{-1} \text{ s}^{-1}$  (32).

<sup>b</sup> $I_f/I_o$  is the ratio of the fluorescence intensity of the product ( $I_f$ ) and the substrate ( $I_o$ ), as in Table 1.

<sup>c</sup>Sensitivity ( $S$ ) for catalysis by human angiogenin is defined by equation 5.

of all data using equation 1 or by linear least squares regression analysis of initial velocity data using equation 2. In both analyses, we assume that the assays were done at substrate concentrations below the  $K_m$  (*vide infra*). Values of  $k_{\text{cat}}/K_m$  for RNase A derived with equations 1 and 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  $k_{\text{cat}}/K_m$  for RNase A derived with equation 1 (Table 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 equation 2 (Table 2), determining  $I_f$  by adding RNase A to the reaction after ~10 min.

### $K_m$ of substrate 6

The value of  $K_m$  for the cleavage of substrate 6 by RNase A was determined by evaluating its ability to inhibit the turnover of substrate 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 (26). Assays were carried out with stirring in 2.00 ml of 0.10 M MES/NaOH buffer, pH 6.0, containing 0.10 M NaCl, 0.60  $\mu\text{M}$  substrate 2 and 0.57 pM RNase A. After 5 min, an aliquot (0.4  $\mu\text{l}$  of a 0.35 mM solution) of substrate 6 was added. Additional substrate was added at 5 min intervals until the substrate decreased the reaction rate by ~10-fold. The maximum substrate competition was achieved prior to the reaction progressing to >10% of completion. The addition of large volumes (>20  $\mu\text{l}$ ) of substrate caused a slight, but noticeable, loss of fluorescence (<1% of the total change in fluorescence). This phenomenon was caused by 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 ( $\Delta I/\Delta t$ ) in units of c.p.s./s. Values of  $K_m$  were determined by non-linear least squares regression analysis of data fitted to equation 3:

$$\Delta I/\Delta t = (\Delta I/\Delta t)_o \{K_m/(K_m + [S])\} \quad 3$$

In equation 3,  $(\Delta I/\Delta t)_o$  is the activity prior to the addition of substrate 6. The use of equation 3 assumes that the cleavage of substrate 6 is competitive with the cleavage of substrate 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 those for the determination of  $K_m$ . Here, aliquots of 3'-UMP (0.50  $\mu\text{l}$  of a 4.9 mM solution) or 5'-ADP (0.50  $\mu\text{l}$  of a 2.3 mM solution)

were added to the reaction mixture. Values of  $K_i$  were determined by non-linear least squares regression analysis of data fitted to equation 4:

$$\Delta I/\Delta t = (\Delta I/\Delta t)_o \{K_i/(K_i + [I])\} \quad 4$$

In equation 4,  $(\Delta I/\Delta t)_o$  is the activity prior to the addition of inhibitor. The use of equation 4 requires that the inhibitor is competitive and that substrate 2 is at concentrations below the  $K_m$  (*vide infra*).

## RESULTS

### Efficacy of substrates

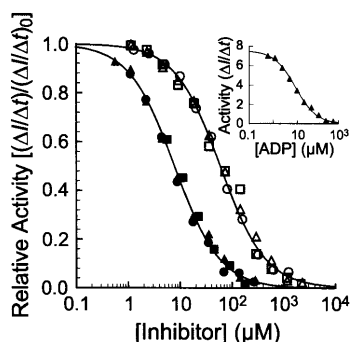
Spectroscopic and kinetic parameters for substrates 1–5 were determined in a continuous assay system. As shown for substrate 2 in Figure 2, the 6-TAMRA label almost completely quenches the fluorescence emission at 515 nm of the 6-FAM label. The emission maximum of intact substrate 2 at 577 nm is likely the result of fluorescence resonance energy transfer (FRET) from the 6-FAM label to the 6-TAMRA label. Cleavage of substrate 2 produces a large increase in fluorescence emission at 515 nm, with  $I_f/I_o = 180$  (Fig. 2 and Table 1). Replicate synthetic preparations of substrate 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. We could not detect cleavage of this deoxynucleotide by RNase A (data not shown), suggesting that the cleavage of substrate 2 is caused by transphosphorylation to the 2'-hydroxyl group of its single ribonucleotide.

Values of  $k_{\text{cat}}/K_m$  for RNase A acting on substrates 1–5 are listed in Table 1. All the substrates have similar values of  $k_{\text{cat}}/K_m$  (i.e. within 3-fold). Substrate 5 has the largest value of  $k_{\text{cat}}/K_m$  at  $6.6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ . These values are insensitive to the changes in substrate concentration and enzyme concentration used in our assays.

Values of  $k_{\text{cat}}/K_m$  for angiogenin acting on substrates 2 and 5 are listed in Table 2. The two substrates have similar values of  $k_{\text{cat}}/K_m$  (i.e. within 2-fold), with substrate 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  $I_f/I_o$ , as in equation 5:

$$S = (k_{\text{cat}}/K_m)(I_f/I_o) \quad 5$$



**Figure 3.** Dependence of the relative ribonucleolytic activity of RNase 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) Dependence of absolute RNase 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 0.10 M NaCl. Data were analyzed using equation 4.

According to this analysis, substrate **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** is a better substrate for angiogenin than is substrate **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 **6** is an unlabeled analog of substrate **2**. The value of  $K_m$  for substrate **6**, as determined in a continuous assay by monitoring inhibition of the cleavage of substrate **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 3. The effect of 5'-ADP concentration on absolute activity  $[(\Delta I/\Delta t)]$  is shown in the inset of Figure 3. The  $K_i$  values for inhibition by 3'-UMP and 5'-ADP were  $60 \pm 3$  and  $8.4 \pm 1.0 \mu\text{M}$ , respectively.

## DISCUSSION

#### Substrate sensitivity with RNase A

6-FAM-(dA)rU(dA)<sub>2</sub>-6-TAMRA (substrate **2**; Fig. 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** is accompanied by a 180-fold increase in fluorescence (Fig. 2 and Table 1). This increase is the largest reported for a ribonuclease substrate.

The kinetic parameters of the substrates follow a predictable trend. The value of  $k_{\text{cat}}/K_m$  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 (16). 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 (27). Thus as substrate length increases, the value of  $k_{\text{cat}}/K_m$  increases. The value of  $k_{\text{cat}}/K_m$  for the cleavage of the dinucleotide substrate of Hofsteenge and co-workers is  $2.06 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ . This value is similar to that for the dinucleotide substrate **1** (Table 1). The  $k_{\text{cat}}/K_m$  value for the nonanucleotide substrate of James and Woolley,  $6.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ , is similar to that for octanucleotide substrate **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) (28). Accordingly, we synthesized substrate **5**, which is identical to substrate **2** but with cytidine in place of uridine. Substrate **5** has a greater  $k_{\text{cat}}/K_m$  value than does substrate **2**. However, substrate **5** has a 2-fold lower  $I_f/I_0$  value. The structural reason for this discrepancy is not readily apparent. One possibility is that protonation of cytidine at N3 ( $pK_a$  4.3; 29) 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  $k_{\text{cat}}/K_m$  and  $I_f/I_0$ , of substrate **2** cleaved by RNase A is larger at  $S_{\text{RNase A}} = 6.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$  than that of the other substrates listed in Table 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** because of its large change in fluorescence intensity.

We also synthesized substrates analogous to substrates **1–4** 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 (12). The ribonucleolytic activity of angiogenin is essential for its angiogenic activity (30). Indeed, variants of angiogenin with greater ribonucleolytic activity are more effective at promoting neovascularization (31). 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 (32). This nucleotide specificity is apparent, though less pronounced, in the cleavage of substrates **2** and **5** (Table 2). Most remarkable, however, is our finding that angiogenin cleaves substrate **2** 100-fold faster than it does UpA. Additional interactions with substrates **2** and **5** apparently have a profound effect on catalysis by angiogenin. Thus, substrates **2** and **5** are exceptional substrates with which to analyze catalysis by angiogenin.

#### Value of $K_m$

The  $K_m$  value for substrate **6** determined herein is  $22 \mu\text{M}$ . This value is similar to the value of  $K_d = 88 \mu\text{M}$  for the RNase A-d(AUAA) complex, which was studied under similar conditions (17). Likewise,  $K_m = 33 \mu\text{M}$  for the nonanucleotide substrate of James and Woolley (11). Using the values of  $k_{\text{cat}}/K_m$  for substrate **2** and  $K_m$  for substrate **6**, we can estimate that the value of  $k_{\text{cat}}$  for substrate **2**

is  $6 \times 10^2 \text{ s}^{-1}$ . This value of  $k_{\text{cat}}$  is similar to that for cleavage of the dinucleotide UpA ( $350 \text{ s}^{-2}$ ; 33).

### Values of $K_i$

Substrate **2** enables an efficient method to evaluate inhibition of ribonucleolytic activity. Because the entire assay is performed by cumulative addition of inhibitor to one 2 ml solution, determining the potency of an inhibitor requires a little over 1 h 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 and 8.4  $\mu\text{M}$ , respectively.

In the RNase A–3'-UMP complex, the phosphoryl group of 3'-UMP interacts directly with the active site residues (33). The value of  $K_i = 60 \mu\text{M}$  for 3'-UMP is in gratifying agreement with the value of  $K_d = 54 \mu\text{M}$  for the RNase A–3'-UMP complex determined under identical conditions by isothermal titration calorimetry (33). In the RNase A–5'-diphosphoadenosine 3'-phosphate complex, the pyrophosphoryl group of the 5'-diphosphoadenosine moiety interacts directly with the active site residues (34). The value of  $K_i = 8.4 \mu\text{M}$  for 5'-ADP is somewhat larger than a value of  $K_d = 1.2 \mu\text{M}$  determined in a solution of lower ionic strength (35), as expected for an interaction that relies on Coulombic forces (17).

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 (5). 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 was the site-directed variant, then the  $K_i$  would match the  $K_d$  determined by other methods, such as isothermal titration calorimetry.

### Conclusions

We report the systematic optimization of a fluorogenic substrate for RNase A. We find that the tetranucleotide 6-FAM–dArUdAdA–6-TAMRA (Fig. 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 enzymatic or small-molecule catalysts of RNA cleavage.

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